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Activation and phosphorylation of the 'dense-vesicle' high-affinity cyclic AMP phosphodiesterase by cyclic AMP-dependent protein kinase

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Incubation of a hepatocyte particulate fraction with ATP and the isolated catalytic unit of cyclic AMP-dependent protein kinase (A-kinase) selectively activated the high-affinity 'dense-vesicle' cyclic AMP phosphodiesterase. Such activation only occurred if the membranes had been pre-treated with Mg^{2+}. Mg^{2+} pre-treatment appeared to function by stimulating endogenous phosphatases and did not affect phosphodiesterase activity. Using the antiserum DV4, which specifically immunoprecipitated the 51 and 57 kDa components of the 'dense-vesicle' phosphodiesterase from a detergent-solubilized membrane extract, we isolated a ^{32}P-labelled phosphoprotein from ^{32}P-labelled hepatocytes. $MgCl_2$ treatment of such labelled membranes removed ^{32}P from the immunoprecipitated protein. Incubation of the Mg^{2+}-pre-treated membranes with [^{32}P]ATP and A-kinase led to the time-dependent incorporation of label into the 'dense-vesicle' phosphodiesterase, as detected by specific immunoprecipitation with the antiserum DV4. The time-dependences of phosphodiesterase activation and incorporation of label were similar. It is suggested (i) that phosphorylation of the 'dense-vesicle' phosphodiesterase by A-kinase leads to its activation, and that such a process accounts for the ability of glucagon and other hormones, which increase intracellular cyclic AMP concentrations, to activate this enzyme, and (ii) that an as yet unidentified kinase can phosphorylate this enzyme without causing any significant change in enzyme activity but which prevents activation and phosphorylation of the phosphodiesterase by A-kinase.

INTRODUCTION

Many hormones exert their actions on target cells by elevating the intracellular concentration of cyclic AMP. The only known mechanism for the degradation of cyclic AMP is by the action of phosphodiesterases; these therefore provide potentially important regulatory sites. At least three high-affinity cyclic AMP phosphodiesterase species are activated by insulin in hepatocytes (Heyworth *et al.*, 1983, 1984). However, only one of these enzyme activities, the so-called 'dense-vesicle' phosphodiesterase, which is located in a unique intracellular vesicle fraction, is also stimulated by increased intracellular cyclic AMP. This can be achieved by exposing cells to glucagon (Heyworth *et al.*, 1983). Indeed, this enzyme is the only phosphodiesterase identified in liver that is known to be stimulated by glucagon.

We have purified this enzyme to apparent homogeneity from rat liver (Pyne *et al.*, 1987*a*), by two different procedures. These yield a 112 kDa apparently dimeric protein which, on SDS/PAGE, exhibits a major band of 57 kDa and a minor band of 51 kDa. The kinetic properties of this enzyme, which serve to characterize it, are a high specificity for cyclic AMP as a substrate, a potent ability of cyclic GMP to inhibit the hydrolysis of cyclic AMP, and the apparently unique ability to be inhibited by the compound ICI 118233 (Pyne *et al.*, 1987*b*).

We have also prepared a number of antisera against this purified enzyme (Pyne *et al.*, 1987*a*,*b*; Houslay *et al.*, 1988). These appear to be highly specific for this enzyme and do not cross-react with other phosphodiesterases in the liver or other tissues. They specifically immunoabsorb, in an active form, the purified 'dense-vesicle' enzyme. Furthermore, they can also immunoabsorb this enzyme in a solubilized state when it is released by hypo-osmotic-shock treatment of hepatocyte membranes (Pyne *et al.*, 1987*a*; Houslay *et al.*, 1988) prepared from both control and hormonally stimulated hepatocytes.

The effects of cyclic AMP on metabolic pathways are mediated by cyclic AMP-dependent protein kinase (hereafter designated A-kinase), which phosphorylates and hence modifies the activity of various enzymes such as glycogen synthase and acetyl-CoA carboxylase (for review see Cohen, 1985). Therefore we have investigated the effect of A-kinase on cyclic AMP phosphodiesterase activity in a particulate fraction from rat hepatocytes. We show that the catalytic subunit of A-kinase activates high-affinity cyclic AMP phosphodiesterase activity, and that this is entirely due to an increase in the activity of the 'dense-vesicle' phosphodiesterase.

MATERIALS AND METHODS

Materials

All biochemicals, unless stated otherwise, were from Sigma, Poole, Dorset, U.K. Cyclic [^3H]AMP and [γ-^{32}P]ATP were from Amersham International, Amersham, Bucks., U.K. Cyclic AMP, cyclic GMP and collagenase were from Boehringer Corp. (U.K.), Lewes, East Sussex, U.K. Protein A was from Bethesda Research

Abbreviations used: A-kinase, cyclic AMP-dependent protein kinase; PAGE, polyacrylamide-gel electrophoresis.

Laboratories, Life Technologies Inc., Gaithersburg, MD, U.S.A., and anti-rabbit horseradish-peroxidase-linked IgG was obtained from the Scottish Antibody Production Unit, Law Hospital, Carluke, Scotland, U.K. ICI 118233 was kindly given by Dr. M. Collis, ICI Pharmaceuticals, Alderley Edge, Macclesfield, Cheshire, U.K. Protein kinase inhibitor was from Sigma. Unless otherwise stated, a commercial preparation of the catalytic subunit of A-kinase from bovine heart was obtained from Sigma. In some experiments an apparently homogeneous preparation of the A-kinase catalytic subunit from bovine heart was used. This was prepared by the method of Reimann & Beham (1983), and was kindly given by Dr. D. Carling and Dr. D. G. Hardie, Department of Biochemistry, University of Dundee, Scotland.

Assay methods

Cyclic AMP phosphodiesterase activity was measured by a modification (Marchmont & Houslay, 1980) of the two-step procedure of Thompson & Appleman (1971). All assays were performed at 30 °C in the presence of 1 μM-cyclic AMP. To ascertain the contribution of 'dense-vesicle' phosphodiesterase to total enzyme activity, the specific inhibitor ICI 118233 was used at a final concentration of 100 μM in the assays (see Pyne *et al.*, 1987*b*) and cyclic GMP at a final concentration of 2 μM (see Pyne *et al.*, 1987*a*).

Preparation of hepatocytes

Isolated hepatocytes were prepared from 225–250 g male Sprague–Dawley rats (Elliott *et al.*, 1976) and incubated as previously described (Smith *et al.*, 1978).

Preparation of a particulate fraction (P_1) from hepatocytes and its subsequent preincubation with Mg^{2+} (buffer B)

Cells (4–5 mg dry wt./ml) were incubated at 37 °C in Krebs–Henseleit (1932) buffer containing 2.5 % (w/v) bovine serum albumin, 10 mM-glucose and 2.5 mM-$CaCl_2$ in a total volume of 5 ml and were gassed with O_2/CO_2 (19:1). After 10 min, 5 ml of ice-cold buffer A, containing 0.25 M-sucrose, 10 mM-Tris/HCl, pH 7.4, 1 mM-EDTA, 2 μM-leupeptin, 0.1 mM-phenylmethanesulphonyl fluoride and 2 mM-benzamidine, was added, and the cells were harvested by centrifugation at 50 g for 2 min. The supernatant was decanted and the cells were washed by resuspension in 5 ml of buffer A and centrifugation as before. The cells were then resuspended in 5 ml of buffer A and homogenized by 30 up-and-down strokes of a Teflon pestle in a glass homogenizer (Camlab), and the homogenate was centrifuged at 22000 $g_{av.}$ for 15 min at 4 °C. The supernatant was decanted and, unless stated otherwise, the pellet (P_1) was resuspended in 0.8 ml of ice-cold buffer B, containing 0.25 M-sucrose, 20 mM-Tris/HCl, pH 7.4, 5 mM-$MgCl_2$, 2 μM-leupeptin, 0.1 mM-phenylmethanesulphonyl fluoride and 2 mM-benzamidine, and incubated at 30 °C. After 10 min NaF plus β-glycerophosphate were added in a total volume of 0.2 ml to give final concentrations of 50 mM and 10 mM respectively (buffer C). Two separate 0.45 ml portions were removed from each sample and incubated in the presence of 0.1 mM-ATP without or with 50 units of Sigma A-kinase at 30 °C for 10 min. Samples were then assayed for phosphodiesterase acitivity. The A-kinase obtained from Sigma, and not the pure preparation, was reconstituted in 0.3 M-dithiothreitol before use, and dithiothreitol was added to all control samples to a final concentration of 16 mM. This had no effect on enzyme activity. When the apparently homogeneous preparation of A-kinase, from Hardie and Carling, was used, 20 units was added to the assays.

Solubilization of the 'dense-vesicle' cyclic AMP phosphodiesterase

This was performed by a modification of the hypo-osmotic-shock method of Loten *et al.* (1978). After the appropriate treatment, the membrane suspension was centrifuged at 22000 $g_{av.}$ for 15 min at 4 °C. The pellet was then extracted with 3 ml of 1 mM-EDTA/10 mM-Tris/HCl, pH 7.4, with four strokes of a Teflon pestle in a glass homogenizer. The extract was left on ice for 30 min before centrifugation as above to obtain the supernatant containing solubilized 'dense-vesicle' cyclic AMP phosphodiesterase activity.

Preparation of immune serum

The 'dense-vesicle' phosphodiesterase was purified to apparent homogeneity, yielding a major band at 57 kDa and a minor band at 51 kDa on SDS/PAGE, by the method of Pyne *et al.* (1987*a*). Analysis on non-denaturing gels gave a single band, staining for protein, which co-migrated with phosphodiesterase activity as assessed by extraction of gel slices for assay (Marchmont & Houslay, 1980). After freeze-drying, the sample was reconstituted in 100 μl of electrophoresis sample buffer (Laemmli, 1970) and boiled for 2 min before SDS/PAGE performed as described by Pyne *et al.* (1987*a*). After location of protein (57 kDa band) by Coomassie Blue staining, the gel bands containing the 'dense-vesicle' enzyme were excised and ground up under liquid N_2 with a pestle and mortar. The ground gel chips (10–20 μg of protein) were homogenized in 0.5 ml of phosphate-buffered saline, pH 7.5, and added to 0.5 ml of Freund's complete adjuvant and then injected into a New Zealand White adult rabbit (Pyne *et al.*, 1987*a*). Injections were repeated over a period of 3 months with incomplete adjuvant. Immune serum was purified as described by Pyne *et al.* (1987*a*) and stored at −70 °C. The properties of this antiserum were as described for antisera DV1 and DV2 (Pyne *et al.*, 1987*a*,*b*; Houslay *et al.*, 1988), and such antisera, including DV3, could substitute for DV4 with identical results.

Preparation of immunoabsorbent matrix

The purified polyclonal antibody DV4 was covalently linked to Protein A–Sepharose by the method of Werner & Machleidt (1978).

Immunoprecipitation of 'dense-vesicle' phosphodiesterase from 'hypotonic extracts'

A 0.5 ml portion of the immunoabsorbent matrix (antibody DV4/protein A–Sepharose) was washed four times in 10 mM-Tris/HCl, pH 7.4, containing 1 mM-EDTA, by centrifuging in an Eppendorf micro-centrifuge (15000 g) for 15 s and finally resuspended in the same buffer. A sample (0.5 ml) of 'hypotonic extract' thus obtained was incubated with an equal volume of the immunoabsorbent at room temperature with occasional shaking. After 30 min the mixture was centrifuged as above, and the supernatant was removed for assay of phosphodiesterase activity. The complex was washed twice as above and finally resuspended in the same buffer

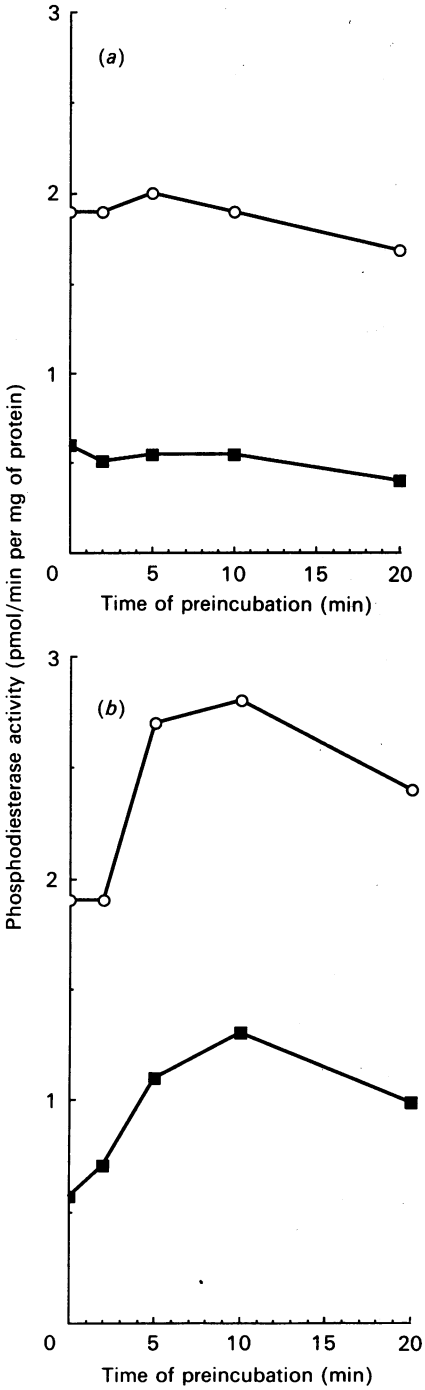

Fig. 1. Effect of time of preincubation with $MgCl_2$ on activation of phosphodiesterase by A-kinase

(*a*) The effect of preincubation of membranes with $MgCl_2$ on phosphodiesterase activity was investigated here. Particulate fraction P_1 was prepared as described in the Materials and methods section; a sample (3.5 mg of protein) was suspended in 0.8 ml of buffer B, containing 5 mM-$MgCl_2$. Samples were taken for the assay of cyclic AMP phosphodiesterase activity at the indicated times. ICI 118233 (100 μM) was included in some assays to ascertain the contribution of the 'dense-vesicle' phosphodiesterase activity (■) compared with the total activity (○) seen in its absence. A representative experiment of one performed at least three times is shown. (*b*) Preincubation of membranes with $MgCl_2$ prior to activation with A-kinase was investigated here. Thus, a particulate fraction P_1 was prepared as described in the Materials and methods section; a sample (3.5 mg of protein) was then suspended in 0.8 ml of buffer B (which contained 5 mM-$MgCl_2$) and was preincubated at 30 °C for the times indicated. NaF and β-glycerophosphate were then added, and two 450 μl portions were removed from each sample and incubated at 30 °C for 10 min in the presence of 0.1 mM-ATP with or without 50 units of A-kinase. Samples were then assayed for phosphodiesterase activity. ICI 118233 (100 μM) was included in some assays to ascertain the contribution to the 'dense-vesicle' phosphodiesterase (■) to total (○) enzyme activity. Phosphodiesterase activity was also assessed at the end of each incubation time and before treatment with A-kinase. This basal activity did not change (< 5%) throughout the experiments. Indeed, it was the same as that noted when incubations were done in buffer A, which did not contain Mg^{2+} (results not shown). A representative experiment of one performed at least three times is shown.

for the assay of phosphodiesterase activity. As a control, 0.5 ml of 'hypotonic extract' was incubated for 30 min at room temperature, then assayed for enzyme activity.

Immunoblotting procedure

Proteins were separated by SDS/PAGE, then transferred to nitrocellulose and immunoblotted, by using the antibody DV4, as described by Milligan *et al.* (1986). Labelled bands were identified by using anti-rabbit peroxidase-linked IgG.

Phosphorylation procedure

Particulate fractions were prepared from hepatocytes and treated with A-kinase as described above, except that 0.1 mM-[γ-^{32}P]ATP (200 mCi/mmol) was included in the incubations. The reaction was terminated by the addition of an ice-cold detergent mixture which gave final concentrations of 60 mM-Tris/HCl, pH 7.5, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 50 mM-NaF, 10 mM-β-glycerophosphate, 1 mM-EDTA, 0.15 M-NaCl and 100 μg of protein kinase inhibitor in a total volume of 1 ml. The 'dense-vesicle' phosphodiesterase was immunoprecipitated at 4 °C in this detergent mixture with the antiserum DV4. After overnight incubation, 50 μl of a 10% (w/v) suspension of Protein A was added, and the incubations were continued for 90 min at 4 °C. The precipitate was then pelleted by centrifugation for 2 min in a micro-centrifuge (16000 $g_{av.}$ for 10 min), and was washed once with a detergent mixture consisting of 50 mM-Tris/HCl, pH 7.5, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM-EDTA and 0.15 M-NaCl. This process was repeated with this detergent mixture but with the NaCl concentration increased to 0.5 M, and lastly performed by washing twice with phosphate-buffered saline, pH 7.5. All centrifugal washes were performed at 4 °C. The final pellet was resuspended in Laemmli (1970) buffer, and the phosphoproteins were separated by SDS/PAGE and identified by autoradiography of the fixed and dried gel. The labelled bands were quantified by excising them from the dried gel and then counting for radioactivity, and also by scanning densitometry of the autoradiograph with a Bio-Rad gel scanner driven by an Olivetti M24 microcomputer and using the Bio-Rad 'I-D' software package.

Labelling of isolated hepatocytes with [^{32}P]phosphate

Hepatocytes were incubated at 37 °C as described above, except that the Krebs–Henseleit (1932) buffer was adjusted to contain 50 μM-[^{32}P]phosphate (0.1 mCi/ml). The incubations were continued for 1 h, the optimum time to achieve labelling of the intracellular ATP pool (Hopkirk & Denton, 1986). Particulate fraction P_i was then prepared and resuspended in 0.8 ml of buffer B. The 'dense-vesicle' phosphodiesterase was immunoprecipitated from 0.4 ml portions of the suspension as described above.

RESULTS

Incubation of the hepatocyte particulate fraction P_1 with 5 mM-$MgCl_2$ for 10 min had little effect on cyclic AMP phosphodiesterase activity (Fig. 1*a*). However, addition of A-kinase, after incubation of the hepatocyte particulate fraction (P_1) with 5 mM-$MgCl_2$, resulted in a marked increase of the cyclic AMP phosphodiesterase activity (Table 1, Expt. 1; $P < 0.05$).

The inhibitor ICI 118233 was used to assess the contribution of the 'dense-vesicle' phosphodiesterase to this activation of total phosphodiesterase activity, as ICI 118233 has been shown to inhibit selectively the 'dense-vesicle' phosphodiesterase in liver (Pyne *et al.*, 1987*b*). Such experiments showed that activation of the dense-vesicle' enzyme accounted entirely (over 95%) for the stimulation of phosphodiesterase activity in the membrane fraction (Table 1). The magnitude of the increase in 'dense-vesicle' phosphodiesterase activity, after incubation of fraction P_1 with A-kinase, varied between preparations, but was always 2–3-fold. This effect is likely to be mediated via a phosphorylation reaction because, in the absence of ATP, A-kinase failed to stimulate phosphodiesterase activity (Table 1, Expt. 6). Furthermore, treatment of membranes with ATP alone had no discernible effect on phosphodiesterase activity (Table 1).

Maximal stimulation of phosphodiesterase activity occurred when the membrane fraction had been preincubated with $MgCl_2$ before incubation with A-kinase (Table 1). The optimum time required for preincubation with $MgCl_2$ was 10 min (Fig. 1*b*), during which there was no change in either the total or the 'dense-vesicle' cyclic AMP phosphodiesterase activity (Fig. 1*a*).

Preincubation of samples without $MgCl_2$ decreased the percentage stimulation ($P < 0.05$) of the 'dense-vesicle' phosphodiesterase activity during subsequent incubation with A-kinase (Table 1, Expt. 4). As before, this stimulation was completely abolished when protein phosphatase inhibitors were present during the preincubation period (Table 1, Expt. 5). No activation of phosphodiesterase activity occurred when samples were preincubated with 5 mM-EDTA before addition of A-kinase (Table 1, Expt. 3).

When the protein phosphatase inhibitors, NaF and β-glycerophosphate were included along with $MgCl_2$ during the preincubation, treatment with A-kinase failed to stimulate phosphodiesterase activity (Table 1, Expt. 2). Nevertheless, protein phosphatase inhibitors had to be added at the end of the preincubation period and during incubation with A-kinase and ATP, in order for stimulation of phosphodiesterase activity to be achieved (Table 1, Expt. 7).

Table 1. Effect of preincubation conditions on the stimulation of phosphodiesterase activity by A-kinase

Hepatocyte fraction P_1 was prepared as described in the Materials and methods section and was suspended in 10 mM-Tris/HCl, pH 7.4, containing 0.25 M-sucrose and the additions indicated. After preincubation at 30 °C for 10 min, all samples were made up to 1.0 ml total volume containing 5 mM-$MgCl_2$ and, where indicated, the phosphatase inhibitors (PTI) 50 mM-NaF and 10 mM-β-glycerophosphate (BGP). Two separate 0.45 ml portions were removed from all samples and incubated for a further 10 min at 30 °C in the absence or presence of 0.1 mM-ATP and with or without A-kinase as indicated. Phosphodiesterase activity was then assayed in the absence and presence of 100 μM-ICI 118233. Results are means ± S.E.M. for three observations: *, ** indicate that the value is significantly different from that for the appropriate incubation in the absence of A-kinase (*$P < 0.05$, **$P < 0.01$).

Expt. no.	Additions to preincubation	Additions post-incubation: ATP	A-kinase	PTI	Total phosphodiesterase (pmol/min per mg)	Total phosphodiesterase (% stimulation)	'Dense-vesicle' phosphodiesterase (pmol/min per mg)	'Dense-vesicle' phosphodiesterase (% stimulation)
1	$MgCl_2$	+	+	+	3.4±0.3*	28.3±6.9	2.2±0.2**	102.0±13.6
	$MgCl_2$	+	−	+	2.7±0.1		1.0±0.04	
2	$MgCl_2$ + NaF + BGP	+	+	+	2.3±0.2	−5.7±5.7	1.0±0.03	−16.0±8.0
	$MgCl_2$ + NaF + BGP	+	−	+	2.5±0.1		1.2±0.1	
3	EDTA (5 mM)	+	+	+	2.3±0.2	−1.0±3.0	1.0±0.1	0.7±6.0
	EDTA (5 mM)	+	−	+	2.3±0.2		0.9±0.1	
4	–	+	+	+	3.0±0.2	14.0±4.0	1.4±0.1	20.1±12.0
	–	+	−	+	2.6±0.1		1.2±0.1	
5	NaF + BGP	+	+	+	2.6±0.1	8.8±4.4	1.1±0.1	2.7±2.7
	NaF + BGP	+	−	+	2.4±0.2		1.1±0.1	
6	$MgCl_2$	−	+	+	2.5±0.1	4.7±4.7	1.0±0.1	−0.7±11.0
	$MgCl_2$	−	−	+	2.4±0.1		1.0±0.3	
7	$MgCl_2$	+	+	−	2.6±0.3	5.7±2.8	0.8±0.2	3.3±2.9
	$MgCl_2$	+	−	−	2.4±0.3		0.8±0.2	

Table 2. Phosphodiesterase activation by a homogeneous A-kinase preparation and blockade of activation of the partially purified preparation by A-kinase inhibitor

Particulate fraction P_1 was preincubated with Mg^{2+} in buffer B before being treated with A-kinase and ATP in the absence or presence of A-kinase inhibitor (2.5 mg) as described in the Materials and methods section. A-kinase either was from Sigma or was purified to apparent homogeneity from bovine heart by the method of Reimann & Beham (1983). Phosphodiesterase activity was measured in the absence or presence of 100 μM-ICI 118233 to ascertain the contribution of the 'dense-vesicle' enzyme to the total activity. Results are means ± S.E.M. for three observations: * indicates that the value is significantly different from that for the appropriate incubation in the absence of A-kinase ($P < 0.01$).

Additions to incubation	Total phosphodiesterase		'Dense-vesicle' phosphodiesterase	
	(pmol/min per mg)	(% stimulation)	(pmol/min per mg)	(% stimulation)
No addition	1.9±0.04	–	0.62±0.05	–
A-kinase (Sigma)	2.5±0.1*	29.3±3.9	1.25±0.1*	102.0±8.0
A-kinase (Sigma)+ kinase inhibitor	1.8±0.1	−8.3±3.6	0.7±0.5	22.0±3.0
No addition	1.7±0.03	–	0.55±0.06	–
A-kinase (purified)	2.4±0.06*	41.0±6.0	1.23±0.04*	128.5±30.0

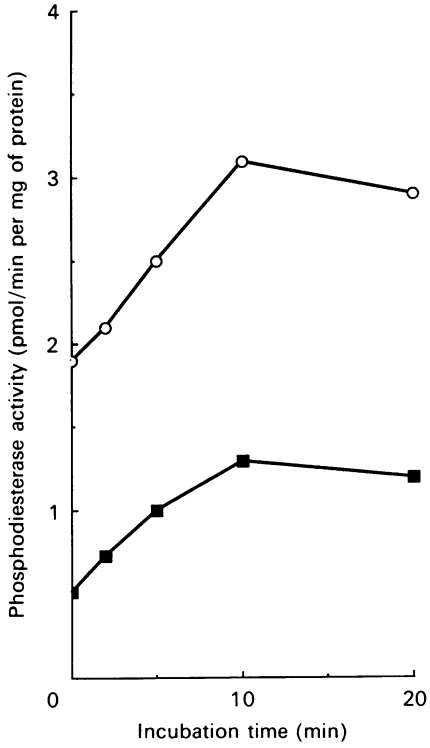

Fig. 2. Time course for the activation of phosphodiesterase by A-kinase

The experimental procedure was as described in the legend to Fig. 1, except that samples were preincubated for 10 min with buffer B before being incubated with A-kinase and ATP for the times indicated. Activity of the 'dense-vesicle' phosphodiesterase (■) and total (○) phosphodiesterase are shown. Basal enzyme activity, i.e. that in the absence of either A-kinase or ATP, did not vary throughout the incubation (< 5% change). A representative experiment of one performed at least three times is shown.

The stimulatory effect of the partially purified A-kinase preparation used in these studies on the cyclic AMP phosphodiesterase could be blocked by the addition of the specific peptide inhibitor of A-kinase (Table 2). Furthermore, activation of the phosphodiesterase could be mimicked by using a homogeneous preparation of A-kinase (Table 2).

Treatment of the preincubated membranes with A-kinase led to optimal activation of the 'dense-vesicle' enzyme after 10 min (Fig. 2).

If A-kinase-treated membranes were washed by repeated centrifugation (15 min at 15000 *g*) in the NaF- and β-glycerophosphate-containing buffer C, and were then incubated for 10 min at 30 °C, then little change in specific activity occurred. However, if the same treatment was performed in the absence of phosphatase inhibitors (buffer B), the cyclic AMP phosphodiesterase specific activity returned to that exhibited by membranes which had not been treated with A-kinase (Table 3). This demonstrated that the stimulatory effect of A-kinase was reversible.

The 'dense-vesicle' enzyme can be released from its membrane environment by a hypo-osmotic-shock procedure (Loten *et al.*, 1978). When such a procedure was applied to a hepatocyte particulate fraction P_1 which had been treated with A-kinase and ATP, in order to activate the 'dense-vesicle' enzyme, then all of the activated enzyme was found in the hypo-osmotically shocked, solubilized fraction (Table 4). This solubilized activated phosphodiesterase activity was completely inhibited upon addition of either ICI 118233 or low concentrations of cyclic GMP to the assays (Table 4), as expected for the 'dense-vesicle' phosphodiesterase.

The polyclonal antiserum DV4, as shown previously with antisera DV1 and DV2 (Pyne *et al.*, 1987*a*,*b*; Houslay *et al.*, 1988), recognized the purified 'dense-vesicle' cyclic AMP phosphodiesterase specifically in Western (immuno-) blotting (results not shown). It also identified the near-identical 51 kDa minor and 57 kDa major subunits of this protein, which have been shown (Pyne *et al.*, 1987*a*) to be released by hypo-osmotic-shock treatment of the membrane fraction (results not shown). No such recognition was observed with pre-immune serum.

An immunoabsorbent matrix was prepared by linking antiserum DV4 covalently to Protein A–Sepharose gel (Werner & Machleidt, 1978). Hypo-osmotic extracts were

Table 3. Reversal of the activation of the 'dense-vesicle' enzyme by A-kinase

Particulate fraction P_1 was preincubated with Mg^{2+} in buffer B before being treated with A-kinase and ATP as described in the Materials and methods section. Samples were then divided into two equal portions and centrifuged at 22000 $g_{av.}$ for 15 min, and the pellets were washed by resuspension in 5 ml of buffer B with or without phosphatase inhibitors (50 mM-NaF and 10 mM-β-glycerophosphate) and centrifuged as before. Pellets were finally resuspended in buffer B with or without phosphatase inhibitors as before and incubated at 30 °C. After 10 min phosphodiesterase activity was measured. ICI 118233 was included in some assays to ascertain the contribution of the 'dense-vesicle' phosphodiesterase to total enzyme activity. The recovery of both total and 'dense-vesicle' phosphodiesterase activity after the centrifugation procedures was 88–96%. Results are means ± S.E.M. for three observations: *, ** indicate that the value is significantly different from that for the appropriate incubation in the absence of A-kinase (*$P < 0.05$, **$P < 0.01$).

Expt. no	Treatment	A-kinase	Total phosphodiesterase (pmol/min per mg)	Total phosphodiesterase (% stimulation)	'Dense-vesicle' phosphodiesterase (pmol/min per mg)	'Dense-vesicle' phosphodiesterase (% stimulation)
1	Before washing	−	3.4 ± 0.1		1.7 ± 0.07	
		+	4.8 ± 0.1*	41.5 ± 8.5	4.0 ± 0.1**	135.0 ± 14.2
2	After washing with phosphatase inhibitors	−	3.1 ± 0.1		1.8 ± 0.1	
		+	4.7 ± 0.2*	52.1 ± 11.6	3.4 ± 0.2*	89.0 ± 15.5
3	After washing without phosphatase inhibitors	−	2.8 ± 0.1		1.8 ± 0.06	
		+	3.0 ± 0.1	6.5 ± 1.0	1.9 ± 0.1	5.5 ± 2.2

Table 4. Effect of A-kinase pre-treatment on the phosphodiesterase activity released by a hypo-osmotic-shock procedure

Particulate fraction P_1 was pre-incubated with Mg^{2+} in buffer B (total vol. 0.8 ml) before being treated with A-kinase and ATP as described in the Materials and methods section. The samples were then solubilized by the hypo-osmotic-shock procedure, and phosphodiesterase activity was measured. ICI 118233 (100 μM) and cyclic GMP (2 μM) were included in some assays to ascertain the contribution of 'dense-vesicle' phosphodiesterase activity to total enzyme activity, by selectively inhibiting this enzyme (Pyne *et al.*, 1987*a*,*b*). The 'ICI-118233-inhibitable' and 'cyclic-GMP-inhibitable' fraction makes up over 85% of the total hypo-osmotic-shock released phosphodiesterase activity. After the hypo-osmotic-shock procedure the recovery of ICI-118233- and cyclic GMP-inhibitable activity was 95 ± 4%; that of total phosphodiesterase (PDE) activity was 84.5 ± 7.5%. Results are means ± S.E.M. for four observations: *** indicates the value is significantly different from the appropriate value for the incubation without A-kinase ($P < 0.001$).

Analysis	Cyclic AMP phosphodiesterase activity (pmol/min per mg of protein): No treatment with A-kinase	After membrane treatment with A-kinase
Total PDE activity	7.5 ± 0.92	16.3 ± 0.71***
ICI-118233-inhibitable fraction	6.4 ± 0.66	14.9 ± 0.30***
Cyclic GMP-inhibitable fraction	6.3 ± 0.82	15.1 ± 0.43***

Table 5. Antiserum DV4 can specifically immunoabsorb the solubilized 'dense-vesicle' enzyme in both native and activated activity states

Particulate fraction P_1 was incubated with or without A-kinase and then 'hypotonic extracts' were prepared as described in the Materials and methods section. A sample of each extract was incubated with DV4-immunoabsorbent matrix at room temperature. After 30 min the DV4 complex was pelleted by spinning in an Eppendorf centrifuge for 15 s, and the supernatant (unbound) was removed. Both the unbound material (supernatant) and the immunoabsorbed material were assayed for phosphodiesterase activity. Control samples of 'hypotonic extract' were incubated at room temperature for 30 min, then assayed for enzyme activity. ICI 118233 (100 μM) and cyclic GMP (2 μM) were included in some assays to ascertain the contribution of the 'dense-vesicle' phosphodiesterase to total enzyme activity. Further details of the experimental procedure are given in the Materials and methods section.

Fraction	A-kinase	Phosphodiesterase activity (nmol/min): Total	ICI 118233-inhibited	Cyclic GMP-inhibited
'Hypotonic extract'	−	286	215	220
	+	524	449	459
Bound to immunoabsorbent	−	193	192	195
	+	410	407	400
Unbound material	−	106	18	18
	+	124	32	21

incubated with the immunoabsorbent matrix for 30 min at room temperature and then centrifuged to yield pellet and supernatant fractions (see the Materials and methods section). In control experiments, i.e. in the absence of the immunoabsorbent and when membranes had not been challenged with A-kinase, there were no differences (less than 5%) between extracts that were left on ice for 30 min

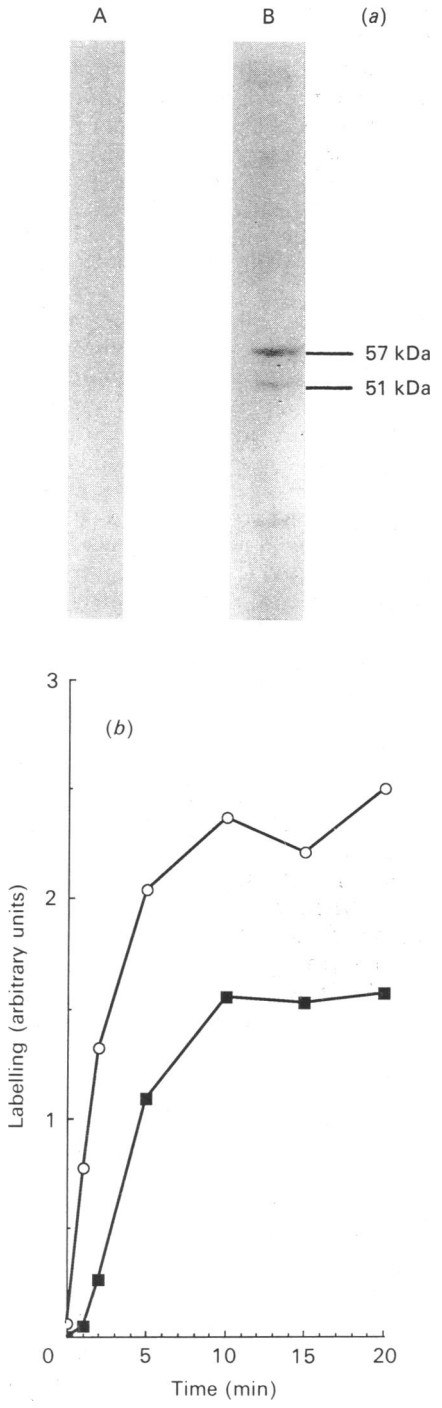

Fig. 3. Phosphorylation of the 'dense-vesicle' phosphodiesterase by A-kinase

(*a*) Particulate fraction P_1 was preincubated in buffer B for 10 min. NaF and β-glycerophosphate were then added, and the incubation was continued for a further 10 min in the presence of [γ-^{32}P]ATP either without A-kinase for 10 min (track A) or with A-kinase for 10 min (track B). The 'dense-vesicle' phosphodiesterase was solubilized and subsequently immunoprecipitated with antiserum DV4. The proteins were separated by SDS/PAGE, and incorporation of label was identified by autoradiography. Tracks from an autoradiograph obtained after 48 h exposure of the fixed and dried gel are shown. (*b*) This experiment was performed over a time course, and labelling was analysed either by scanning densitometry of the autoradiography or by cutting out the labelled bands from the gel and counting them for radioactivity. This plot shows the time-dependent increase in labelling of the 51 kDa (■) and 57 kDa (○) bands, and show a typical experiment of one performed three times.

and those incubated at room temperature, in total phosphodiesterase activity or in the amount of activity inhibited by ICI 118233 and cyclic GMP (results not shown). In the absence of any immunoabsorbent, the increase in 'dense-vesicle' phosphodiesterase activity caused by treatment with A-kinase was, however, decreased by some 25–30% after incubation of extracts at room temperature for 30 min. When control extracts were incubated with the immunoabsorbent matrix, the phosphodiesterase activity associated with it was totally (over 95%) inhibited by both ICI 118233 and cyclic GMP, showing that the matrix specifically bound the 'dense-vesicle' phosphodiesterase (Table 5). After incubation of hypo-osmotic extracts with the immunoabsorbent matrix, the phosphodiesterase activity that had been stimulated by A-kinase treatment was removed (over 95%) from the extracts by being bound to the matrix, leaving the unstimulated activity in the supernatant. This provides further evidence to show that it is the 'dense-vesicle' isoenzyme that is activated by A-kinase treatment.

Solubilization of membranes by hypo-osmotic-shock treatment or detergents liberates the 110 kDa 'dense-vesicle' enzyme, consisting of a major 57 kDa subunit and a minor 51 kDa subunit, which can be immunoprecipitated by the antiserum DV4 as well as being recognized by Western blotting. The antisera DV1 and DV2 also selectively immunoabsorb 'dense-vesicle' phosphodiesterase activity (Pyne *et al.*, 1987*b*; Houslay *et al.*, 1988). Incubation of fraction P_1 with A-kinase and [γ-^{32}P]ATP was followed by immunoprecipitation with antiserum DV4, separation of phosphoproteins by SDS/PAGE and subsequent autoradiography of the fixed and dried gel. Such a procedure revealed that A-kinase increased the phosphorylation of both the 57 kDa and 51 kDa species (Fig. 3). Analysis of the autoradiograph by densitometry and of the dried gel by counting the radioactive content of the excised phosphoproteins revealed that the time course for phosphorylation of the 'dense-vesicle' enzyme (Fig. 3) was very similar to that for activation of the enzyme by A-kinase (Fig. 2). Thus near-maximal incorporation of phosphate and maximal activation of the enzyme were achieved after incubation with A-kinase for approx. 10 min. We calculated that 0.79 ± 0.15 mol of ^{32}P was incorporated per mol of enzyme ($n = 3$, S.D.).

Thus exposure of $MgCl_2$-pretreated membranes to A-kinase increased the labelling of the 57 kDa band some 28.5 ± 2.5-fold and the 51 kDa band some 26.3 ± 3.2-fold, as detected by densitometric scanning. However, if the membranes had not been pretreated with $MgCl_2$, then A-kinase exerted no effect on the fold difference in labelling, being 1.1 ± 0.3-fold for the 57 kDa band and 0.95 ± 0.15-fold for the 51 kDa band ($n = 3$).

The antiserum DV4 was also used to investigate whether the 'dense-vesicle' phosphodiesterase in hepatocyte membranes was indeed phosphorylated. In order to do this, we labelled hepatocytes with [^{32}P]P_i, as described in the Materials and methods section, before membrane preparation. The membranes were then solubilized, immunoprecipitated with antiserum DV4

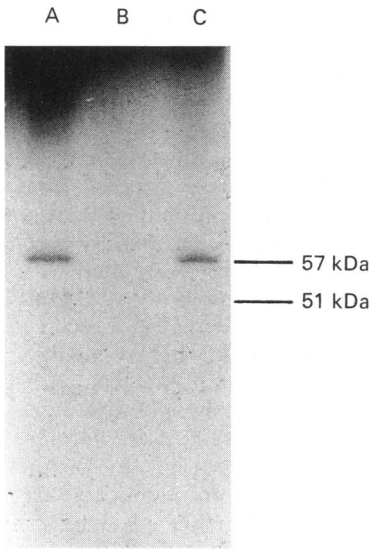

Fig. 4. ^{32}P labelling of intact hepatocytes and the phosphorylation of the 'dense-vesicle' phosphodiesterase

Hepatocytes were incubated with [^{32}P]P_i as described in the Materials and methods section. A particulate fraction (P_1) was made exactly as in the experiments used to study phosphodiesterase activation. Membranes were then used (track A) with no further treatment, (track B) after 10 min preincubation at 30 °C in 10 mM-Tris/HCl (pH 7.4) with 5 mM-$MgCl_2$, and (track C) as in track B, except that 50 mM-NaF and 10 mM-β-glycerophosphate were also present. Thus tracks B and C correspond respectively to the preincubation conditions of Expts. 2 and 1 in Table 1. The membranes were then solubilized and immunoprecipitated with antiserum DV4 as described in the Materials and methods section. This shows a typical experiment of one done three times.

and then subjected to SDS/PAGE and autoradiography. From such experiments we could show incorporation into the 57 kDa (major species) and 51 kDa (minor species) subunits of the enzyme (Fig. 4, track A). If, however, membranes were treated with 5 mM-$MgCl_2$ for 10 min at 30 °C, before solubilization and immunoprecipitation, then no labelled bands were evident (Fig. 4, track B), implying that dephosphorylation had occurred. This was supported by our observation that if, together with $MgCl_2$ also 50 mM-NaF and 10 mM-β-glycerophosphate were added, we observed no loss of label from these bands compared with the untreated state (Fig. 4, track C).

DISCUSSION

We and others have shown that both in hepatocytes (Heyworth *et al.*, 1983; Boyes *et al.*, 1981) and in adipocytes (Manganiello, 1987) there is a high-affinity membrane-bound cyclic AMP phosphodiesterase which is activated by hormones such as glucagon and by β-adrenoceptor activation (see also Francis & Kono, 1982). Both of these hormones activate adenylate cyclase and increase the intracellular concentration of cyclic AMP. It has been long considered that such activation was mediated via A-kinase, as activation could also be mimicked by treating such cells with permeant analogues of cyclic AMP such as dibutyryl cyclic AMP and 8-bromo cyclic AMP (Heyworth *et al.*, 1983; Corbin *et al.*, 1986). However, despite the purifications of such enzymes from adipose tissue (Degerman *et al.*, 1987), heart (Harrison *et al.*, 1986) and liver (Pyne *et al.*, 1987*a*) to apparent homogeneity, there has been no evidently successful demonstration that the purified enzymes can be phosphorylated by A-kinase and that this could lead to activity changes.

We have called this high-affinity intracellular-membrane enzyme the 'dense-vesicle' phosphodiesterase (Heyworth *et al.*, 1983). With the thought that the membrane environment may be important for the activation process, we have attempted here to determine whether the 'dense-vesicle' enzyme could be phosphorylated and activated, in an isolated membrane fraction from hepatocytes, by the free activated catalytic unit of A-kinase. The identification of 'dense-vesicle' enzyme activity can be resolved by assaying the membrane fraction either in the presence of the compound ICI 118233 or at low cyclic GMP concentrations, which selectively inhibit this enzyme (Pyne *et al.*, 1987*a*,*b*). Furthermore, hypo-osmotic shock-treatment, which has been shown by many investigators (see, e.g., Loten *et al.*, 1978; Heyworth *et al.*, 1983) to release a solubilized form of this enzyme in an activated state, can also be used to resolve this hormone-regulated species.

However, we discovered here that treatment of this membrane fraction with ATP and A-kinase was singularly unsuccessful in altering the activity of the 'dense-vesicle' enzyme, whether or not phosphatase inhibitors were present. Thus, as with treatment of the purified homogeneous enzyme with A-kinase (N. J. Pyne, E. Kilgour, N. G. Anderson & M. D. Houslay, unpublished work), no activation of the phosphodiesterase resulted.

In contrast with such observations, we observed that, if the membrane fraction was preincubated with Mg^{2+} for a short time, then the subsequent addition of A-kinase and ATP resulted in activation of the 'dense-vesicle' enzyme (Table 1). It would appear that the action of Mg^{2+} during the preincubation period is thus to stimulate endogenous phosphatases which act on the 'dense-vesicle' enzyme. This was deduced from our observation that, if protein phosphatase inhibitors such as NaF and β-glycerophosphate were included with Mg^{2+} during the preincubation period, then A-kinase failed to activate the 'dense-vesicle' enzyme (Table 1). This was not due to any effect of such compounds in interfering with the action of A-kinase on the 'dense-vesicle' enzyme, because, if, after preincubation with Mg^{2+} alone, these compounds were subsequently included in the incubation with A-kinase, then activation was potentiated. Indeed, a phosphorylated form of this enzyme could be immunoprecipitated from membranes isolated from ^{32}P-labelled hepatocytes (Fig. 4). The treatment with $MgCl_2$ apparently removed ^{32}P from this protein in a fashion which was blocked by the addition of phosphatase inhibitors.

The role of protein phosphatases during the preincubation could have been to dephosphorylate, and hence make available for rephosphorylation, the site on the 'dense-vesicle' enzyme that was responsible for activation of the enzyme during subsequent treatment with A-kinase. If this were the case, a fall in phospho-

diesterase activity would have been expected to occur during the preincubation period. As no such decrease in enzyme activity occurred, we propose that protein phosphatases dephosphorylated the 'dense-vesicle' phosphodiesterase at a site distinct from that phosphorylated during subsequent treatment with A-kinase. Thus it is possible that the 'dense-vesicle' phosphodiesterase contains at least two regulatory sites which can be phosphorylated: one being a site for phosphorylation by A-kinase, which enhances enzyme activity, and the other a site which prevents phosphorylation and activation by A-kinase. Certainly other enzymes, such as glycogen synthase, pyruvate dehydrogenase and acetyl-CoA carboxylase, are known to be regulated by multi-site phosphorylation mechanisms (for reviews see Cohen, 1985; Krebs, 1985). Indeed, Carling *et al.* (1987) have purified a kinase which they propose could account for the high basal phosphorylation and low activity state of acetyl-CoA carboxylase isolated from rat liver. The kinase, which is activated by 5'-AMP, has been shown to be stimulated when cells are homogenized, owing to the subsequent anoxia and hence increased concentration of 5'-AMP (Carling *et al.*, 1987). Intriguingly, they have shown that this 5'-AMP kinase prevents A-kinase from phosphorylating acetyl-CoA carboxylase (Munday *et al.*, 1988).

Solubilization of the membrane fraction, in order to release the 'dense-vesicle' enzyme, allowed it to be specifically immunoprecipitated with the antibody DV4. Incubation of the untreated membranes with [^{32}P]ATP and A-kinase failed to show any increase in the incorporation of phosphate into the enzyme, which was in agreement with our failure to detect any change in phosphodiesterase activity under such conditions. However, if membranes were pre-treated with Mg^{2+}, before incubation with A-kinase and [^{32}P]ATP, then we observed the time-dependent incorporation of label into both 51 and 57 kDa components of the 'dense-vesicle' enzyme (Fig. 3). The rate of incorporation of label showed a remarkably good correlation with the increase in activity elicited (Figs. 1 and 3).

We propose that phosphorylation of the 'dense-vesicle' enzyme accounts for its activation by A-kinase. A-kinase could have phosphorylated the 'dense-vesicle' enzyme directly, or alternatively could have phosphorylated another kinase, acting as an intermediary, which then resulted in an increase in the phosphorylation of the 'dense-vesicle' enzyme. If the latter mechanism was correct, the intermediary phosphatase or kinase would have to be membrane-bound or tightly associated with the 'dense-vesicle' phosphodiesterase.

Our results provide the first evidence to support the contention that activation of A-kinase mediates the activation of the 'dense-vesicle' phosphodiesterase which occurs when intracellular cyclic AMP is increased (Heyworth *et al.*, 1983). They further indicate that the 'dense-vesicle' enzyme is regulated by multi-site phosphorylation. This may include a site for phosphorylation by A-kinase which results in enhanced phosphodiesterase activity, and a site for phosphorylation by an unidentified kinase which, although not having any obvious effect on activity, prevents phosphorylation and activation by A-kinase. It is possible that the latter kinase may be the 5'-AMP kinase described by Carling *et al.* (1987). As insulin can also activate this enzyme by a mechanism distinct from that used by glucagon (Wilson *et al.*, 1983), it is possible that a third site of phosphorylation occurs on this enzyme.

As the 'dense-vesicle' phosphodiesterase appears to be regulated by highly sophisticated control mechanisms, it may be that it plays a pivotal role in controlling cellular processes which are regulated by cyclic AMP. Indeed, it would seem that this enzyme is expressed in relatively high amounts in cardiac tissue (Pyne *et al.*, 1987*b*), where it may provide the site of action of cardiotonic drugs which act to inhibit it selectively.

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