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EVALUATION OF KEMPTIDE, A SYNTHETIC SERINE-CONTAINING HEPTAPEPTIDE, AS A PHOSPHATE ACCEPTOR FOR THE ESTIMATION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITY IN RESPIRATORY TISSUES

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Abstract—In the search for a phosphate acceptor to estimate cyclic AMP-dependent protein kinase (A-kinase) activity in respiratory tissues devoid of the disadvantages inherent with the use of histones, we compared and contrasted a conventional substrate, histone IIa, with that of a novel heptapeptide phosphate acceptor, Kemptide (Kemp *et al.*, *J Biol Chem* **252**: 4888–4894, 1977). The specific activities of soluble A-kinase isolated from guinea-pig lung parenchyma and from bovine tracheal smooth muscle were significantly (12–16-fold) greater when Kemptide was used as substrate when compared to histone IIa. Moreover, studies with a specific inhibitor of A-kinase demonstrated that whilst the cyclic AMP-stimulated phosphorylation of Kemptide was catalysed exclusively by A-kinase, only ~89% of the phosphate incorporated into histone IIa was attributed to the activity of this enzyme. The activation constant (K_{act}) of cyclic AMP for A-kinase was estimated to be 1.5 μM when Kemptide was used as substrate, 25-fold higher than that obtained using histone IIa (60 nM) under identical conditions. In a complementary series of experiments it was found that both the basal and isoprenaline-stimulated A-kinase activity ratios were significantly higher when histone IIa was used as substrate when compared with that obtained using Kemptide. Elevating the ionic strength of the homogenization buffer or assay cocktail with NaCl produced a reversible (following dialysis), concentration-dependent, mixed uncompetitive, inhibition of cyclic AMP-stimulated histone phosphorylation; phosphokemptide formation was unaffected. The ability of salt to inhibit A-kinase activity also affected the elution profile of A-kinase isoenzymes following their separation by DEAE-cellulose chromatography. With histone IIa as substrate the Type I:Type II isoenzyme ratio in guinea-pig lung and in bovine tracheal smooth muscle was 9:91 and 35:65, respectively. Changing the phosphate acceptor to Kemptide reduced these respective ratios to 5:95 and 29:71. Although the effect on the isoenzyme ratio was small, the specific activities of the two isoenzymes isolated from both tissues were markedly attentuated by *ca.* 9% (Type I) and 36% (Type II). It is concluded that Kemptide is without many of the disadvantages inherent with histone IIa and is, thus, a preferable phosphate acceptor for estimating soluble A-kinase activity and determining the isoenzyme ratio in guinea-pig lung and in bovine tracheal smooth muscle.

Cyclic AMP-dependent protein kinase (EC 2.7.1.37) is believed to represent the sole intracellular acceptor for cyclic AMP in all cell types so far studied. Analysis of cyclic AMP-stimulated phosphotransferase activity in tissues after their fractionation over anion-exchange columns usually reveals two main peaks of catalytic activity which correspond to isoenzymatic forms of native enzyme [1, 2]. These isoenzymes, termed Type I and Type II, differ in a number of ways including their susceptibility to autophosphorylation, the molecular weight of their respective regulatory subunits and their sensitivity to activation by cyclic AMP [2, 3]. In addition, the subunits of the Type I and Type II holoenzymes contrast dramatically with respect to their dissociation and reassociation kinetics [1–3], differences that must be appreciated if accurate estimates of the *in vivo* activation state of A-kinase are to be made. In tissues, therefore, which contain predominantly the Type I isoenzyme, homogenization buffers of low ionic strength must be used for this condition prevents further enzyme activation. In contrast, high salt-containing homogenization buffers must be employed in tissues containing predominantly the Type II isoenzyme since a high ionic strength is necessary to effectively limit subunit reassociation (inactivation).

Traditionally, A-kinase activity was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into one of a family of phosphate acceptors collectively known as histones. Mixed histones have been almost exclusively used since they are inexpensive and are readily available from various commercial sources. Over the last 15 years, however, it has become apparent that there are a number of potential disadvantages in using histones in A-kinase assays (see Refs 2 and 4). The most significant of these are that: (1) histones can be phosphorylated by many different protein kinases that may be present in a tissue extract; (2) A-kinase-stimulated phosphohistone formation is inhibited by physiological concentrations of salt in some tissues [5–8]; (3) the

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inactive holoenzyme subunit structure of A-kinase is dissociated by histones [9]; and (4) histones are relatively poor substrates for A-kinase [10].

Although some of the above mentioned drawbacks can be minimized, no satisfactory modification has yet been developed to prevent the inhibitory effect induced by salt. Indeed, the necessity to use homogenization buffers of high ionic strength when measuring A-kinase activity in tissues which contain significant quantities of the Type II isoenzyme (see above) renders the use of histones completely unsuitable. This problem is especially pronounced with A-kinase extracted from respiratory tissues (e.g. canine, guinea-pig and bovine trachealis, human, bovine and murine lung) where a large proportion (60–90%) of this enzyme is present as the Type II species [5, 11–13, 14].

In the search for a phosphate acceptor devoid of the above mentioned disadvantages we have compared and contrasted a conventional substrate, histone IIa, with that of Kemptide, a serine-containing heptapeptide (Leu-Arg-Arg-Ala-Ser*-Leu-Gly) that corresponds very closely to that present at the active site of porcine hepatic pyruvate kinase [15]. Although this substrate was described more than 10 years ago [15], histones are still almost exclusively employed in protein kinase assays. One reason, other than the commercial cost of Kemptide, that may explain the apparent reluctance of investigators to use this substrate may be due to the fact that no systematic study, to our knowledge, has been performed to show that Kemptide is without the disadvantages identified in histone substrates. In this paper, therefore, we described the results of detailed studies designed to assess the suitability of Kemptide as a phosphate acceptor for the estimation of soluble A-kinase activity isolated from guinea-pig lung and from bovine tracheal smooth muscle.

MATERIALS AND METHODS

Chemicals, drugs and analytical reagents. The following were purchased from the Sigma Chemical Co. (St Louis, MO): ATP (free acid), bacitracin, benzamidine, bovine serum albumin (BSA, grade III) cyclic AMP, (*dl*)1,4-dithiothreitol (DTT), EDTA-(tetrasodium salt), glycerol, β-glycerol phosphate, histone IIa, histone H2b, heparin, 3-isobutyl-1-methyl-xanthine (IBMX), (−)isoprenaline-D-bitartrate, Kemptide, magnesium acetate, phenylmethylsulphonylfluoride (PMSF), protein kinase inhibitor (PKI, code P-5015, lots 93F 9580 and 105F 9705), IP_{20}(Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp), sodium acetate, soybean trypsin inhibitor, Type I A-kinase holoenzyme (code P-4890, Lot 34F 9515) and Type II A-kinase holoenzyme (code P-3891, Lot 46C 9540). All other chemicals, drugs and analytical reagents were obtained from the following sources: [γ-^{32}P]ATP (Amersham, Bucks, U.K.), DEAE-cellulose (DE-52) and P81 phosphocellulose ion exchange paper (Whatman), phosphoric acid and salts for buffers and constituents of the assay cocktail (BDH, Poole, U.K.).

* Phosphorylatable serine residue.

Isolation, preparation and treatment of respiratory tissues. Lungs from male Camm–Hartley guinea-pigs (250–300 g) and tracheae from cows (*ca.* 18 months) were used. Guinea-pigs were heparinized (1400 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 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) Krebs–Henseleit Solution (KHS). The lower lung lobes were isolated from the major airways and blood vessels and tissue showing significant haemorrhage was discarded. Tracheal smooth muscle from the bovine tissue was dissected free of cartilage, epithelium and all connective tissue. Fragments (20–40 mg) of sub-pleural lung parenchyma and small strips (*ca.* 3 × 2 × 10 mm) of bovine cervical trachealis were then cut from the remaining tissue and incubated, free-floating, in oxygenating KHS maintained at 37°. Tissue was left to equilibrate for 60 min and then rapidly removed from the KHS, blotted on dry absorbent paper and quick-frozen by submersion in liquid nitrogen. 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. The composition of the KHS was as follows (mM): NaCl 118, KCl 4.7, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7, $CaCl_2.6H_2O$ 2.5.

Preparation and assay of soluble A-kinase. Unless otherwise stated 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, 500 mM NaCl). The resulting homogenate was centrifuged (Beckman J2 21) for 20 min at 31,000 g_{max} (4°) in a fixed arm JA 17 rotor (r_{av} = 8.95 cm) to form soluble (supernatant) and particulate (pellet) fractions. The supernatant fraction was then used as the source of the soluble enzyme. When Kemptide was used as substrate (see below) the supernatant was diluted 16-fold in buffer A supplemented with 2 mg/ml BSA.

Soluble A-kinase activity was measured using a modification [16] of the method originally described by Witt and Roskoski [17]. Assays were performed in triplicate at 30° and initiated by the addition of 25 μl (40–60 μg protein with histones; 3–4 μg protein with Kemptide) of the soluble extract to 75 μl of a reaction medium (pH 6.8) containing (final concentration): 10 mM KH_2PO_4, 10 mM K_2HPO_4 pH 6.8, 10 mM $Mg.(CH_3COO)_2$, 500 μM IBMX, 500 μg/ml BSA, 100 μM ATP supplemented with 100 cpm/pmol [γ-^{32}P]ATP (sp. act. 20–40 Ci/mmol) and 100 μM Kemptide, 1 mg/ml histone IIa or 1 mg/ml histone H2b (see text) in the absence and presence of cyclic AMP (lung, 10 μM; trachealis, 2 μM). The heat-stable PKI originally isolated by Walsh *et al.* [18], or the purified synthetic icosapeptide inhibitor, IP_{20} [19], was used to define A-kinase activity [5, 11, 20]. Preliminary studies established that PKI gave identical results to IP_{20}. 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 75 mM phosphoric acid (10 ml per paper square). 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 radioactivity (representing phosphohistone/Kemptide) was quantified by liquid scintillation spectrometry (Packard TriCarb 460 CD) in ASC (Amersham). The above conditions allowed A-kinase activity to be estimated utilizing less than 7% of the total ATP concentration. The availability substrate did not, therefore, become rate-limiting. Unless otherwise indicated one unit of enzyme activity is defined as that amount of A-kinase which catalysed the incorporation of 1 nmol of phosphate from ATP into Kemptide/histone IIa/histone H2b 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. Total soluble protein was determined by the method of Lowry *et al.* [21], using BSA as standard, and corrected for the protein contributed by contaminating haemoglobin [5].

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 approximately 100 mg of frozen tissue were homogenized in 20 vol. (w/v) of buffer B (5 mM KH_2PO_4, 5 mM K_2HPO_4 pH 6.8, 1 mM EDTA, 2 mM DTT), containing 10 μM PMSF, 10 μg/ml soybean trypsin inhibitor, 2 mM benzamidine and 100 μg/ml bacitracin, and centrifuged to form soluble and particulate enzyme fractions as described above. The soluble enzyme was diluted in buffer B such that the conductivity was <3 mS (at 4°) and then applied to a column (Bio-Rad; 1 × 10 cm) of DEAE-cellulose (DE-52) pre-equilibrated with buffer B. The column was then washed with a further 40 ml of the same buffer to remove unbound protein and A-kinase isoenzymes were eluted 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 76% 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. 22). 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 by DEAE-cellulose chromatography essentially as described by Walsh and his colleagues [23, 24]. Three peaks of PKI activity, corresponding to different charged species of PKI and denoted I_1, I_2 and I_3 [23, 24], 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 litres each) of buffer D (500 μM β-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, re-constituted in 1 ml of buffer B containing 40% (v/v) glycerol, aliquoted and stored at −80°. When required PKI was diluted 1:15 in buffer B.

Statistical analysis. Values in the text represent the mean ± SE of N determinations. Where statistical evaluation was required data were analysed non-parametrically using the statistical package 'Number Cruncher Statistical System' (written by Dr J. H. Hintze, 865 East 400 North Kaysville, Utah, U.S.A.). The Wilcoxon matched pairs sign-ranks test and Mann–Whitney U-test were used to test significance between paired and unpaired variates, respectively. When more than two groups of unpaired data were compared the Kruskal Wallace analysis of variance (ANOVA) was used for multiple comparisons. Significance was accepted when $P < 0.05$.

RESULTS

Preliminary experiments revealed that the phosphorylation of histone IIa and that of Kemptide by A-kinase under the conditions described in Materials and Methods was linear with respect to time (up to 10 min) and protein concentration (histone IIa: up to 40 μg; Kemptide: up to 10 μg). No measurable endogenous protein phosphorylation was detected in the absence of exogenous substrate.

Kinetics and specificity of A-kinase-induced protein phosphorylation

Table 1 shows calculated V_{max} values of A-kinase-induced phosphorylation of histone IIa, histone H2b and Kemptide using the soluble enzyme isolated from guinea-pig lung and from bovine tracheal smooth muscle. The rate of exogenous protein phosphorylation catalysed by A-kinase in the presence of a saturating concentration of cyclic AMP was significantly higher for both the lung (*ca.* 16-fold) and tracheal smooth muscle (*ca.* 12-fold) enzyme when Kemptide was used as phosphate acceptor compared to either histone IIa or histone H2b.

It is well documented that in many tissues a large proportion of basal histone IIa phosphorylation and that induced by exogenous cyclic AMP is not catalysed by A-kinase [5, 11, 20]. This has given rise to the consensus that histones can act as substrates for many different protein kinases [5, 11, 20]. Having established that cyclic AMP-stimulated phospho-Kemptide formation occurred at a rate significantly greater than the phosphorylation of histones it was necessary to determine how selective this former phosphorylation was (i.e. is phosphoKemptide formation attributable solely to the activity of A-kinase?). To answer this question the elegant method

Table 1. Comparison of cyclic AMP-stimulated, PKI/IP_{20}-sensitive phosphorylation of histone IIa, histone H2b and Kemptide by soluble A-kinase isolated from guinea-pig lung and from bovine tracheal smooth muscle

Tissue	Substrate	V_{max} (pmol/min/mg protein)	
Guinea-pig lung	Histone IIa	523 ± 27	(13)
Guinea-pig lung	Histone H2b	663 ± 37	(6)
Guinea-pig lung	Kemptide	8271 ± 237*	(19)
Bovine trachealis	Histone IIa	571 ± 62	(6)
Bovine trachealis	Kemptide	7254 ± 474**	(16)

Soluble A-kinase was prepared from guinea-pig lung parenchyma and from bovine tracheal smooth muscle as described in Materials and Methods in the absence of NaCl. Phosphotransferase activity that was stimulated by 2 μM cyclic AMP and inhibited by 20 μl PKI (lung) or 10 μM IP_{20} (trachealis) was assumed to represent A-kinase activity. Values in parentheses represent the number of independent determinations.

* and ** $P < 0.001$: significantly greater specific activity than that achieved with histone IIa. Data were analysed by Kruskal Wallace ANOVA with multiple comparisons (*) and Mann–Whitney U-test (**), respectively.

described by Torphy *et al.* [11] was used which makes use of the now well characterized specific eicosapeptide inhibitor (IP_{20}) of A-kinase [19].

Figure 1 illustrates the effect of IP_{20} on the phosphorylation of histone IIa and of Kemptide catalysed by bovine tracheal smooth muscle A-kinase in the absence and presence of 2 μM cyclic AMP. Consistent with previous investigations using post-DEAE PKI extracted from rabbit skeletal muscle [5, 11, 20], IP_{20} inhibited, in a concentration-dependent manner, the ability of the tracheal smooth muscle enzyme to phosphorylate histone IIa (Fig. 1a) with an IC_{50} of 73.4 ± 8.3 nM (N = 4) when the assay was performed in the presence of cyclic AMP (2 μM). Significantly, however, neither the basal (A + D) nor cyclic AMP-stimulated (B + C + D) phosphorylation of this substrate was abolished by IP_{20} (Fig. 1b) suggesting that phosphotransferase activities other than A-kinase were also being expressed (C and D). In the presence of a maximally effective concentration of IP_{20} (10 μM), the basal and cyclic AMP (2 μM)-stimulated phosphorylation of histone IIa was inhibited by only 37% (A) and 89% (B), respectively (Fig. 1b). It became apparent that failure to subtract these IP_{20}-resistant activities away from the total measured phosphorylation significantly over-estimated the A-kinase activity ratio. This problem is illustrated in Fig. 1b where the crude −cyclic AMP/+cyclic AMP ratio (0.166 ± 0.03, N = 4), given by (A + D)/(B + C + D), was significantly higher (*ca.* 2-fold) than the 'true' A-kinase activity ratio (0.082 ± 0.01, N = 4) calculated after subtraction of the respective IP_{20}-resistant activities away from the total activities (A/B).

These results with the trachealis were qualitatively identical to those obtained with PKI on soluble A-kinase isolated from guinea-pig lung where basal and cyclic AMP-stimulated histone phosphorylation were inhibited by 60 and 92%, respectively (IC_{50} for the cyclic AMP-stimulated activity: 76.3 ± 12.3 nM, N = 4). The basal A-kinase activity ratio in the lung determined in the absence of NaCl was reduced from 0.12 ± 0.01 (N = 4) to 0.063 ± 0.01 (N = 4) when the PKI-resistant activities were taken into consideration.

When identical experiments were performed using Kemptide as the substrate IP_{20} abolished basal and cyclic AMP-stimulated phosphotransferase activity. As with the histone IIa experiments IP_{20} inhibited the phosphorylation of Kemptide in a concentration-dependent manner and was equally effective with both the trachealis- (Fig. 1c) and lung-derived enzyme (data not shown). The respective IC_{50} values of IP_{20} for soluble A-kinase isolated from these tissues were 32.7 ± 6.5 nM (N = 4) and 41.1 ± 7.7 nM (N = 4). Interestingly, these values were significantly ($P < 0.05$) lower than the corresponding inhibitory constants obtained when histone IIa was utilized as substrate (see above). It is likely that this disparity is due to the higher (16-fold) concentration of A-kinase used in the experiments using histones since the efficacy of IP_{20} is reduced as the concentration of A-kinase is increased [25]. The basal A-kinase activity ratio in bovine tracheal smooth muscle and in guinea-pig lung calculated using Kemptide as substrate (A/B in Fig. 1d) was 0.092 ± 0.02 (N = 4) and 0.059 ± 0.02 (N = 4), respectively, essentially the same as the IP_{20}-sensitive ratios calculated from the histone IIa data in Fig. 1b (see above).

It is pertinent to point out that the above ratios do not represent the actual basal activation state of A-kinase thought to be found *in vivo*. This is because the majority of A-kinase (>75%) in guinea-pig lung and in bovine tracheal smooth muscle is present as the Type II isoenzyme (see below). As such, conditions of high ionic strength are necessary to maintain A-kinase in an active state. This in itself poses no problem. However, as described in some detail below, histone phosphorylation is markedly inhibited by salt, negating any accurate comparison with Kemptide which is not subject to inhibition by conditions of high ionic strength. The above data, therefore, serve only to illustrate the limitations of using histone IIa as a substrate in the assay of A-kinase. More representative activity ratios determined using Kemptide as substrate are given in Table 2.

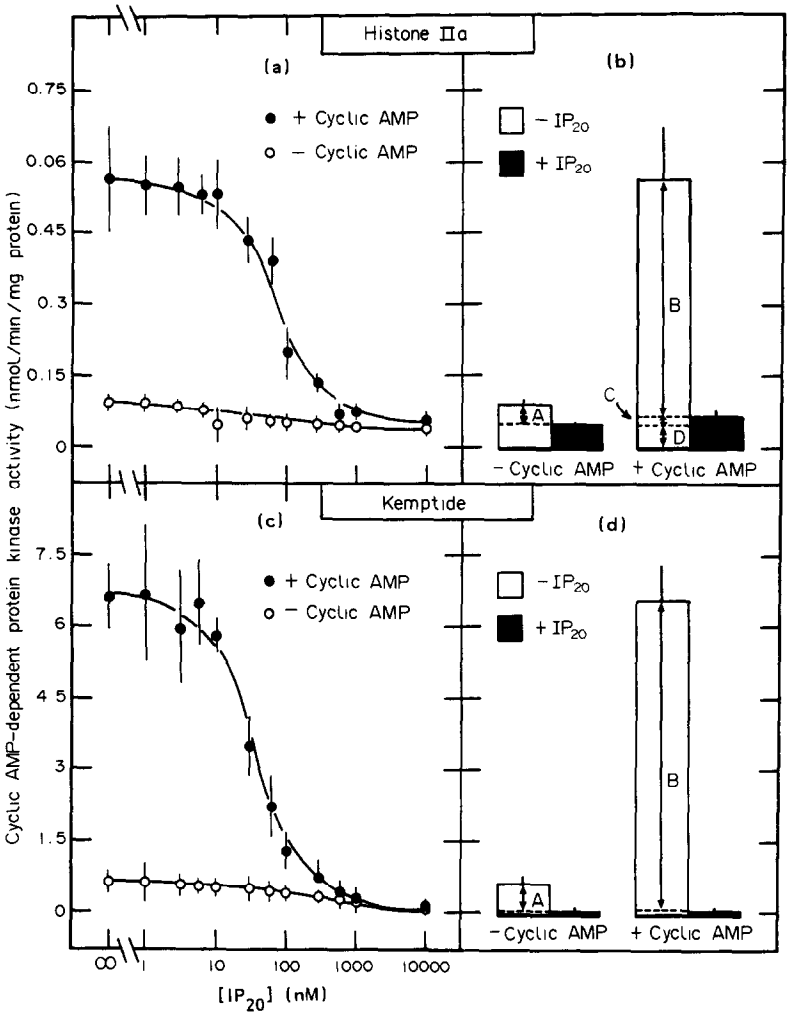

Fig. 1. Effect of IP_{20} on basal and on the cyclic AMP-stimulated phosphorylation of histone IIa and of Kemptide using soluble A-kinase isolated from bovine cervical trachealis. A-Kinase induced phosphorylation of histone IIa (a) and of Kemptide (c) was determined with increasing concentrations of IP_{20} in the absence and presence of cyclic AMP (2 μM) as described in Materials and Methods. In (b) and (d) basal and cyclic AMP-stimulated enzyme activity were determined in the absence (open bars) and presence (filled bars) of a maximally effective concentration (10 μM) of IP_{20} using histone IIa and Kemptide as substrate, respectively. Basal A-kinase, total A-kinase, G-kinase and cyclic nucleotide-independent protein kinase activities are given by A, B, C and D, respectively (see text and Ref. 11 for further details). Since elevated ionic strength inhibited the phosphorylation of histone IIa (see text and Fig. 3 for details) NaCl was omitted from the homogenization buffer in these experiments. It is important to note that the activity ratio determined using histone IIa as substrate ([A + D]/[B + C + D] in panel b) was an over-estimation of the true activation state seen when Kemptide was employed ([A/B] in panel d) due to the expression of protein kinase activities other than A-kinase. Both substrates, however, gave comparable results if IP_{20}-resistant phosphorylation (basal: D, cyclic AMP-stimulated: C + D) was subtracted away from the total measured activities, i.e. (A/B) in panel b = (A/B) in panel d. Values represent the mean ± SE of four independent observations.

Effect of the exogenous phosphate acceptor in the determination of the activation constant (K_{act}) *for cyclic AMP*

The data shown in Fig. 2 illustrate the effect of exogenous cyclic AMP on PKI-sensitive phosphotransferase activity present in the soluble fraction of guinea-pig lung homogenates using histone IIa and Kemptide as substrate. With the former phosphate acceptor the K_{act} of cyclic AMP for A-kinase was *ca.* 60 nM. This value is in good agreement with K_{act} obtained for cyclic AMP with histone IIa in other tissues [5, 9, 11, 20]. In contrast, the K_{act} for cyclic AMP-stimulated Kemptide phosphorylation determined using the same enzyme preparation at the same protein concentration and under identical conditions was *ca.* 1.5 μM.

Effect of ionic strength on cyclic AMP-stimulated, PKI/IP_{20}-sensitive phosphohistone and phosphokemptide formation

There are a number of reports in the literature demonstrating that the phosphorylation of histones by A-kinase is inhibited by salt in a concentration-dependent manner [5–8, 25–28]. Although it is generally believed that salt directly affects the ability

Table 2. Effect of the phosphate acceptor on the estimation of the basal and isoprenaline-stimulated A-kinase activity ratio

	Activity ratio† × 100 Histone IIa	Kemptide
Guinea-pig lung		
Basal	39.8 ± 1.1* (19)	26.4 ± 1.3 (19)
Isoprenaline	64.3 ± 1.4* (19)	47.5 ± 0.9 (19)
Bovine trachealis		
Basal	46.3 ± 2.1* (12)	29.8 ± 0.8 (12)
Isoprenaline	60.3 ± 1.9* (12)	50.5 ± 1.4 (12)

Soluble A-kinase from control and from isoprenaline (10 μM)-treated guinea-pig lung and bovine tracheal smooth muscle was isolated in buffer A containing 500 mM NaCl. Activity ratios were then determined as described in Materials and Methods after accounting for PKI-resistant activity. Values in parentheses refer to the number of independent determinations.

* $P < 0.05$: significantly greater ratio than that obtained with Kemptide. Data were analysed by Wilcoxon matched pairs signed rank tests.

† (A/B) in Fig. 1b and d.

using soluble A-kinase isolated from guinea-pig lung. As shown in Fig. 3b the specific activity of PKI-sensitive histone IIa phosphorylation observed in the absence of exogenous cyclic AMP was higher when lung tissue was homogenized in buffer A supplemented with 2 M NaCl (actual NaCl concentration in assay cocktail: 500 mM). These data contrasted dramatically with A-kinase activity assayed in the presence of a maximally effective concentration of cyclic AMP (10 μM), where phosphohistone IIa formation was progressively inhibited (IC_{50} = 16.1 ± 7.4 mM; N = 7) as the ionic strength of the homogenization buffer was increased (Fig. 3b). As a direct consequence of these two opposing effects of salt on A-kinase activity there was an apparent, but artifactual, increase in the basal activity ratio from *ca.* 10% at NaCl concentrations up to 10 mM to a value of >60% at 500 mM NaCl (Fig. 3a). The ability of NaCl to inhibit the phosphorylation of histone IIa was also observed when NaCl was added directly to the assay cocktail (data not shown).

The nature of the salt-induced inhibition of histone IIa phosphorylation was investigated using commercial *bona fide* Type I and Type II A-kinase purified, through the DEAE-cellulose step, from rabbit

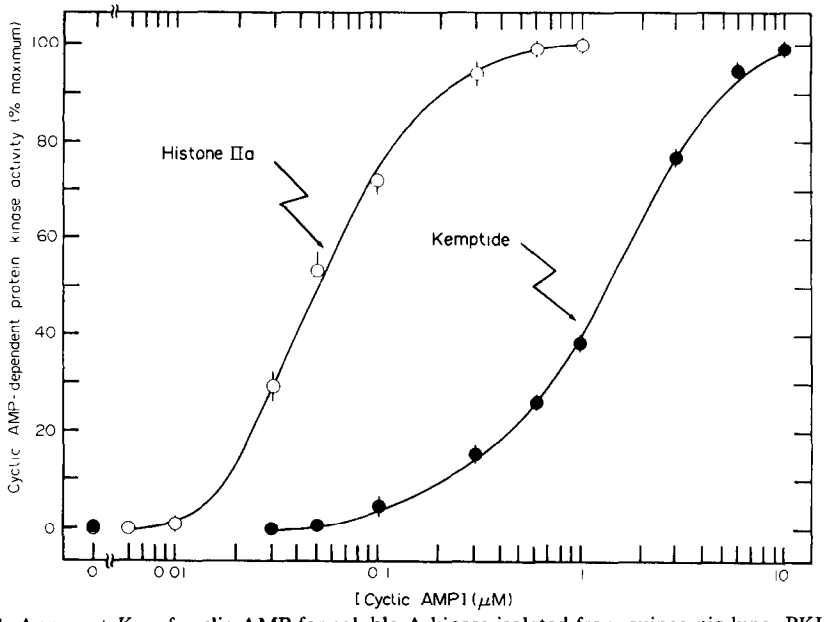

Fig. 2. Apparent K_{act} of cyclic AMP for soluble A-kinase isolated from guinea-pig lung. PKI-sensitive A-kinase activity was determined in the presence of increasing concentrations of cyclic AMP using histone IIa (○) and Kemptide (●) as substrate as described in Materials and Methods. In these experiments NaCl was omitted from the homogenization buffer. One hundred per cent activity represents 0.53 ± 0.03 and 7.12 ± 0.38 nmol phosphate transferred/min/mg soluble protein for histone IIa and Kemptide, respectively. Values denoted by the symbols refer to the mean ± SE of four independent observations.

of the substrate to be phosphorylated [5–8, 25–28], convincing evidence is also available to suggest that A-kinase, itself, undergoes a significant conformational change at physiological ionic strength and pH [29] resulting in enzyme inactivation.

In view of these latter observations the effect of the ionic strength of the homogenization buffer on basal and cyclic AMP-stimulated phosphohistone IIa and phosphokemptide formation and on the resultant resting A-kinase activity ratio were compared skeletal muscle. Figure 4a shows that increasing the ionic strength of the assay cocktail with NaCl (2–500 mM) produced a concentration-dependent inhibition of cyclic AMP (10 μM)-stimulated phosphohistone IIa formation with IC_{50} values of *ca.* 80 mM for both isoenzymes. To rule out the possibility that this inhibition was due to competition between NaCl and phosphorylated histone for binding sites on the ion exchange paper, parallel experiments were performed whereby the NaCl was added

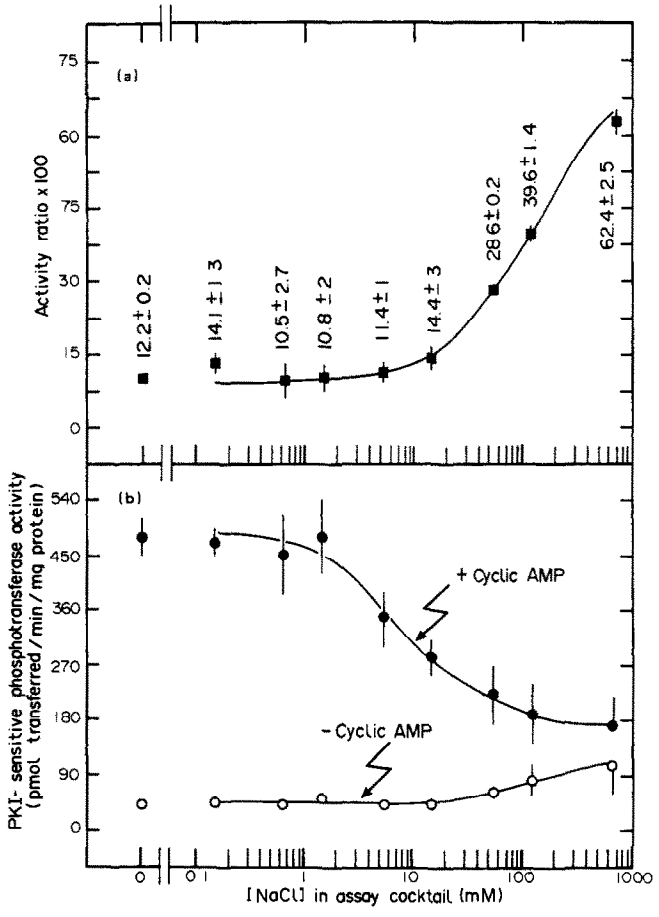

Fig. 3. Effect of NaCl on A-kinase activity and on the A-kinase activity ratio in guinea-pig lung using histone IIa as substrate. Guinea-pig lung was homogenized in buffer A supplemented with NaCl (0–2 M). PKI-sensitive A-kinase activity (b) was then estimated in the absence (○) and presence (●) of cyclic AMP (10 μM) using histone IIa as the substrate as described in Materials and Methods. The A-kinase activity ratios calculated at each ionic strength are shown in (a). Values denoted by the symbols refer to the mean ± SE of seven independent observations.

to each incubate either before initiation of the reaction or at its termination (i.e. immediately before spotting the 70 μl aliquots of the reaction medium onto the P81 ion exchange paper). Reference to Fig. 4a clearly shows that the observed inhibition of histone phosphorylation was due to a direct effect of salt on A-kinase and/or histone. Kinetic analysis of this phenomenon showed the salt-induced inhibition to be reversible (see below) and of the mixed uncompetitive type (Fig. 4b).

To determine if the observed inhibitory effect of salt on histone phosphorylation was a general phenomenon, the same series of experiments were performed employing Kemptide as substrate (Fig. 5). Like that observed with histone IIa, the basal rate of Kemptide phosphorylation increased as a function of the ionic strength of the homogenization buffer. This effect, however, was significantly greater when Kemptide was used. Thus, the rate of Kemptide phosphorylation catalysed by A-kinase isolated in buffer A containing 2 M NaCl was *ca.* 4-fold greater than that measured in buffer A alone, whilst the rate of histone IIa phosphorylation, under identical conditions, was only doubled. The reason for this difference was attributed to the fact that, although not apparent in Fig. 3b, the basal IP_{20}-sensitive phosphorylation of histone IIa was also inhibited by high ionic strength. In stark contrast to the histone experiments, Kemptide phosphorylation measured in the presence of cyclic AMP (2 μM) was unaffected by NaCl at any concentration examined. As a consequence, the A-kinase activity ratio increased from 8% at concentrations of NaCl up to 10 mM to only *ca.* 27% at 500 mM NaCl (Fig. 5a, c.f. Fig. 3a).

Taken together, the above results have significant implications for the estimation of the A-kinase activation state in tissues where the Type II isoenzyme is present in a high concentration (e.g. lung parenchyma, airway smooth muscle [5, 11–14]), for accurate estimates of the Type II A-kinase activity ratio can be made only if this enzyme is isolated under conditions of high ionic strength [1, 3]. This problem is amply illustrated by the data in Table 2 which shows that following homogenization of tissue from both species in buffer A supplemented with 500 mM NaCl (a salt concentration known to effectively prevent the reassociation of the regulatory and catalytic

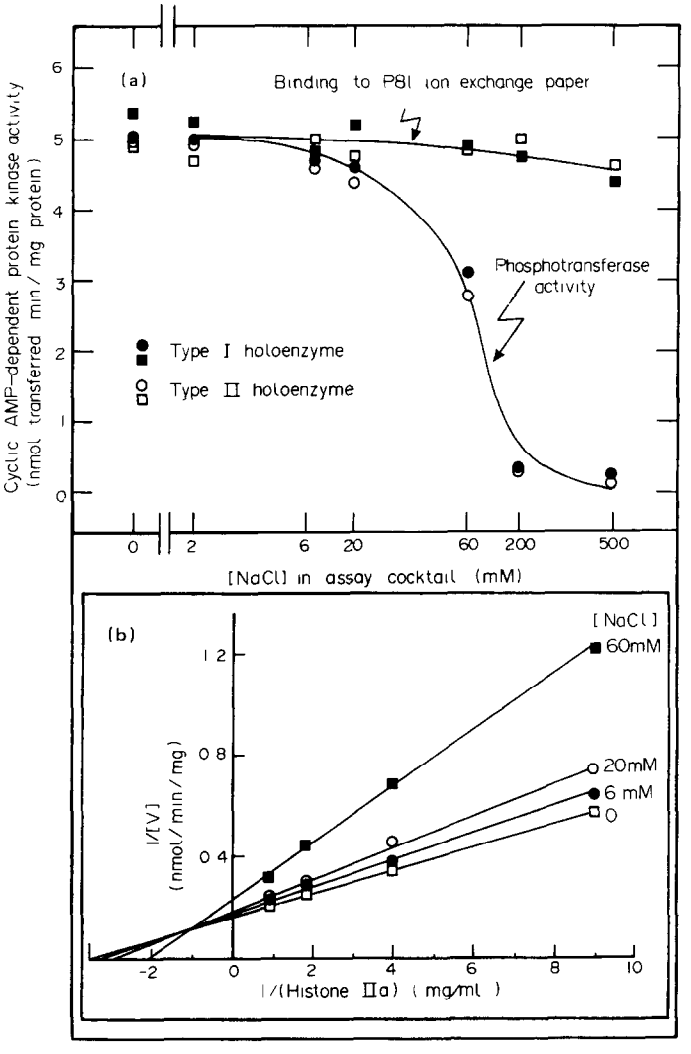

Fig. 4. Kinetic analysis of the effect of NaCl on the phosphorylation of histone IIa. The data in panel (a) show the effect of NaCl on the ability of *bona fide* Type I, (●, ■) and Type II (○, □) A-kinase isolated from rabbit skeletal muscle to phosphorylate histone IIa and to compete with phosphohistone for binding sites on the ion exchange paper. In (b) the data for inhibition of histone phosphorylation are expressed in the form of a Lineweaver–Burk plot. Values denoted by the symbols refer to the mean of two independent observations.

subunits of the Type II holoenzyme [30]) both the basal and isoprenaline (10 μM)-stimulated activity ratio of soluble A-kinase were significantly higher when histone IIa was used as substrate than when Kemptide was employed. This increase in the A-kinase activity ratio induced by salt was in addition to that calculated if the PKI-resistant protein kinase activity was ignored (Fig. 1b,d).

Suitability of histone IIa as a substrate for the quantification of the A-kinase isoenzyme ratio

The A-kinase isoenzymes present in the soluble fraction of guinea-pig lung and of bovine tracheal smooth muscle were separated by anion-exchange chromatography over DEAE-cellulose and the relative proportions of each species were determined using Kemptide as the substrate. Cyclic AMP-stimulated, IP_{20}/PKI-sensitive phosphotransferase activity was resolved into two main peaks eluting at 45 mM (Type I) and 195 mM NaCl (Type II) and 85 mM (Type I) and 210 mM NaCl (Type II) for the lung- and trachealis-derived isoenzymes, respectively. In four separate experiments the Type I:Type II isoenzyme ratio was 24.9 ± 2.6%:74.1 ± 3.2% (trachealis) and 4.6 ± 0.2%:95.4 ± 4.5% (lung).

Given the inhibition that salt exerted on the phosphorylation of histone IIa we decided to determine if the use of this substrate significantly affected the estimation of the Type I:Type II isoenzyme ratio in respiratory tissues when anion exchange chromatography was employed as the method of isoenzyme separation. It was reasoned that some distortion of the isoenzyme ratio would occur since the linearly increasing NaCl gradient would be expected to produce a differentially greater inhibition of the Type II A-kinase activity (eluting at high ionic strength) when compared with the phosphorylation catalysed by the Type I isoenzyme (eluting at low ionic

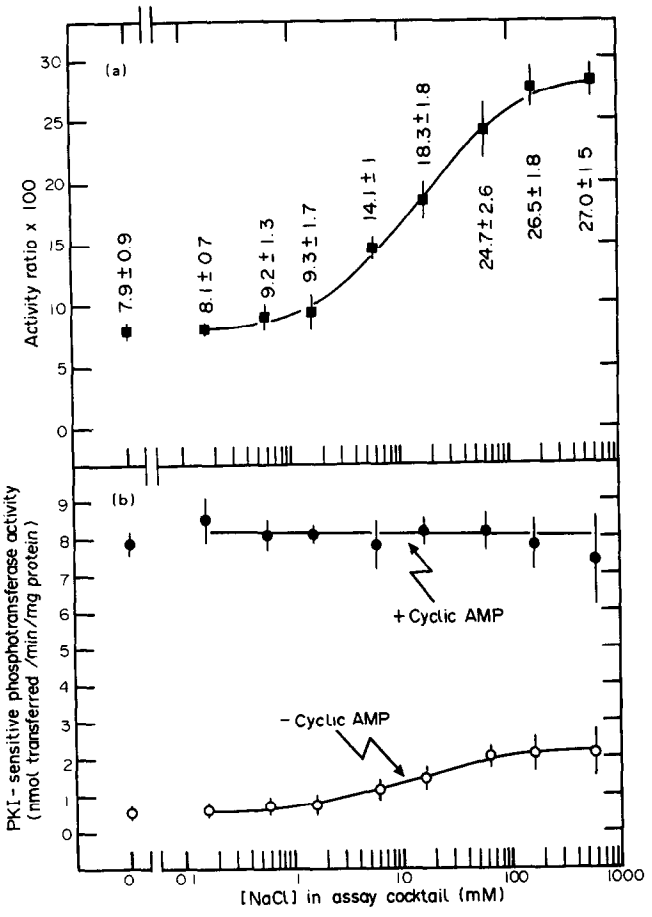

Fig. 5. Effect of NaCl on A-kinase activity and on the A-kinase activity ratio in guinea-pig lung using Kemptide as substrate. Experiments were performed exactly as described in the legend to Fig. 3 using Kemptide as the substrate. Values denoted by the symbols refer to the mean ± SE of six independent observations.

strength). Reference to Fig. 6 illustrates that this supposition is indeed, correct. Following dialysis of the peak fractions containing A-kinase to remove solute, the specific activities of the Type I and Type II isoenzymes were increased by *ca.* 9 and 36%, respectively, when compared with the activities of aliquots of the same isoenzyme fractions that were not subject to dialysis. The effect of NaCl on histone IIa phosphorylation was a reversible phenomenon for readdition of NaCl to the post-dialysed isoenzyme fractions once more resulted in enzyme inhibition (data not shown).

Having established that NaCl exerted a greater inhibition of Type II A-kinase activity than of its sister isoenzyme, the potential effect of NaCl on the A-kinase isoenzyme elution profile was determined. Table 3 shows the soluble isoenzyme ratios of cyclic AMP-stimulated, PKI/IP_{20}-sensitive A-kinase activity isolated from guinea-pig lung and from bovine tracheal smooth muscle following fractionation of soluble extracts of these tissues over DEAE-cellulose. Using histone IIa as substrate the Type I:Type II isoenzyme ratio was 9:91 and 35:65 for the lung and tracheal A-kinase, respectively, significantly higher than the ratios obtained when Kemptide was used to assess phosphotransferase activity (see above). Although this difference in the isoenzyme ratio was small it was, nevertheless, significant and readily reproducible.

In a complementary series of experiments it was found that the curve depicted in Fig. 4a could be used effectively as a standard to correct the elution profile of A-kinase isoenzymes if histone IIa was employed to measure catalytic activity. When phosphotransferase activity of the Type I and Type II holoenzymes shown in Fig. 4a was plotted as a linear function of the NaCl concentration the observed inhibition of histone IIa phosphorylation was adequately described by a single exponential function of the form:

$$y = Ae^{(-Bt)}$$

where y = response variable (sp. act. of A-kinase), t = NaCl concentration (mM), A = sp. act. of A-kinase when $t = 0$ and B = first order coefficient for A-kinase inhibition. The concentration of NaCl required to inhibit histone phosphorylation by 50% (IC_{50}) was calculated from the expression $IC_{50} = 0.693/t$. The equation describing the linearized data in Fig. 4a was $y = 5.14e^{(-0.009t)}$ ($r^2 = 0.998$) from which the IC_{50} for NaCl at inhibiting histone IIa phosphorylation was calculated to be 77.3 mM.

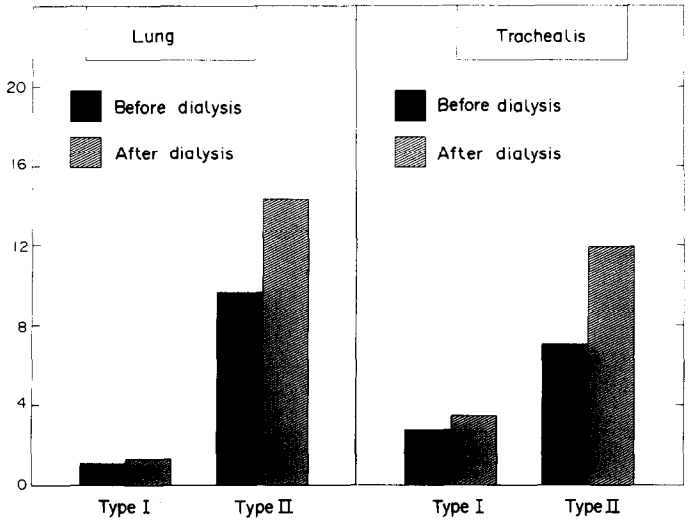

Fig. 6. Effect of dialysis on the specific activities of Type I and of Type II A-kinase isolated, by DEAE-cellulose chromatography, from guinea-pig lung and from bovine tracheal smooth muscle. The Type I and Type II isoenzymes of A-kinase from guinea-pig lung and from bovine tracheal smooth muscle were separated by DEAE-cellulose chromatography. The peak fractions corresponding to each isoenzyme were pooled and an aliquot was subjected to dialysis in Spectrapor 3 dialysis tubing (MWCO 3500) for 10 hr in buffer B to remove solute. The A-kinase activity of the non-diffusable material was then determined as described in Materials and Methods and compared to the activity of the same pooled isoenzyme fractions that were not subjected to dialysis. Values represented by the histograms refer to the mean of two independent observations.

Table 3. Ratio of soluble A-kinase isoenzymes in respiratory and other tissues before and after correction for the inhibitory effect of NaCl on histone phosphorylation

	Isoenzyme ratio		Corrected ratio		
Tissue	Type I	Type II	Type I	Type II	Reference
Guinea-pig lung	9	91	5	95	This study
Bovine trachealis	35	65	29	71	This study
Canine trachealis	54	48	47	53	[11]
Murine lung*	10	90	8	92	[13]
Human lung*	30	70	24	76	[12]
Bovine coronary artery	46	51	39	58	[31]
Opossum lower oesophageal sphincter	10	90	4	96	[20]

The curve in Fig. 4a was used as a standard to correct the soluble A-kinase isoenzyme elution profile and, therefore, isoenzyme ratio in guinea-pig lung and bovine tracheal smooth muscle when histone IIa was used as substrate. Also shown are corrected isoenzyme ratios reported by other investigators in a number of tissues. In these latter cases the ionic strength† of the homogenization buffer and assay cocktail was estimated for each experiment and were found to be approximately 25 and 70 mM, respectively. Since there was little variability in the starting ionic strength correction was made only on the basis of (1) the NaCl concentration at which peak isoenzyme activities were eluted and (2) the volume of each fraction assayed with respect to the total incubation volume (i.e. the effective dilution of the NaCl concentration).

* Ratio estimated from elution profile.

† The ionic strength (I) of the homogenization buffers and assay cocktails was determined from the standard equation, $I = \frac{1}{2} \Sigma c_i . z_i^2$ where c_i is the ion concentration in molarity and z_i is the valency of the contributing ionic species.

The data in Table 3 illustrate the use of this correction for soluble A-kinase isolated from guinea-pig lung and from bovine tracheal smooth muscle. It can be seen that after adjustment for the inhibitory effect induced by salt the isoenzyme ratios determined using histone IIa were the same as those obtained with Kemptide (see above). Also shown in Table 3 are the corrected ratios in a number of other tissues where histones were used to detect phosphotransferase activity. It is clear from these data that despite the marked inhibitory effect that NaCl exerted on A-kinase isoenzyme activity (see Fig. 6) this effect was reflected by a much smaller increase in the percentage of the Type II isoenzyme

in the sample. Indeed, from our own data and that selected from the literature the use of histone IIa underestimates the amount of Type II isoenzyme by, on average, 6% irrespective of the fact that the catalytic activity of this isoenzyme species is inhibited by over 36%.

DISCUSSION

The results of these studies clearly demonstrate that Kemptide appears to be a suitable phosphate acceptor for the measurement of soluble A-kinase activity in bovine tracheal smooth muscle and in guinea-pig lung parenchyma. Kemptide demonstrated none of the limitations that were apparent with the use of histones and, indeed, offered a number of significant advantages over this more traditional substrate. First, it was a far better phosphate acceptor than was histone IIa, a property that markedly improved the sensitivity of the assay. Use of Kemptide, therefore, has the obvious benefit of enabling the measurement of A-kinase in small or limited quantities of tissue. Second, Kemptide was phosphorylated specifically by soluble A-kinase as indicated by the ability of PKI and IP_{20} to abolish basal and cyclic AMP-stimulated phosphoKemptide formation. These latter observations contrast strongly with the phosphorylation of histones where appreciable PKI/IP_{20}-resistant activities were expressed both in the absence and presence of exogenous cyclic AMP. That the A-kinase activity ratio calculated using Kemptide as substrate was essentially the same as the IP_{20}/PKI-sensitive ratio obtained with histone IIa suggests that these ratios reflect the true activation state of A-kinase. Use of Kemptide, therefore, also simplifies the measurement of A-kinase activity since PKI/IP_{20}-resistant activities do not need to be subtracted away from the total activity [11]. Third, cyclic AMP-stimulated Kemptide phosphorylation was not measurably affected by physiological concentrations of salt corroborating the concept that high salt primarily acts to prevent the ability of histones to be phosphorylated [5–8, 26–28]. Thus elevated ionic strength appears to be more effective at altering the properties of histone IIa, such that it is rendered a poorer substrate for A-kinase, than at inhibiting A-kinase *per se* (at least the soluble enzyme present in guinea-pig lung and in bovine tracheal smooth muscle). This conclusion contrasts with results obtained by Moll and Kaiser [6] who in a detailed study provided evidence that high salt exerts a direct inhibitory effect on the enzyme itself. Indeed, these investigators calculated that the inhibition of cyclic AMP-stimulated histone IIa phosphorylation by homogeneous Type I A-kinase purified from rabbit skeletal muscle was directly proportional to the square root of the ionic strength of the assay medium [29]. The reason for this disparity is not clear. Finally, and in stark contrast to histones, Kemptide does not appear to directly activate A-kinase.

It is important to note that the inability of PKI/IP_{20} to completely prevent histone IIa phosphorylation is not peculiar to respiratory tissues. Indeed, similar PKI-resistant activities have been reported in many other tissues including the smooth muscle of the canine trachea [11], opossum lower oesophageal sphincter [20] and bovine coronary artery [31]. Studies with soluble A-kinase isolated from canine trachealis lead Torphy *et al.* [11] to suggest that these IP_{20}-resistant activities represent cyclic GMP-dependent and cyclic nucleotide-independent protein kinases respectively (C and D in Fig. 1b). Caution should, therefore, be taken in the estimation of A-kinase activity ratios especially if histones are to be used as substrate. Ideally, use of PKI or IP_{20} should be routine in preliminary experiments to ascertain what proportion of the net measured phosphotransferase activity is attributable to A-kinase [5, 11, 20]. Such pilot experiments should be performed irrespective of the phosphate acceptor.

Perhaps the most significant disadvantage of mixed histones identified in this study, that has not yet been reported, was that their use resulted in a marked overestimation of the enzymes' activation state under both basal and drug-stimulated conditions. The main reasons for these aberrant results were undoubtedly attributable, in most part, to the concentration-dependent inhibition that salt exerted on the cyclic AMP-stimulated phosphorylation of histone IIa coupled with the intrinsic ability of histone IIa, itself, to directly dissociate, and thereby activate, A-kinase [9]. This latter property was amply illustrated with the observation that the K_{act} of cyclic AMP for soluble A-kinase isolated from guinea-pig lung parenchyma was 25-fold lower when histone IIa was used as phosphate acceptor than when Kemptide was employed under identical conditions.

The inhibitory effect that NaCl exerted on histone IIa phosphorylation also resulted in a marked underestimation of phosphorylating capacity of the Type I and, especially, the Type II isoenzyme in the tissue extract. This gave rise to a small, but nevertheless significant, underestimation of the A-kinase isoenzyme ratio in both bovine tracheal smooth muscle and in guinea-pig lung when the isoenzymes were separated by anion-exchange chromatography. This effect was attributed to the linearly increasing NaCl gradient (necessary to fractionate adsorbed proteins from anion exchange columns) which presumably exerted a progressive inhibition of A-kinase isoenzyme activity when histones are used to assess phosphotransferase activity. Although this differential inhibition was easily overcome by using the curve depicted in Fig. 4a to correct the elution profile, it was tedious and, moreover, unnecessary, since with Kemptide as substrate A-kinase activity was not inhibited by concentrations of NaCl up to 500 mM.

It was a consistent finding that the specific activity of PKI-sensitive histone IIa phosphorylation observed in the absence of exogenous cyclic AMP was significantly higher when lung tissue was homogenized in buffer A supplemented with 2 M NaCl (see Fig. 3b). Although attempts were not made to establish the exact cause of this phenomenon it is likely, based on other studies, that the higher enzyme activity expressed at elevated ionic strength is due to the fact that (1) the basal activation state of Type II A-kinase (the predominant isoenzyme in guinea-pig lung) is maintained only under conditions of elevated ionic strength [3, 30] and (2) high salt prevents the

ionic interaction that occurs between free (active) catalytic subunits and anionic sites on the particulate material demonstrable at low ionic strength [30]. Indeed, these conclusions are supported by the observation that the apparent stimulatory effect of NaCl on basal A-kinase activity, although relatively modest, was concentration-dependent.

One curious observation that became apparent from the studies investigating the inhibitory effect of NaCl on histone phosphorylation was that the IC_{50} values of NaCl differed depending upon the purity of the A-kinase preparation. Thus, with the crude enzyme the IC_{50} for NaCl was *ca.* 16 mM (Fig. 3b) compared with the IC_{50} value of *ca.* 80 mM (Fig. 4a) with the post-DEAE-cellulose-fractionated enzyme. At present we can offer no explanation as to why the crude enzyme displays this partial (~30%) resistance to inhibition by NaCl.

In conclusion, the intrinsic ability of histone IIa to directly activate A-kinase, coupled with the appetency of elevated ionic strength to inhibit phosphohistone formation renders this substrate generally unsuitable for measuring A-kinase activity. Kemptide, however, demonstrated none of the limitations apparent with histone IIa and was, thus, considered to be a preferable substrate for the estimation of A-kinase activity in guinea-pig lung and in bovine tracheal smooth muscle. It is important to emphasize that the use of Kemptide (or other substrates that are not adversely affected at physiological ionic strength) is essential if A-kinase activity is to be estimated in respiratory and other tissues which contain appreciable amounts of the Type II isoenzyme. Although this paper has focussed exclusively on A-kinase, it is critically important to appreciate that histones are used as substrates in many protein kinase assays. It is likely, therefore, that since high salt primarily modifies the structure of the histone molecule (see above), the specific activity of all serine-selective protein kinases will appear to be increasingly attenuated as the ionic strength of the assay medium is elevated. The possibility also exists that high salt may similarly inhibit the ability of histones to be phosphorylated by kinases that act at threonine and tyrosine residues. Collectively, therefore, we believe that the use of histones is appropriate only for protein kinases whose activities can be effectively and accurately determined under conditions of low ionic strength.

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