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Use of a synthetic dodecapeptide (malantide) to measure the cyclic AMP-dependent protein kinase activity ratio in a variety of tissues

Kenneth J. MURRAY,* Paul J. ENGLAND,* James A. LYNHAM,* David MILLS,* Carsten SCHMITZ-PEIFFER† and Martin L. REEVES*

*Department of Cellular Pharmacology, Smith Kline and French Research Limited, The Frythe, Welwyn, Herts. AL6 9AR, and †Department of Biochemistry, University of Bristol, University Walk, Bristol BS8 1TD, U.K.

1. The cyclic AMP-dependent protein kinase activity-ratio assay was investigated by comparing histone and a synthetic peptide, malantide [Malencik & Anderson (1983) Anal. Biochem. **132**, 32–40], as substrates. 2. In several tissues the activity ratio was higher when assayed with histone as the substrate; this result was obtained in control tissues and also in those incubated with agents known to increase cyclic AMP. The effect of these agents to increase the activity ratio was more clearly demonstrated with malantide. 3. The higher activity ratios observed with histone are due to: (*a*) measurement of phosphorylation not catalysed by cyclic AMP-dependent protein kinase; (*b*) activation of cyclic AMP-dependent protein kinase by histone during the assay. 4. When tissues were homogenized in buffers without NaCl, lower activity ratios were found, owing to the catalytic subunit being artifactually removed from the supernatant. 5. We conclude that the measured activity ratio more faithfully reflects that in the tissue when NaCl is included in the homogenization buffer and malantide is used in the assay. This was confirmed in experiments where cyclic AMP-dependent protein kinase was added to the tissue before homogenization, and no dissociation of the exogenous enzyme was observed.

INTRODUCTION

A large number of hormones exert their physiological response by increasing the intracellular levels of cyclic AMP in their target tissues. In the absence of any contradictory evidence, it is generally assumed that the vast majority of effects of cyclic AMP are mediated through activation of cyclic AMP-dependent protein kinase (cAMP-PrK) and subsequent increases in protein phosphorylation. As there is not always an exact correlation between changes in cyclic AMP levels and the measured response, it is useful to determine the degree of activation of the cAMP-PrK. This is achieved by use of the cAMP-PrK activity-ratio assay, first introduced by Corbin *et al.* (1973).

This assay measures the relative proportions of R_2C_2 (R, regulatory subunit; C, catalytic subunit) and C in the tissue, according to the following equation (Corbin *et al.*, 1978):

$$R_2C_2 \text{ (inactive)} + 4 \text{ cyclic AMP} \rightleftharpoons R_2(\text{cyclic AMP})_4 + 2C \text{ (active)}$$

A tissue homogenate is assayed in the absence of added cyclic AMP to measure the activity of free catalytic subunit (C), and in the presence of added cyclic AMP to measure the total kinase activity (R_2C_2+C). This is then expressed as an 'activity ratio' or 'fractional activity' (C/R_2C_2+C).

However, for this activity ratio to be representative of the activity shown in the tissue it is necessary that there is no association or dissociation of the holoenzyme (R_2C_2) during the homogenization and assay of the tissue. In particular, Palmer *et al.* (1980) have reported that, under the typical conditions of the assay, the dissociation of cAMP-PrK is not blocked. Further pitfalls of the assay are discussed by Corbin (1983), who suggested these may lead to the activity ratio assay being 'semiquantitative'. In both of the above reports histone was used as a substrate for cAMP-PrK in the activity ratio assay. More recently, peptide substrates for cAMP-PrK have become available and, indeed, have been used by a number of investigators in the activity-ratio assay (e.g. Seiler *et al.*, 1987; Smith & Manganiello, 1989), although the effects of changing substrate on the assay have not been determined.

In this paper we have systematically compared histone and a peptide substrate in this assay, and we conclude that many of the criticisms are not inherent to the measurement of the activity ratio, but are largely due to the choice of histone as substrate. Similarly, we have investigated the homogenization conditions and have shown that the presence of NaCl in the homogenization buffer is essential for the satisfactory assay of activity in a variety of tissues. The peptide used is malantide (sequence RTKRSGSVYEPLKI), corresponding to the site phosphorylated by cAMP-PrK on the β-subunit of phosphorylase kinase (Yeaman *et al.*, 1977), and first used by Malencik & Anderson (1983). A preliminary report of some of these findings has been published (Murray *et al.*, 1988).

MATERIALS AND METHODS

Tissues

Hearts were perfused as described by England (1976), human platelets were prepared as described by Simpson *et al.* (1988), and rat adipocytes were prepared from epididymal pads as described by Rodbell (1964) and modified by Whitewell & Gliemann (1979). Adenosine and N^6-[*R*-(−)-1-methyl-2-phenethyl]adenosine were used in fat-cell incubations as suggested by Honnor *et al.* (1985). For the experiments shown in Fig. 1, the rat hearts were perfused with 20 nM-(−)-isoprenaline for 30 s, and the guinea-pig hearts with 4 nM-(−)-isoprenaline for 30 s. The platelets were incubated with 20 μM-forskolin for 3 min; the adipocytes were incubated with 10 μM-isoprenaline for 2 min. The hearts were freeze-clamped with Wollenberger tongs; the cell incubations were frozen in liquid N_2. For the experiments

Abbreviations used: cAMP-PrK, cyclic AMP-dependent protein kinase; PKI, cyclic AMP-dependent protein kinase inhibitor protein; cGMP-PrK, cyclic GMP-dependent protein kinase; R, regulatory subunit of cAMP-PrK; C, catalytic subunit of cAMP-PrK.

described in the other Figures and Tables, hearts and adipocytes that had been perfused or incubated under control conditions were used. Freshly dissected rat and guinea-pig tissues were powdered under liquid N_2.

Homogenization

Heart and other tissue powders were homogenized by use of a motor-driven glass/Teflon homogenizer in 10–40 vol. of 10 mM-NaH_2PO_4/10 mM-EDTA/0.5 mM-3-isobutyl-1-methylxanthine (pH 6.8) (buffer A) containing NaCl as described. Supernatants were prepared by low-speed centrifugation (6000 *g* for 5 min, unless stated otherwise). For extraction of adipocytes, 3 vol. of buffer A was added to the frozen cells, followed by vigorous vortex-mixing to ensure complete extraction, and the infranatant obtained after centrifugation (12000 *g*, 4 min) was used for the assay. Platelets were thawed, centrifuged at 450000 *g* for 20 min, and the undiluted supernatant was used in the assay.

cAMP-PrK activity-ratio assay

Tissue extract (10 μl) was mixed with 50 μl of assay mixture containing 70 mM-Na_2HPO_4 (pH 6.8), 14 mM-$MgCl_2$, 1.4 mM-EGTA, 0.014% (v/v) Tween-20, 28 μM-malantide (or 7 mg of histone-IIA/ml), and in the absence or presence of 2.8 μM-cyclic AMP. After a 1 min equilibration at 30 °C, the reaction was initiated by addition of 10 μl of 2.1 mM-[γ-^{32}P]ATP (1–2 Bq/pmol). After incubation for a further 2 min, 10 μl of 1 M-HCl was added to the tubes to terminate the reaction. After this, a 35 μl sample was spotted on to phosphocellulose paper (P81; Whatman, Maidstone, Kent, U.K.). The papers were given four washes of 5–10 min each in 0.5% (v/v) tetraphosphoric acid/38 mM-H_3PO_4 in mesh baskets (Corbin & Reimann, 1974), dried and counted by Čerenkov radiation.

The activity ratio was obtained by dividing the radioactivity (c.p.m.) obtained in the absence of cyclic AMP by that obtained in its presence. Blanks, obtained by adding the HCl before the [γ-^{32}P]ATP, were subtracted from all values. Tissues were processed in numbers that allowed the assay to be completed within 10 min of homogenization. Other additions to the assay mixture are noted in the individual Figure and Table legends. Tween-20 is included in the assay mix to preserve the activity of cAMP-PrK; this is necessary with assay conditions that have low protein concentration (Murray & Leigh, 1986). The reactions are terminated with HCl, rather than by spotting the reaction mixture directly on to the P81 papers, as this allows the use of repeating pipettes, which in itself allows more assays in the 2 min period. Tetraphosphoric acid was included in the washing buffer, as it was required to decrease the assay blank with the combination of certain batches of P81 papers and [γ-^{32}P]ATP. However, we have found that some peptides (e.g. kemptide) no longer stick to the papers under these conditions. The incorporation of ^{32}P into malantide was linear with respect to time and amount of tissue extract in all circumstances when less than 20% of the peptide was utilized.

Effects of histone

For the experiments described in Table 4, tissue extracts were incubated for 5 min at 30 °C with either 5 mg of BSA/ml or 5 mg of histone IIA/ml, and the incubated extract was diluted with 3 vol. of buffer A before assay. This procedure was used to minimize the competition between histone and malantide in the assay. The reaction was terminated by the addition of 10 μl of 100% trichloroacetic acid, and the supernatant after centrifugation (15000 *g*, 1 min) was spotted on the phosphocellulose paper; this allows just the ^{32}P incorporated into malantide to be measured.

Addition of exogenous cAMP-PrK

For the experiments described in Table 6, approx. 50 mg of heart powder was homogenized in 2 ml of buffer A containing 100 mM-NaCl, with or without the addition of 10 μl of partially purified II type cAMP-PrK from bovine heart (added to the buffer just before homogenization). All subsequent steps were as described above, except that the extracts with added cAMP-PrK were diluted 1:1 with homogenization buffer before assay. The added cAMP-PrK had an activity ratio of 0.03; the total added activity was 0.05 μmol of ^{32}P transferred/min (measured with malantide in the presence of cyclic AMP); this represented 2.5–3 times the endogenous activity. The expected activity was calculated by the simple addition of the exogenous and endogenous activities. Variations in the endogenous activities owing to slightly different amounts of powder homogenized were corrected for by measuring the protein content of the extracts. The expected activity ratio is based on the assumption that none of the added cAMP-PrK has dissociated.

Kinetic determinations

The kinetic parameters of the phosphorylation of malantide and histone IIA were determined by using purified catalytic subunit of cAMP-PrK and cGMP-PrK (cyclic GMP-dependent protein kinase). The assay mixture was as above, 10 μM-cyclic GMP being included for the assays using cGMP-PrK. Malantide and histone-IIA concentrations were varied over a 128-fold range and the results analysed by non-linear regression.

Preparation of proteins

Catalytic subunit of cAMP-PrK was prepared from bovine heart (Reimann & Beham, 1983), cGMP-PrK from bovine lung (Lincoln, 1983), and protein kinase inhibitor from rabbit skeletal muscle (Schlender *et al.*, 1983). Type II holoenzyme was partially purified from bovine heart by chromatography on DEAE-cellulose (Whatman) and Mono Q (Pharmacia, Milton Keynes, Bucks., U.K.).

Materials

Malantide was obtained from Ocean Biologicals, Edmonds, WA, U.S.A. The peptide was dissolved in water and the concentration determined by absorbance (ϵ_{275} = 1380 $M^{-1} \cdot cm^{-1}$) (Shugar, 1952). PKI-(5–24)-amide (Cheng *et al.*, 1986) was from Peninsula Labs, St. Helens, Lancs., U.K. (code no. 8899). Histone IIA and most other biochemicals were from Sigma, Poole, Dorset, U.K.

Other methods

Protein was determined by the method of Bradford (1976), with BSA as a standard.

RESULTS AND DISCUSSION

Use of malantide as substrate

Different cAMP-PrK activity ratios are obtained when tissue extracts are assayed with histone and malantide as substrates. Table 1 shows that in four tissues the activity ratios are consistently higher when histone is the substrate. This is true both when tissues have been incubated under control conditions and also when treated with agents known to increase cyclic AMP. In addition, differences between control and treated tissues were more clearly observed when malantide was used as the

Table 1. Cyclic AMP-dependent protein kinase activity ratios assayed with malantide and histone as substrates

The tissues were treated with various agents known to raise intracellular cyclic AMP levels and subsequently processed as described in the Materials and methods section. The activity ratio was determined by assaying the tissue extract in the absence and presence of 2 μM-cyclic AMP with either 20 μM-malantide or 5 mg of histone IIA/ml as substrates. The treatment of the tissues was: rat heart 20 nM-(−)-isoprenaline, 30 s; guinea-pig heart 4 nM-(−)-isoprenaline, 30 s; platelets 20 μM-forskolin, 180 s; adipocytes 10 μM-isoprenaline, 120 s. Results are shown as means ± S.E.M. ($n = 4$ for all observations): $^{a}P < 0.05$, $^{b}P < 0.01$, for assays with malantide compared with assays with histone (i.e. column A versus B, and C versus D); $^{c}P < 0.05$, $^{d}P < 0.01$, for control compared with treated (i.e. A versus C and B versus D).

	Activity ratio			
	Control		Treated	
Tissue	Malantide (A)	Histone (B)	Malantide (C)	Histone (D)
Rat heart	0.10 ± 0.01	0.40 ± 0.02^{b}	0.35 ± 0.01^{d}	0.59 ± 0.02^{b,d}
Guinea-pig heart	0.16 ± 0.01	0.67 ± 0.07^{b}	0.33 ± 0.01^{a}	0.69 ± 0.03^{b}
Human platelet	0.10 ± 0.03	0.62 ± 0.05^{b}	0.54 ± 0.06^{d}	0.76 ± 0.07^{a,c}
Rat adipocytes	0.28 ± 0.05	0.41 ± 0.04^{a}	0.44 ± 0.03^{c}	0.65 ± 0.06^{a,c}

Table 2. Kinetic parameters of the phosphorylation of malantide and histone IIA

The K_m and $V_{max.}$ for the phosphorylation of malantide and histone IIA by the purified catalytic subunit (C) of the cAMP-PrK and the purified cGMP-PrK were determined as shown in the Materials and methods section: 1 unit = 1 μmol of ^{32}P transferred/min. Results are shown as means ± S.E.M.

Substrate	Kinase	K_m	$V_{max.}$(units/mg)
Malantide	C subunit ($n = 3$)	15 ± 2 μM	23.8 ± 1.5
	cGMP-PrK ($n = 1$)	223 μM	6.6
Histone	C subunit ($n = 3$)	2.8 ± 0.6 mg/ml	0.41 ± 0.03
	cGMP-PrK ($n = 2$)	0.13 ± 0.03 mg/ml	0.17 ± 0.05

substrate. In particular, a statistically significant effect of isoprenaline on the activity ratio of guinea-pig heart was only observed with malantide. In light of the difference in the activity ratios measured with histone and malantide, experiments were designed to ascertain which activity ratio more closely reflects that found in the tissue.

One general advantage that malantide has over histone is that kinetically it is a far better substrate for cAMP-PrK (Table 2). This allows the assay of more dilute tissue extracts, thus decreasing the effects of other enzymes (ATPases, phosphodiesterases) and endogenous cyclic AMP on the assay. In addition, small amounts of tissue, or tissues which contain low levels of cAMP-PrK, can be assayed [e.g. we have successfully assayed as little as 10 ng of human heart (Kaumann *et al.* 1989)]. Another advantage of malantide is that it is a more selective substrate for cAMP-PrK. cGMP-PrK is well known to be able to phosphorylate most, if not all, the substrates of cAMP-PrK. However, as shown in Table 2, malantide gives a far better discrimination between the two cyclic-nucleotide-dependent protein kinases than does histone. Malantide has been reported to be a poor substrate for phosphorylase kinase (Malencik & Anderson, 1983).

The selectivity of malantide for cAMP-PrK in a number of rat tissues was assessed by the use of the specific protein inhibitor (PKI) of cAMP-PrK. Table 3 shows that when tissues are assayed in the presence of cyclic AMP approx. 90% (and in most instances > 95%) of the activity towards malantide is inhibited by PKI, showing that most of the malantide phosphorylation is catalysed by cAMP-PrK. In contrast, when histone is used as the substrate there is a far greater variability in the effect of PKI between tissues, and importantly, less activity is inhibited by PKI. The histone phosphorylation not inhibited by PKI could be due to cyclic AMP-independent phosphorylation of histone, or to the presence of increased levels of endogenous substrates resulting from the higher concentration of tissue extract required. In both cases, the effect of using histone as a substrate is to produce a higher activity ratio, owing to the measurement of phosphorylation not catalysed by cAMP-PrK.

It has been reported that histone can cause the dissociation of R_2C_2 (Miyamoto *et al.*, 1973). Table 4 shows the effects of incubating tissue extracts with 5 mg of histone/ml (or BSA as a control) on the activity ratios as determined by the use of malantide. In rat and guinea-pig heart extracts, histone causes an approx. 3-fold activation within 5 min, and this provides another reason why activity ratios are higher when assayed with histone. A similar activation by histone was observed on partially purified R_2C_2 from bovine heart (type II), whereas no reaction was found when the enzyme was preincubated with 20 μM-malantide (results not shown). The activation by histone of cAMP-PrK in tissue extracts clearly shows that the activity ratio reported when histone is used as a substrate is higher than the true activity ratio found in the tissue.

In conclusion, we suggest that activity ratios measured with histone as a substrate are artificially high, owing to measurement of cyclic AMP-independent kinases, and also because histone causes activation of the cAMP-PrK in the assay. Whereas the first problem may be overcome by assays that include PKI (e.g. Torphy *et al.*, 1982), this represents additional assays, and in any case does not overcome the dissociation in the test tube. In contrast, neither problem is encountered when malantide is used

Table 3. Inhibition by PKI of malantide kinase and histone kinase activities in rat tissues

Rat tissues were homogenized in buffer A containing 100 mM-NaCl as described in the Materials and methods section. Supernatants obtained after centrifugation (15000 *g*, 2 min) were diluted 3-fold for the malantide assay, and used undiluted in the histone assay. The supernatants were assayed in the presence of 2 μM-cyclic AMP with either 20 μM-malantide or 5 mg of histone IIA/ml as the substrate and in the absence or presence of PKI-derived peptide (1 μM). The Table shows the percentage of kinase activity that was inhibited by the addition of PKI derived peptide. Results are shown as means ± S.E.M. ($n = 4$): $^*P < 0.05$, $^{**}P < 0.01$ for differences from assays with malantide. Similar results were obtained with partially purified PKI protein.

	Activity inhibited by PKI (%)	
Tissue	Malantide	Histone
Skeletal muscle	96.3±0.1	66.7±11.5*
Liver	95.6±0.7	62.5±2.9**
Kidney	86.7±2.6	42.8±0.9**
Brain	93.1±0.3	41.1±2.8**
Spleen	89.5±1.7	48.5±4.7**
Heart	96.4±0.3	82.7±0.7**
Adipocytes	94.6±2.4	97.8±2.0

Table 4. Effect on the activity ratio of incubation of tissue extracts with histone

Control guinea-pig or rat hearts were homogenized in 10 vol. of buffer A. After centrifugation (6000 *g*, 5 min) the supernatant was immediately assayed for activity ratio (column A). The activity ratio was also determined after incubation of the tissue extract for 5 min with either 5 mg of BSA/ml (column B) or 5 mg of histone IIA/ml (column C). Full details are given in the Materials and methods section. Results are shown as means ± S.E.M. ($n = 3$): $^*P < 0.05$ for differences from column A.

	Activity ratio		
		After 5 min incubation with:	
Tissue	0 min (A)	BSA (B)	Histone (C)
Rat heart	0.07±0.01	0.05±0.01	0.21±0.04*
Guinea-pig heart	0.12±0.01	0.12±0.01	0.43±0.07*

as a substrate. The faster rate of phosphorylation of malantide also produces significant advantages, e.g. in the amount of tissue required, and interference from other enzymes.

Effect of NaCl in the homogenization buffer

Our initial observations showed that guinea-pig hearts homogenized in buffer A containing 100 mM-NaCl had consistently higher activity ratios than did the same hearts homogenized in buffer A alone. To investigate this further, a number of other guinea-pig tissues were homogenized in buffer A alone or in buffer A containing 100 mM-NaCl. To determine whether there was a direct effect on the catalytic subunit, 0.1 mM-cyclic AMP was included in the homogenization buffers (in all cases the measured activity ratio was 1.0, showing that the holoenzyme was fully dissociated). As shown in Table 5, for all the tissues studied the specific activity of cAMP-PrK in the supernatant was decreased if NaCl was not included in buffer A. It has previously been reported that free catalytic subunit binds

Table 5. Effect of NaCl on catalytic-subunit binding to the particulate fraction of various guinea-pig tissues

Guinea-pig tissues were frozen with Wollenberger clamps and powdered under liquid N_2. The tissues were homogenized with a motor-driven glass/Teflon homogenizer in 40 vol. of 10 mM-sodium phosphate/10 mM-EDTA/0.5 mM-3-isobutyl-1-methylxanthine/0.1 mM-cyclic AMP, containing 0 or 100 mM-NaCl. After centrifugation (5000 *g*, 5 min), the supernatant was assayed for cAMP-PrK with malantide as substrate. The cyclic AMP in the homogenization buffer caused complete dissociation of the holoenzyme, as judged by the observation that all activity ratios were 1. Results are shown as mean ± S.E.M. ($n = 2$).

	cAMP-PrK (nmol/min per mg of protein)	
Tissue	0 mM-NaCl	100 mM-NaCl
Brain	23.6±0.8	47.0±2.3
Liver	5.2±0.8	8.0±0.3
Skeletal muscle	6.4±0.7	13.2±0.03
Spleen	15.8±1.0	23.8±1.3
Kidney	6.7±0.3	9.9±0.4

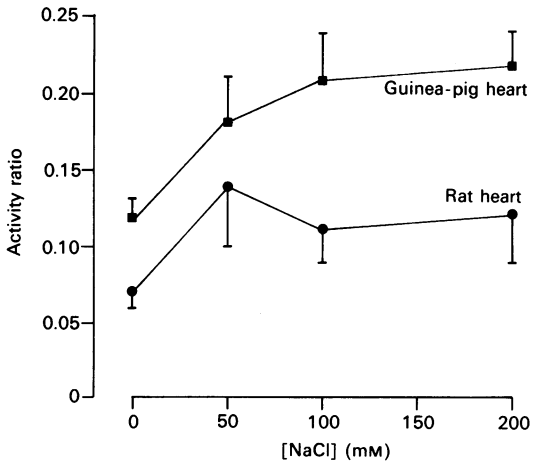

Fig. 1. Effect of addition of NaCl to tissue extracts on the activity ratio

Rat (●) and guinea-pig (■) hearts were homogenized in 10 vol. of buffer A and supernatants were prepared as described in the Materials and methods section: 3 vol. of buffer containing NaCl was added to the supernatants to give the final concentration of NaCl shown. A 10 μl portion of this was assayed in the standard activity-ratio assay. Results are shown as activity ratios (means ± S.E.M., $n = 3$ for all observations). All values were significantly different ($P < 0.05$) compared with the corresponding 0 NaCl value; there was no significant difference between all other values.

to the particulate fraction in the absence of NaCl (Keely *et al.*, 1975). We confirmed this observation by assaying the particulate fractions, and in all cases greater activity was found in the particulate fractions from tissues homogenized in the absence of NaCl (results not shown). These results suggest that homogenization in the absence of NaCl causes catalytic subunit to be removed from the supernatant, resulting in an artifactually low activity ratio.

As these results show that it was necessary to include NaCl in the homogenization buffer to prevent trapping of free catalytic subunit, it was important to ascertain whether including NaCl could cause other effects, e.g. dissociation of holoenzyme by NaCl has been reported (Palmer *et al.*, 1980). As shown in Fig.

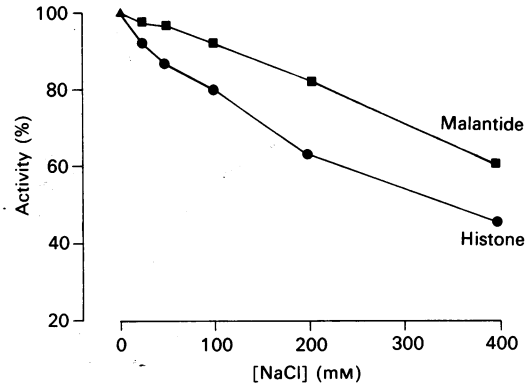

Fig. 2. Inhibition by NaCl of catalytic-subunit activity assayed with malantide and histone

Purified catalytic subunit was assayed with either 20 μM-malantide (■) or 5 mg of histone/ml (●) as substrate and the indicated concentration of NaCl. Results are shown as percentage of the activity obtained in the absence of NaCl, and are means of three separate experiments.

1, addition of NaCl to rat or guinea-pig heart supernatants does cause an increase in the activity ratio. However, as increasing the NaCl concentration from 50 mM to 200 mM had no further effect on the activity ratio in rat and guinea-pig heart, these results are consistent with the view that the true activity ratio is that measured in the presence of NaCl, and that the lack of salt results in an artificially low activity ratio. In the experiments with rat hearts, addition of 50 mM-NaCl caused an increase in both activity assayed with cyclic AMP ($9\pm2\%$, $n=3$) and that in its absence ($25\pm2\%$, $n=3$). The effect on the total, as well as the basal, activity again suggests that in the absence of NaCl not all the cAMP-PrK activity is being measured, possibly owing to free catalytic subunit binding to other proteins. With the guinea-pig hearts, addition of 50 mM-NaCl increased the activity in the absence of cyclic AMP, but did not change the total activity, so in this case NaCl is probably blocking the re-association of the R_2 and C subunits, as has been previously reported for tissues containing the type II holoenzyme (Cherrington *et al.*, 1976; Corbin *et al.*, 1973).

We have investigated this further in rat epididymal fat-cells, where the effects of NaCl in the homogenization buffer on the activity ratio are rather marked. When cells were homogenized in the absence of NaCl, activity ratios of 0.05 ± 0.01 ($n=5$) were obtained for control cells and 0.06 ± 0.01 ($n=5$) for those treated with 10 μM-isoprenaline; the corresponding values for cells homogenized in 0.2 M-NaCl were 0.28 ± 0.03 ($n=4$) and 0.44 ± 0.03 ($n=4$) respectively. Thus, in the absence of added NaCl, not only were much lower ratios evident, but the expected increase in ratio after exposure of cells to isoprenaline was not observed. These observations are compatible with extensive re-association of R_2 and C subunits occurring in the absence of NaCl, and this was confirmed by adding purified catalytic subunit to frozen fat-cell incubations before extraction. The activity of the exogenous catalytic subunit was only fully obtained when the cells were homogenized in 100 μM-cyclic AMP or 0.2 M-NaCl. It is probable that the exogenous C subunit bound to endogenous R_2 subunit, as full activity was recovered in cells homogenized in the absence of NaCl but assayed in the presence of cyclic AMP. It has been reported that NaCl inhibits the phosphorylation of histone by cAMP-PrK (Cherrington *et al.*, 1976; Palmer *et al.*, 1980). As shown in Fig. 2, the phosphorylation of malantide is less sensitive to inhibition by NaCl. At the final concentration of NaCl (14 mM) in the assay, no significant inhibition was observed.

In conclusion, we would recommend that NaCl is included in homogenization buffers to prevent the trapping of free catalytic subunit in the particulate fraction. Omitting NaCl appears to cause artificially low activity ratios, which, interestingly, is the opposite effect of that obtained with histone. Including NaCl does not result in any artificial dissociation of holoenzyme, and is required to prevent re-association with the type II isoenzyme.

Other considerations

A major factor that must be accounted for in these assays is the depletion of the added [γ-^{32}P]ATP by endogenous ATPases. There is considerable tissue variability in this respect; e.g. with rat heart supernatants, 300 μM-ATP is decreased to approx. 100 μM in 2 min, whereas in platelet supernatants less than 10% of 100 μM-ATP is hydrolysed in 5 min. In this respect, the use of malantide as a substrate has several advantages: (i) more dilute supernatants can be assayed; (ii) more radioactivity is incorporated per unit time.

It is important to show the measured activity ratio is that actually present in the tissue, and is not altered by the homogenization procedure. To do this, Palmer *et al.* (1980) have suggested adding exogenous holoenzyme to the tissues. In similar experiments, partially purified type II holoenzyme was added to guinea-pig and rat heart (Table 6). The exogenous cAMP-PrK was added to the buffer immediately before homogenization so that, as far as possible, it was subject to the same manipulations as the tissue cAMP-PrK. Column (C) of Table 6 shows the activity ratio that would be expected if none of the added cAMP-PrK was dissociated during the homogenization and assay procedures. Column (B) shows the activity ratios that were actually measured with the added cAMP-PrK. Comparison of these columns shows that no dissociation of the exogenous cAMP-PrK has occurred. In fact, the observed activity ratios are 0.92 ± 0.05 (mean ± S.E.M., $n=6$) of the predicted values, although this was not statistically significant from unity ($P>0.05$, paired t test). It is particularly important that the same results were obtained with control and isoprenaline-treated hearts, showing that the exogenous cAMP-PrK is not merely reflecting the cAMP content of the tissue. No dissociation of exogenous cAMP-PrK was observed when it was added to

Table 6. Effect of exogenous cAMP-PrK on the activity ratio in rat and guinea-pig heart

Heart powder was homogenized with and without addition of exogenous cAMP-PrK, the activity ratios obtained being shown in columns (A) and (B) respectively. Column (C) shows the calculated activity ratio that would be expected with the addition of exogenous cAMP-PrK, assuming that none of the added cAMP-PrK had dissociated. When treated, the rat hearts were perfused with 20 nM-isoprenaline for 30 s, and the guinea-pig heart with 10 nM-isoprenaline for 35 s. Full details are given in the Materials and methods section.

		Activity ratio		
		Observed		Expected
Tissue	Treatment	−cAMP-PrK (A)	+cAMP-PrK (B)	+cAMP-PrK (C)
Rat heart	Control	0.17	0.05	0.07
		0.15	0.06	0.06
	Isoprenaline	0.41	0.12	0.14
		0.36	0.12	0.14
Guinea-pig heart	Control	0.22	0.09	0.09
	Isoprenaline	0.45	0.16	0.15

guinea-pig brain or human platelets before homogenization (results not shown). Thus dissociation of cAMP-PrK does not occur during these particular homogenization and assay conditions, showing that the activity ratio reported is that present in the tissue.

General discussion

The original cAMP-PrK activity-ratio assay was introduced by Corbin *et al.* (1973) to measure the activation of the enzyme in adipose tissue. The assay was subsequently modified for use in other tissues (see Corbin, 1983), but has also been the subject of criticism (Palmer *et al.*, 1980). Our results indicate that use of a peptide substrate in place of histone and including NaCl in the homogenization buffer would further improve the accuracy of this assay. The advantages of using malantide as a substrate have been discussed above, and to our knowledge there are no disadvantages to this particular substrate. It would be expected that similar results could be obtained with other peptides (e.g. kemptide), although this has not been systematically assessed. Since submission of this manuscript, kemptide has been assessed in the activity-ratio assay, and results similar to those reported here were obtained (Giembycz & Diamond, 1989).

Others workers have included salt (NaCl or KCl) in the homogenization buffer for tissues that contain the type II isoenzyme to prevent its re-association, although it is omitted for tissues that contain type I isoenzyme to prevent its dissociation. This has therefore presented problems for the assay of tissues (e.g. rat liver) that contain an approximately equal distribution of isoenzymes, and in this case an intermediate salt concentration has been suggested (Corbin, 1983). Our data suggest that NaCl should be included in the homogenization buffer for all tissues, to prevent association of catalytic subunit with the particulate fraction (Table 5). The concentration of NaCl used is determined by the tissue to be assayed, e.g. concentrations of NaCl above 200 mM start to cause solubilization of myofibrils in cardiac tissue, whereas this concentration of NaCl is required to prevent re-association of R and C subunits in adipose tissue.

Palmer *et al.* (1980) have reported that NaCl causes dissociation of holoenzyme (especially type I), and is an inhibitor of the catalytic activity. We found no evidence for a dose-dependent dissociation of holoenzyme by NaCl in extracts from rat heart (type I) or guinea-pig heart (type II) (Fig. 1). Possibly the dissociation observed by Palmer *et al.* (1980) is due to their use of histone as a substrate, which, in our hands, causes holoenzyme dissociation in rat and guinea-pig heart extracts (Table 4). Similarly, inhibition of catalytic activity by NaCl is also substrate-dependent, with malantide phosphorylation being more resistant to inhibition (Fig. 2). Therefore, at least with malantide as substrate, there is no apparent disadvantage to including NaCl in the homogenization buffer.

Under the conditions we employed for the activity-ratio assay, we found no dissociation of added holoenzyme (Table 6). In contrast, Palmer *et al.* (1980) reported dissociation of holoenzyme when added to liver before homogenization, and suggested that the measured activity ratio may reflect the cyclic AMP concentration during extraction, rather than the activity ratio actually occurring in the tissue. There are several differences between the assays used in this paper and that by Palmer *et al.* (1980): (i) dilution (we use a final dilution of 1:280 compared with 1:75), and (ii) choice of substrate. In our hands, the histone-induced dissociation of holoenzyme (Table 4) has such a large effect that it makes prediction of activity ratios virtually impossible.

The data presented here suggest that the cAMP-PrK activity ratio can be accurately determined if a peptide is used as the substrate, and if NaCl is included in the homogenization buffer. Tissue variability was observed, showing the importance of conducting control experiments when new tissues are analysed. Pertinent experiments include: (*a*) showing that phosphorylation of malantide is linear with respect to time and tissue extract and is blocked by the addition of PKI; (*b*) determining the rate of [γ-^{32}P]ATP hydrolysis by the tissue extract; (*c*) investigating whether exogenous holoenzyme is activated during the assay.

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