Biochemical Pharmacology, Vol. 39, No. 8, pp. 1365–1374, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

DETERMINATION OF SOLUBLE cAMP-DEPENDENT PROTEIN KINASE ACTIVITY IN GUINEA-PIG TRACHEAL SMOOTH MUSCLE

PREFERENTIAL USE OF KEMPTIDE AS A PHOSPHORYLATING SUBSTRATE

JOHN M. LANGLANDS* and IAN W. RODGER

Department of Physiology and Pharmacology, Royal College, University of Strathclyde, Glasgow G1 1XW, U.K.

(*Received* 2 *March* 1989; *accepted* 7 *September* 1989)

Abstract—A method is described for the determination of soluble (cytosolic) cyclic AMP (cAMP)-dependent protein kinase (A-kinase) activity in guinea-pig tracheal smooth muscle. The method relies upon the use of either histone or kemptide as a phosphorylating substrate. The relative merits of each of these substances were compared by studying the effects of a protein kinase inhibitor (PKI) and of Na^+ on the phosphorylation of each substrate. PKI induced a concentration-dependent inhibition of basal and cAMP-stimulated phosphohistone formation but could not abolish it. Phosphokemptide formation was abolished by equivalent concentrations of PKI. Elevations in Na^+ concentration in the reaction buffer inhibited cAMP-stimulated phosphohistone formation in a concentration-dependent manner with concomitant elevations in the enzyme activity ratio. Basal or cAMP-stimulated phosphokemptide formation was not inhibited by elevated Na^+ concentrations. When tissues were homogenized in high Na^+ concentration buffers, an increase in the basal A-kinase activity was observed using kemptide as the substrate. No apparent change in cAMP-stimulated activity was observed. Concomitant with this was an elevation in the enzyme activity ratio. However, a high Na^+ concentration in the homogenizing buffer elevated basal phosphokemptide formation and the activity ratio. Separation of the isoenzymes of the enzyme yielded three peaks of activity upon assay of the fractions, which comprised free catalytic subunits (5% total activity), type I holoenzyme (5% total activity), and type II holoenzyme (90% total activity). Enzyme activity was increased upon pretreatment of tissues with isoprenaline and forskolin using both histone IIa and kemptide as phosphorylating substrates. The data support the preferential use of kemptide over histone IIa as a phosphorylating substrate during the determination of A-kinase activity in guinea-pig trachealis. The potential benefits of the use of kemptide are discussed.

The use of β-adrenoceptor agonists in the therapy of asthma is now so widespread that this class of drug is the primary bronchodilator therapy in use today [1]. However, development of refractoriness to the beneficial effects of β-agonists, possibly by homologous desensitization, has produced a research effort aimed at finding alternative means of inducing cyclic AMP (cAMP)-mediated bronchodilatation. To this end, the recent identification of several phosphodiesterase isoenzymes [2] has led to a search for new chemical entities that are isoenzyme-selective inhibitors of phosphodiesterase [3–5]. Such compounds raise cAMP levels within cells by inhibiting the isoenzymes responsible for the degradation of cAMP, thereby effectively bypassing both the β-adrenoceptor and adenylate cyclase.

The acceptance of a role for cAMP in the mechanism(s) underlying airway smooth muscle relaxation has developed from studies demonstrating that β-adrenoceptor agonists elevate cAMP during relaxation of airway smooth muscle [6–9]. Additional support for this view is derived from recent studies using certain isoenzyme-selective phosphodiesterase inhibitors [10, 11]. In contrast, several studies have shown that some bronchoconstrictors, for example methacholine and leukotrienes C_4 and D_4, inhibit cAMP accumulation in airway smooth muscles [12–14].

It is widely accepted that the cellular effects of cAMP are mediated via cAMP-dependent protein kinase (A-kinase) [15]. The A-kinase holoenzyme is an inactive tetrameric complex which, after interaction between cAMP and the regulatory (R) subunit, dissociates to yield two catalytic (C) subunits. It is these catalytic subunits that express the phosphotransferase activity [16, 17].

As part of an ongoing investigation of the effects of different contractile and relaxant agents on cAMP-dependent cellular events in airway tissues, it was important for us to establish a reliable method for measuring the functional activity of A-kinase. Thus, this paper describes a method for determining soluble (cytosolic) A-kinase activity in guinea-pig tracheal tissues and the partial characterization of the A-kinase isoenzymes. Furthermore, it identifies several reasons supporting the preferential use of kemptide (a synthetic heptapeptide [18]) over the more commonly used phosphorylating substrate histone.

* Author for correspondence at current address: Faculty of Pharmaceutical Sciences, University of British Columbia, 2146 East Mall, Vancouver, British Columbia, Canada V6T 1W5.

METHODS

Reagents and solutions

Adenosine 5′-trisphosphate (ATP-Tris salt), bovine serum albumin (grade III), charcoal, Folin Ciocalteau's reagent, cyclic adenosine 3′,5′-monophosphate (cAMP-free acid), copper sulphate, DL-dithiothreitol (DTT), dimethyl formamide, EDTA, forskolin, histone (type IIa), 3-isobutyl-1-methylxanthine (IBMX), isoprenaline hydrochloride, kemptide, magnesium acetate, phenylmethylsulphonyl fluoride (PMSF), potassium/sodium tartrate, protein kinase inhibitor (code P-8140, Lot 34F 9625), sodium fluoride, sodium pyrophosphate and trichloracetic acid were all obtained from the Sigma Chemical Co., Poole, U.K. Diethyl ether, ethanol, phosphoric acid, salts and buffers for Krebs–Henseleit solution and assay buffers were obtained from BDH. [γ-^{32}P]ATP (code PB132) was obtained from Amersham International, Buckinghamshire, U.K. Flurbiprofen was a gift from Boots PLC, Nottingham, U.K.

Stock solutins of IBMX and PMSF were prepared in 10% ethanol and 10% dimethyl formamide, respectively, and stored at 4°. Subsequent dilutions were made with distilled water such that the concentration of organic solvent in the assay medium did not exceed 0.5%. All other reagents were dissolved in distilled water.

Tissue preparation

Male Dunkin–Hartley guinea-pigs (300–400 g) were stunned by a blow to the head and killed by exsanguination. The trachea was rapidly excised and placed in Krebs–Henseleit solution (KHS) of the following composition (mM): NaCl, 118; KCl 4.7; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; and D-glucose, 11.7. The trachea was dissected free of extraneous connective tissue cut into rings and mounted in 10-mL water-jacketed tissue baths containing KHS at 37° and aerated with 5% CO_2 in O_2. Tissues were treated with the cyclooxygenase inhibitor flurbiprofen (1 μM) to inhibit the formation of prostanoids [19], e.g. thromboxane A_2 (TXA_2), prostacyclin, and prostaglandins E_2 and $F_{2\alpha}$ (PGE_2 and $PGF_{2\alpha}$ respectively), known to be produced during contraction induced by some agonists [20, 21]. Formation of such substances not only alters tissue contractility but also biochemical events within the cell. Tissues were allowed to equilibrate for 45 min under an initial resting tension of 20 millinewtons (mN) and were then freeze-clamped using tongs pre-cooled in liquid nitrogen. In some experiments, after the equilibration period, isoprenaline and forskolin were added to the tissues for 20 min prior to freeze-clamping. Tissues were weighed and stored at −20° until A-kinase activity was determined.

A-kinase activity assays

A-kinase activity was measured using a modification of the methods described by Corbin and Reimann [22], Cook *et al.* [23] and Torphy *et al.* [24]. By including a protein kinase inhibitor (PKI) in the assay system, the contribution of A-kinase activity to total phosphotransferase activity can be calculated for accurate assessment of enzyme activity. Ideally a substrate that is phosphorylated by A-kinase alone should be used. Therefore, the synthetic heptapeptide kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was used as a phosphate acceptor specific for A-kinase [18].

Method 1: Use of histone as a phosphorylating substrate. Frozen tracheal rings were placed in 10 vol. (w/v) of homogenizing buffer (pH 6.8) containing 10 mM potassium phosphate (KH_2PO_4/K_2HPO_4), 10 mM EDTA, 0.5 mM IBMX, 1 mM DTT, 5 mM NaCl and 10 mg/mL charcoal. The tissues were homogenized using an Ultraturrax homogenizer (Jankel Kunken KG, Staufen) for 2 × 10 sec bursts, and the homogenate was centrifuged immediately at 23,000 *g* (MSE high speed centrifuge) for 15 min at 4° to form the soluble (supernatant) and particulate (pellet) fractions.

Assay of the enzyme was initiated by the addition of 24.2 μL of the supernatant fraction to 65.8 μL of reaction buffer (pH 6.8) containing 20 mM potassium phosphate (KH_2PO_4/K_2HPO_4), 0.1 mM [γ-^{32}P]ATP (100 cpm/pmol), 10 mM magnesium acetate, 0.5 mM IBMX, 10 mM sodium fluoride (NaF) and 1 mg/mL histone (Sigma type IIa) in the absence and presence of 10 μM cAMP and protein kinase inhibitor. The incubation was carried out at 30° for 8 min. The reaction was terminated by spotting 65-μL aliquots of reaction mixture onto filter discs (Whatman 3MM, 24 mm diameter) which were dropped immediately into an ice-cold mixture containing 10% trichloroacetic acid (TCA)/2.5% sodium pyrophosphate. The filter circles were washed once in hot (85°) 5% TCA/2.5% pyrophosphate for 30 min and twice in cold 5% TCA/2.5% pyrophosphate for 15 min each. After a final wash in 95% ethanol and diethyl ether (5 min each), the discs were allowed to dry in the atmosphere.

Method 2: Use of kemptide as a phosphorylating substrate. As described in Method 1, frozen tracheal rings were homogenized in 25 vol. (w/v) of homogenizing buffer (pH 6.8 containing 10 mM potassium phosphate (KH_2PO_4/K_2HPO_4), 10 mM EDTA, 0.5 mM IBMX, 1 mM DTT, 10 μM PMSF, 20 mM NaCl, 140 mM KCl and 10 mg/mL charcoal. The tissues were homogenized and separated into soluble and particulate fractions as described in Method 1. For studies examining the effect of [Na^+] on A-kinase phosphorylation of kemptide, the enzyme was prepared in the low [Na^+] (6 mM) homogenizing buffer described in Method 1.

The assay reaction was initiated by the addition of 24.2 μL of the supernatant fraction to 65.8 μL of the reaction buffer (pH 6.8) containing 20 mM potassium phosphate (KH_2PO_4/K_2HPO_4), 0.1 mM [γ-^{32}P]ATP (100 cpm/pmol), 10 mM magnesium acetate, 0.5 mM IBMX, 10 mM NaF and 68 μM kemptide in the absence and presence of 10 μM cAMP. The reaction, carried out at 30°, was terminated after 8 min by spotting 65 μL aliquots of the reaction mixture onto filter squares (Whatman P81 phosphocellulose cation exchange paper, 25 mm), which were dropped immediately into 75 mM phosphoric acid at room temperature. The filter papers were washed five times (10 min each) in 75 mM phosphoric acid (at room temperature), followed by one wash in 95%

ethanol and one one wash in diethyl ether (5 min each). The filter papers were allowed to dry in the atmosphere.

Measurement and expression of A-kinase activity

Using both assay methods the radioactivity associated with the filter discs (a measure of the amount of phosphotransferase activity) was quantified by liquid scintillation spectrometry (Tricarb 1500). Each filter paper was counted for 4 min in 5 mL scintillation fluid (Cocktail K, BDH).

One unit of A-kinase activity was expressed as 1 pmol of phosphate transferred from ATP to histone IIa or kemptide in 1 min at 30° per mg protein in the enzyme fraction. The extent of A-kinase activation was assessed by calculating the activation ratio, which is the ratio of specific activity in the absence of added cAMP to that in the presence of sufficient cAMP to fully activate the enzyme (−cAMP/+cAMP activity ratio).

Separation of the isoenzymes of A-kinase

For the separation and identification of the isoenzymes of A-kinase, and the free catalytic subunits associated with the enzyme, approximately 50 mg of trachealis (dissected free of cartilage) was incubated for 60 min in KHS containing 1 μM flurbiprofen, maintained at 37° and aerated with 5% CO_2 in O_2. The tissue was blotted on filter paper and rapidly frozen in liquid nitrogen. The frozen tissue was homogenized in 20 vol. (w/v) of a homogenizing buffer (pH 6.8) containing 10 mM potassium phosphate (KH_2PO_4/K_2HPO_4), 1 mM EDTA, 10 mM DTT, 10 μM PMSF and 0.5 mM IBMX and centrifuged to form soluble and particulate enzyme fractions. The enzyme preparation was diluted further to maintain the conductivity of the preparation below 1 millisiemens (mS) (at 4°). The crude enzyme preparation was applied to a column (1.5 × 8 cm) of DEAE-cellulose (DE-52) pre-equilibrated with homogenizing buffer. The column was then washed with 50 mL of the same buffer to remove unbound proteins. The free catalytic subunits and isoenzymes of A-kinase were eluted from the column using a linear gradient of NaCl (0–350 mM) in a total volume of 40 mL. The flow rate was set at 180 μL/min and forty 1 mL fractions were collected. The temperature of the buffers, separation column and fractions was maintained at <5° throughout.

The A-kinase activity of each fraction was assayed as described in Method 2 using kemptide as the phosphorylating substrate. This activity was expressed as the amount of PO_4 transferred per minute per millilitre of eluate at 30°.

Protein determination

The protein concentration in the supernatant fraction was determined by the method of Lowry *et al.* [25] and with the aid of a "Lowry curve fitting program" (Biosoft, Elsevier, U.K.). Tissue samples of approximately 20–30 mg wet weight were used and yielded between 40 and 60 μg protein/100 μL of enzyme preparation.

Statistical analysis

Values in the text and figures represent the mean ± standard error of the mean (SE), N = 5. Where required, data were analysed nonparametrically using the Mann–Whitney sign rank test. In all figures, standard error bars are shown where they are greater than the symbol size denoting the mean value. Significance was accepted when $P < 0.05$.

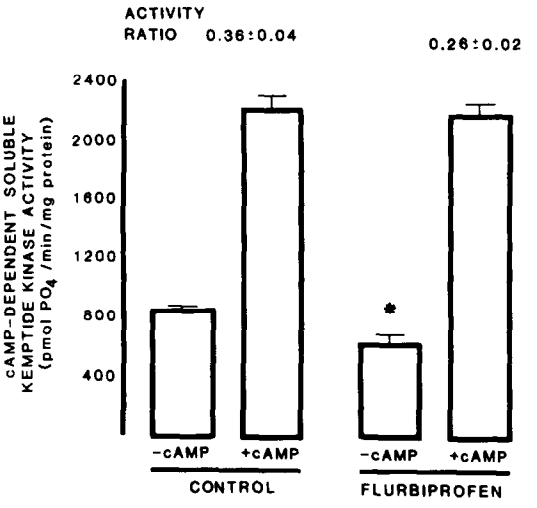

Fig. 1. Effect of flurbiprofen (1 μM) on basal (−cAMP) and stimulated (+cAMP) cytosolic A-kinase activity in guinea-pig trachealis. Tissues were equilibrated under 20 mN resting tension for 45 min in the absence or presence of flurbiprofen. An asterisk (*) indicates significant difference from control, $P < 0.05$ (Mann–Whitney).

RESULTS

Initial studies indicated that A-kinase from guinea pig trachealis was present predominantly (80%) in the supernatant fraction, and for this reason our investigations focussed on soluble A-kinase. Over the first 9 min of assay reaction, in which soluble A-kinase catalysed the transfer of phosphate from ATP to protein substrate (histone IIa or kemptide), the kinetics were linear with respect to time both in the absence and in the presence of exogenous cAMP (10 μM).

Effect of flurbiprofen on A-kinase activity

In our studies, all tension recordings were performed in the presence of the cyclooxygenase inhibitor flurbiprofen (1 μM). In addition to reducing the basal tension in the tissue from 20 mN to approximately 7.5 mN, flurbiprofen reduced basal A-kinase activity measured in the absence but not in the presence of exogenous cAMP (10 μM). Consequently, the basal enzyme activity ratio was found to be lower in flurbiprofen-treated tissues (Fig. 1).

Effects of protein kinase inhibitor on phosphotransferase activity

When histone IIa was used as a substrate for determining A-kinase activity by phosphocellulose adsorption and TCA precipitation, a proportion of the phosphotransferase activity expressed was due to cAMP-*independent* protein kinase activity. By inclusion of a heat-stable protein kinase inhibitor

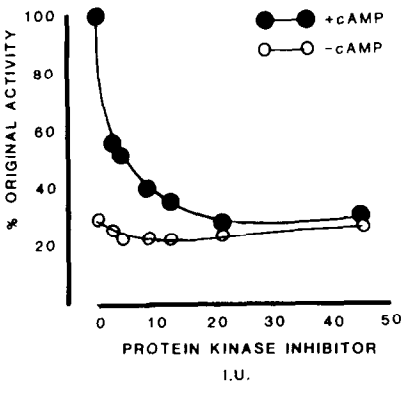

Fig. 2. Effect of increasing concentrations of protein kinase inhibitor (Sigma P8140, Lot No. 34F 9625) on cytosolic cAMP-dependent histone kinase activity in the absence and presence of added cAMP (10 μM). Control values: −cAMP 172 ± 7 and +cAMP 609 ± 38 pmol PO_4/min/mg protein (mean ± SE, N = 5).

(PKI) [26] in the assay medium, this cAMP-*independent* protein kinase activity can be estimated and accounted for. This simple method used to quantify accurately A-kinase activity was described by Torphy *et al.* [27]. The activity of A-kinase in the absence of added cAMP was calculated by subtracting the enzyme activity in the presence of PKI from the enzyme activity in the absence of PKI. The activity of A-kinase in the presence of added cAMP was calculated in a similar fashion.

A commercially available PKI preparation from bovine heart muscle (Sigma P8140, Lot No. 34F 9625) produced a concentration-dependent inhibition of both basal and cAMP-stimulated phosphotransferase activity when histone IIa was used as the substrate (Fig. 2). When the phosphotransferase activity was assayed in the absence of added cAMP, PKI inhibited approximately 50% of phosphohistone formation (Fig. 3A). This fraction of enzyme activity represents free catalytic subunits and reflects basal A-kinase activation. In the presence of cAMP (10 μM), phosphotransferase activity increased approximately 5-fold. This was inhibited incompletely by PKI. In the presence of 25 inhibitory units per tube of PKI, a small but significant increase (6%) in phosphohistone formation was seen independent of A-kinase activation (Fig. 3A).

The specific activity of A-kinase using kemptide was found to be approximately five times that obtained with histone IIa (Fig. 3, A and B). Inclusion of PKI (25 I.U./assay tube) abolished phosphokemptide formation in the absence and presence of exogenous cAMP (Fig. 3B).

Effect of Na^+ *on A-kinase activity*

Increasing the Na^+ concentration (0–15 mM) in the reaction medium produced a concentration-dependent inhibition of phosphohistone formation in the absence of added cAMP. However, above this level recovery to basal phosphohistone formation occurred. In the presence of cAMP (10 μM), marked inhibition of phosphohistone formation was observed when the Na^+ concentration in the assay medium was elevated from 0 to 400 mM. A concomitant elevation in the A-kinase activity ratio (0.21 ± 0.02 to 0.69 ± 0.09) was also observed (Fig. 4, top panel). Basal or cAMP-stimulated phosphorylation of kemptide was not inhibited by Na^+ concentrations up to 400 mM (Fig. 4, bottom panel).

This lack of inhibitory effect by Na^+ on phosphokemptide formation allowed the study of the effects of Na^+ during homogenization. Increasing the salt concentration (6–400 mM) in the homogenizing buffer produced a concentration-dependent elevation in the basal phosphokemptide formation without any apparent effect on cAMP-stimulated phosphotransferase activity (Fig. 5). A concomitant elevation in the A-kinase activity ratio was also observed.

This elevation in basal phosphotransferase activity, and thus the activity ratio, represents an increase in the amount of free catalytic subunits present in the enzyme preparation. A similar effect was observed with K^+, though an inhibition of cAMP-stimulated phosphokemptide formation was observed at concentrations of K^+ greater than 250 mM (data not shown).

Separation of the isoenzymes of A-kinase by DEAE-cellulose chromatography

There is substantial evidence from chromatographic and electrophoretic studies supporting the idea of at least two isoenzymes of A-kinase, both thought to operate through the same mechanism of activation [28]. These species, designated type I and type II due to their order of elution by anion exchange chromatography, differ only in the structure of their regulatory subunits. It is important to identify and quantify the relative proportions of each of these species within the trachealis since the isoenzymes differ not only in their molecular weight [29] and net charge, but also in their susceptibility to autophosphorylation, effects of altered $[Mg^{2+}]$, dissociation–reassociation kinetics and sensitivity to activation by cAMP [17, 30].

The isoenzymes of cytosolic A-kinase obtained from homogenates of guinea-pig tracheal smooth muscle eluted from a DE-52 column in three main peaks (Fig. 6). The activity present in the first peak eluted at a conductivity of approximately 3 mS and was seen irrespective of whether the fractions were assayed in the absence or presence of exogenous cAMP (10 μM). The activity was abolished completely by PKI, suggesting that this phosphotransferase activity was attributable to free catalytic subunits. The second and third peaks eluted from the column at conductivities of approximately 7 and 13 mS respectively. The second peak was considerably smaller than the third and both were magnified several-fold in the presence of exogenous cAMP (10 μM), suggesting that they represented inactive type I and type II holoenzyme respectively. Using a *bona fide* holoenzyme preparation from bovine cardiac muscle (containing significant quantities of both type I and type II isoenzymes), it was found that some type I co-eluted with catalytic subunit (fraction 11) and continued to elute over nine further fractions adding further difficulty in the evaluation

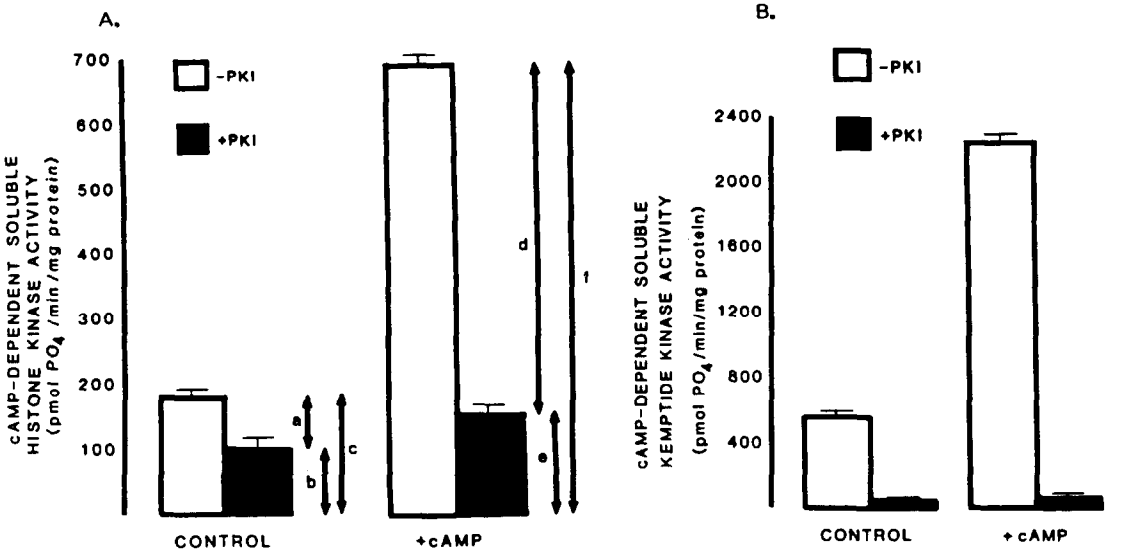

Fig. 3. Effect of addition of 25 inhibitory units (I.U.) of PKI (Lot No. 34F 9625) to the reaction buffer using histone IIa (A) and kemptide (B) as a phosphorylating substrate. In the absence of protein kinase inhibitor (PKI), A-kinase activities (c and f) were observed. The true A-kinase activities (a and d) were calculated by subtraction of cAMP-*independent* kinase activities observed in the presence of PKI (b and e).

of the relative contribution of type I to total cAMP-stimulated A-kinase activity. In two separate experiments, the free catalytic subunits, and type I and type II holoenzymes represented 5.5, 4.2 and 90.2%, respectively, of the total cAMP-stimulated A-kinase activity.

Mechanical- and drug-induced alterations in A-kinase activity

It is important in such studies that the A-kinase activity determined is that which is present in the tissue under basal conditions or as a result of drug treatment, and not as a result of homogenization *per se*. When added to tracheal tissue and to homogenizing buffer alone, *bona fide* holoenzyme (R_2C_2) and catalytic subunit (C) did not dissociate or reassociate upon homogenization (Fig. 7). However, loss of both basal and stimulated activity associated with the holoenzyme was observed after homogenization for periods greater than 15 sec. This was prevented by increasing the concentration of DTT in the buffer to 2.5 mM. This concentration of DTT did not alter the enzyme activity associated with the tissue preparations (results not shown).

The use of charcoal in the homogenizing buffer has been advised, in some reports, to adsorb any cAMP that may be released by the homogenization process itself [31, 32]. In the present study charcoal (10 mg/mL) did not affect significantly the basal or cAMP-stimulated A-kinase activity or the enzyme activity ratio. In addition, it was found that isoprenaline (10^{-7}–10^{-5} M) added to tissues prior to assay induced relaxation of the tissues and produced a concentration-dependent increase in the A-kinase activity ratio with both histone IIa (0.19 ± 0.02 to 0.46 ± 0.06) and kemptide (0.32 ± 0.01 to 0.58 ± 0.07) as substrates. Forskolin (10^{-6} M) induced full activation of the enzyme activity ratios using both substrates (Fig. 8).

DISCUSSION

To date, only a few studies have been carried out to examine the effects of various pharmacological agents on cAMP-dependent protein kinase in smooth muscle preparations [33, 34]. The activity of A-kinase has not been determined previously in guinea-pig tracheal smooth muscle. Thus, during the development of the assay as an accurate, routine method for the physiological evaluation of changes in the cAMP-generating system of this tissue, it was necessary to characterize several aspects of the enzyme.

Several differences in the methods for measuring phosphohistone and phosphokemptide formation are outlined in the Methods. These general conditions for running the assay were obtained as a result of examination of the effects of altering [Na^+] in the homogenizing and reaction buffers on A-kinase activity using histone IIa and kemptide as substrates. In addition, since greater specific activity was obtained using kemptide as a phosphorylating substrate, the enzyme was diluted by homogenizing the tissue in 25 vol. instead of 10 vol. of buffer. Thus, assay of enzyme activity using kemptide was permitted in smaller tissues samples. The concentrations of histone IIa and kemptide used in the protocols described were chosen such that the substrate would not be depleted during the reaction. It was noted that by doubling or reducing by half the substrate concentration, the rates of phosphohistone and phosphokemptide formation were not altered. As a precaution, PMSF was included in the buffer during separation of the isoenzymes in order to prevent enzyme degradation. As a purely cautionary

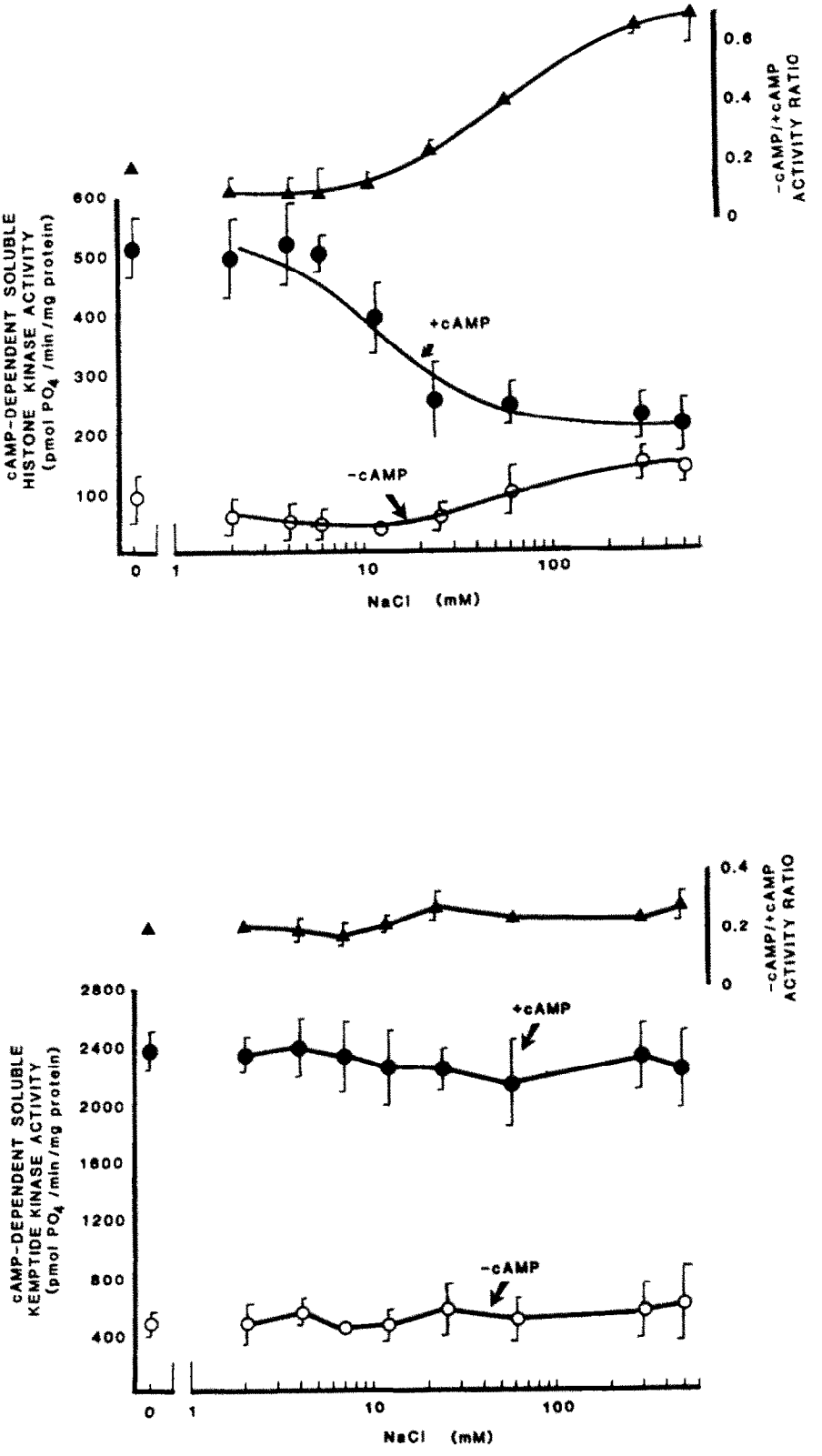

Fig. 4. Effect of addition of NaCl to the A-kinase assay buffer (see Methods) on (top panel), cytosolic cAMP-dependent histone kinase activity and (bottom panel), cytosolic cAMP-dependent kemptide kinase activity measured in the absence and presence of added cAMP (10 μM). The histone kinase activity and activity ratio were corrected for the interference from cAMP-*independent* protein kinase activity.

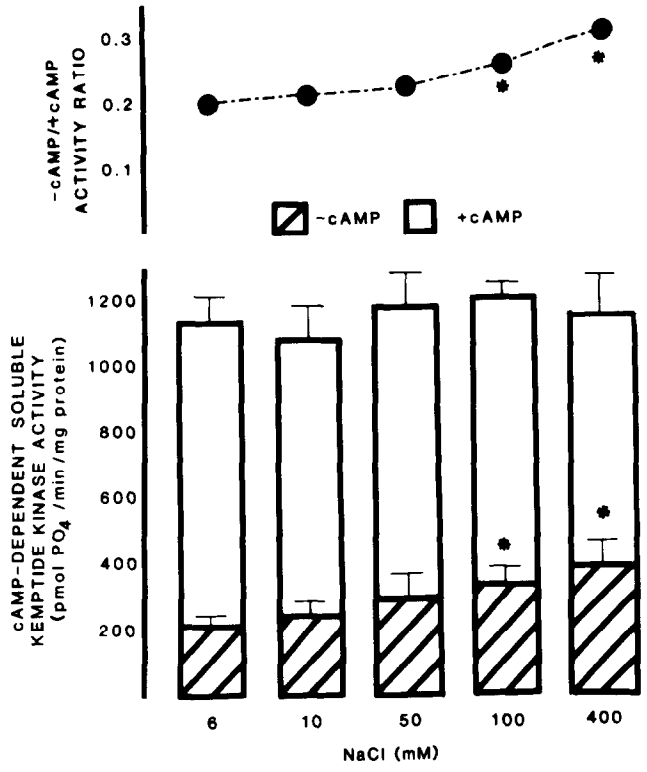

Fig. 5. Effect of addition of NaCl to the A-kinase activity assay homogenizing buffer, during the tissue preparation, on cytosolic cAMP-dependent kemptide kinase activity, measured in the absence and presence of added cAMP (10 μM). From these values the enzyme activity ratio was calculated. An asterisk (*) indicates a significant difference from 6 mM NaCl, $P < 0.05$ (Mann–Whitney).

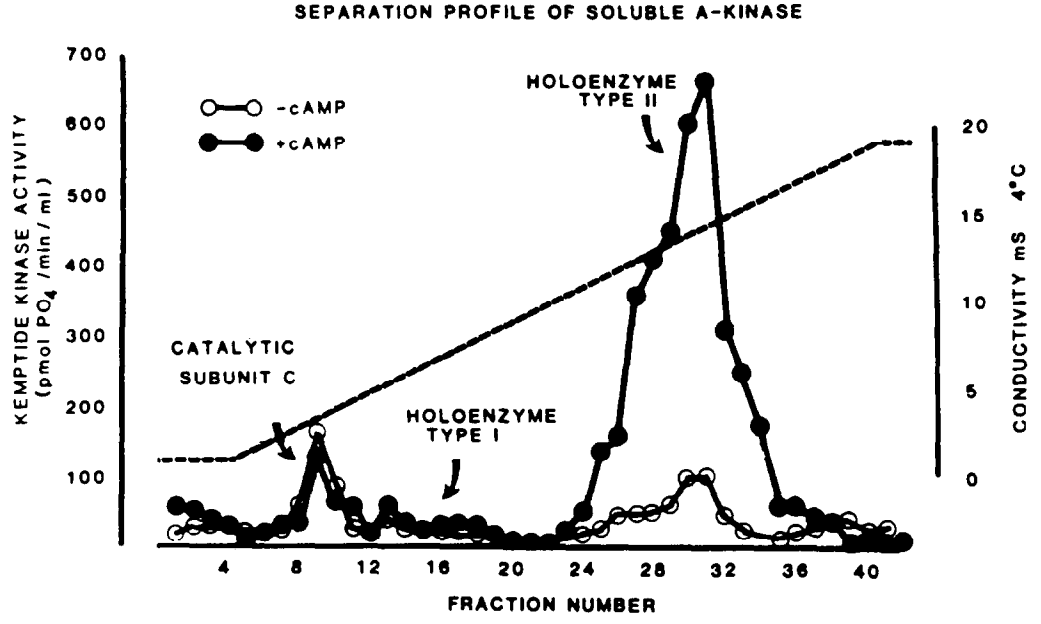

Fig. 6. Separation profile of cAMP-dependent protein kinase (cytosolic) obtained from guinea-pig tracheal smooth muscle. For details of the separation procedure see Methods. Each fraction (1 mL) was assayed for A-kinase activity in the absence and presence of added cAMP (10 μM).

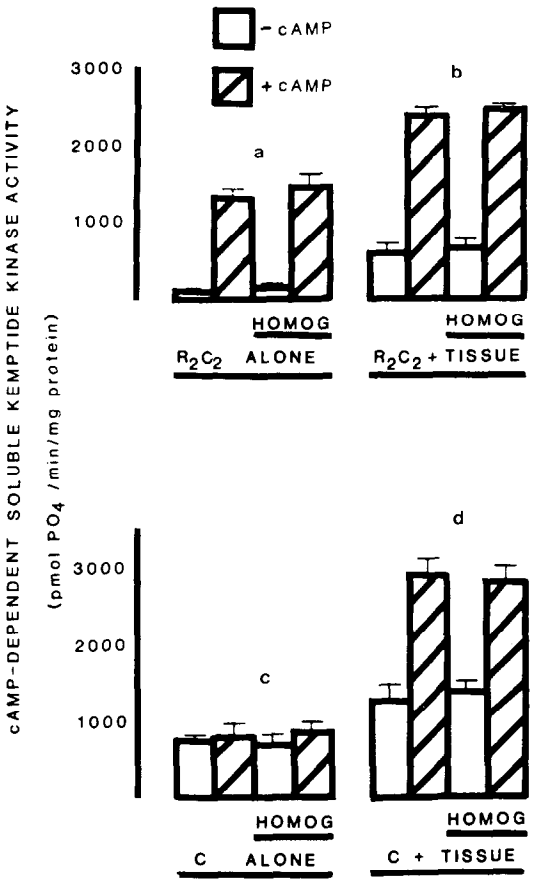

Fig. 7. Effect of homogenization on purified A-kinase holoenzyme (R_2C_2) and free catalytic subunit (C). R_2C_2 or C was added to buffer alone (a and c) and to tissue (b and d) prior to and post homogenization.

measure, PMSF was included in the buffer during measurement of phosphokemptide formation. PMSF itself did not alter A-kinase activity.

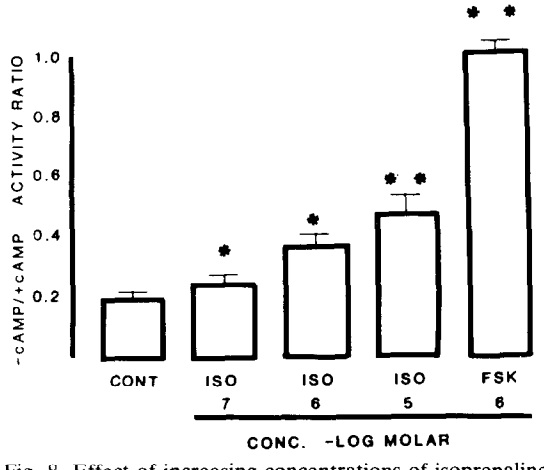

Fig. 8. Effect of increasing concentrations of isoprenaline (10^{-7}, 10^{-6}, 10^{-5} M) and forskolin (10^{-6} M) on guinea-pig tracheal smooth muscle cytosolic A-kinase activity ratio. Isoprenaline and forskolin were added to tissues, pre-equilibrated in KHS containing flurbiprofen (1 μM), for 20 min. Key: (*) and (**) indicate signicant difference from control, $P < 0.05$ and 0.01 respectively (Mann–Whitney).

To prevent changes in cAMP accumulation, resulting in altered A-kinase activity, arising from interference from various prostanoids released under basal conditions and during the contractile reponse, tissues were pretreated with flurbiprofen. Since flurbiprofen did not activate or inhibit A-kinase *per se* (data not shown), the ability of the cyclooxygenase inhibitor to reduce tension and induce a significant decrease in basal A-kinase activity and activity ratio indicates that flurbiprofen not only removed the ability of the tissue to produce prostanoids which induce tone [20, 21], but also inhibited formation of substances which elevate cAMP content. Such observations are consistent with the hypothesis that under basal conditions guinea-pig airway preparations continually release low levels of prostanoids, some of which stimulate cAMP accumulation, e.g. PGE_2, and others which possess the ability to induce tissue tone, e.g. TXA_2 and $PGF_{2\alpha}$ [20].

One of the problems associated with the determination of A-kinase activity when using histone IIa is that a proportion of the activity expressed is due to cAMP-*independent* protein kinase activity. Thus, in order to quantify the activity of the enzyme, a heat-stable protein kinase inhibitor (PKI) was used to determine the proportion of cAMP-*independent* phosphotransferase activity. Using histone IIa, PKI inhibited only a fraction of the A-kinase activity, corroborating the findings of Torphy *et al.* [27] and Silver *et al.* [35] who also found similar PKI-resistant phosphotransferase activities in canine trachealis and bovine coronary artery respectively. Although not investigated in the present study, it is likely that cyclic nucleotide-*independent* protein kinases and cGMP-dependent protein kinases are responsible for this PKI-resistant activity observed in both the absence and the presence of exogenous cAMP. Use of kemptide is clearly superior since greater phosphotransferase activity is exhibited and no PKI-resistant activity is apparent. It would appear that under the conditions the assay was performed phosphorylation of kemptide is exclusively catalysed by A-kinase. Addition of cyclic GMP (10 μM) instead of cyclic AMP to the reaction medium failed to elevate basal phosphokemptide formation (data not shown). Thus, it would appear that cGMP does not activate A-kinase or any activation of G-kinase which may occur does not induce phosphokemptide formation. This observation is not surprising since previous studies have demonstrated tremendous difficulty in measuring G-kinase activity in smooth muscle [36], unless enzyme activity is measured rapidly and at 4°. Unfortunately, no purified G-kinase is available to test its ability to induce phosphokemptide formation. It is possible that such improved specificity and activity observed with kemptide may be related to its high serine content and its amino acid sequence which corresponds closely to the active site of porcine pyruvate kinase, an endogenous A-kinase substrate [18]. A similar synthetic protein phosphate acceptor, malantide [37], has shown similar advantages to kemptide over the use of histones as a phosphorylating substrate [38].

In addition to being a relatively poor substrate for A-kinase-induced phosphotransferase activity, the

phosphorylation of histone was found to be subject to inhibition by high concentrations of salt. One consequence of this is an over-estimation of the enzyme activity ratio. For this reason, determination of A-kinase activities using histone IIa used reaction buffers containing low salt (6 mM Na^+). Using kemptide, high concentrations of salt did not affect the phosphotransferase activity and so permitted the use of more physiologically-relevant salt concentrations in the assay media. Inclusion of a high salt concentration in the homogenizing buffer elevated the unstimulated A-kinase activity, representing an increase in the amount of free catalytic subunits present. A similar observation has been reported by Murray and co-workers [38] for cardiac tissue. This group noted that in low [Na^+] homogenizing buffer, free catalytic subunits became bound to the particulate fraction during enzyme preparation. Thus, the need to use physiologically relevant salt concentrations in this buffer in order to obtain accurate quantitation of the enzyme activity is reinforced. Since using histone IIa in the assay forbids the use of high salt concentrations, loss of a proportion of free catalytic subunits is inevitable and, therefore, results in an under-estimation of the enzyme activity ratio.

The insensitivity of phosphokemptide formation to high salt concentrations allowed the separation of the isoenzymes of cytosolic A-kinase. The relative quantities of each isoenzyme appear to vary considerably from tissue to tissue and among species [29, 39, 40]. Cytosolic A-kinase prepared from guinea-pig trachealis was composed predominantly of type II holoenzyme with levels comparable to those found in guinea-pig lung parenchyma [41]. It has been demonstrated that subunits of type II protein kinase in extracts may reassociate upon dilution in buffers of low ionic strength [31], thus leading to underestimation of the activity ratio. In high ionic strength buffers, equivalent to physiological ionic conditions, reassociation can be prevented and, thus, the activity ratio raised [39, 42]. Thus, for guinea-pig trachea which contains predominantly type II A-kinase, this would allow a more accurate quantitation of activity of the enzyme. The opposite appears to be true for the type I isoenzyme which rapidly dissociates in high salt solutions and also in the presence of histones [31]. Thus, if the type I isoenzyme is present in significantly large quantities, problems may arise during the assay of phosphotransferase activity using high salt solution, and be further magnified by use of histones as the phosphorylating substrate. Therefore, in order to optimise the recovery of free catalytic subunits of A-kinase, obtained from guinea-pig trachealis during the assay using kemptide, the use of a solution with physiological concentrations of salt is favoured.

Upon separation of the isoenzymes of guinea-pig tracheal A-kinase, very little activity was associated with the holoenzyme fraction until exogenous cAMP was added. Taken together with the stability of *bona fide* holoenzyme and free catalytic subunit during homogenization, this indicates that very little spontaneous activation of the enzyme occurs during the assay procedure. Such effects are important in relating modulations in A-kinase activity to mechanical events within the tissue upon administration of various pharmacological agents. In the present study, both isoprenaline and forskolin elevated A-kinase activity in guinea-pig trachealis. We have also demonstrated the ability of cholinomimetics to decrease A-kinase activity in airway smooth muscle [14]. Since A-kinase activity may be increased or decreased in response to various spasmogens and relaxant agents, this assay provides a useful method for evaluating the physiological importance of alterations in cAMP content associated with changes in tissue contractility.

In conclusion, this study has demonstrated the preferential use of kemptide over histone IIa as a phosphorylating substrate when determining A-kinase activity in guinea-pig trachealis. Use of kemptide in this assay had several advantages: (1) it was a much better substrate for A-kinase compared with histone IIa, (2) phosphokemptide formation was not inhibited by high concentrations of Na^+, (3) physiological concentrations of Na^+ were used effectively to prevent binding of catalytic subunits to the particulate fraction during enzyme preparation, (4) no cAMP-*independent* phosphotransferase activity was expressed, and (5) reassociation of the enzyme was prevented by use of buffer containing high [Na^+]. Thus, activity of guinea-pig tracheal A-kinase can be determined under physiological conditions with greater ease.

Acknowledgements—We are grateful to the Asthma Research Council for financial support and to Drs J. Diamond and M. A. Giembycz for helpful discussion of the work.

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