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Microheterogeneity of Type II cAMP-dependent Protein Kinase in Various Mammalian Species and Tissues*

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Excluding autophosphorylated species, at least six forms of the regulatory subunit of type II cAMP-dependent protein kinase (R^{II}) from various mammalian tissues were identified by sodium dodecyl sulfate (SDS) gel electrophoresis of purified samples and of crude preparations photoaffinity labeled with 8-azido[^{32}P] cAMP and by gel filtration. After autophosphorylation some heart R^{II} forms termed type IIA (bovine, porcine, equine, and dog) shifted to a more slowly migrating band on SDS gels while others termed type IIB (rat, guinea pig, rabbit, and monkey) did not detectably shift. Both subclasses of R^{II} exhibited variation in apparent M_r on SDS gels. Bovine and porcine heart non-autophosphorylated R^{II} had M_r 56,000 and the autophosphorylated R^{II} had M_r 58,000, while dog and equine heart R^{II} had M_r 54,000 and 56,000 for these bands, respectively. Rat heart R^{II} had M_r 56,000 while rabbit and guinea pig heart R^{II} had M_r 52,000. More than one R^{II} was found in different tissues of the same species. Rabbit skeletal muscle contained a M_r 56,000 IIB form. Bovine lung contained almost equal amounts of a IIA form apparently identical to that of bovine heart and a M_r 52,000 IIB form similar to that which predominated in bovine brain. Rat adipose tissue, brain, and monkey heart contained predominantly a M_r 51,000 IIB form. The rat liver M_r 56,000 IIB form chromatographed differently from all other R^{II} tested by gel filtration. Several lines of evidence indicated that the various forms of R^{II} were not derived from one another through proteolysis or other processes. Each of the type II forms rapidly incorporated 0.3–1.0 mol of ^{32}P per mol of subunit when incubated with [γ-^{32}P]ATP and C subunit. Four of the forms tested were similar in the cAMP concentration dependence for activation of their corresponding holoenzymes and inhibited C subunit about equally. Each exhibited two components of [^{3}H]cAMP dissociation, indicating two intrachain cAMP-binding sites, and the dissociation rates for the respective sites were similar.

In the past it has been widely believed that the regulatory subunit of type II cAMP-dependent protein kinase (R^{II}) from various mammalian tissues is very similar, if not identical, in its properties.[1] However, when the existence of the two types of cAMP-dependent protein kinase was first described (1), it was pointed out that rat adipose tissue type II eluted differently from rat heart type II on DEAE-cellulose columns. It was also shown that mixtures of the two tissue forms rechromatographed as two separate peaks (1). Subsequently it was found (2) that the human heart type II holoenzyme differed from type II of hearts from five other species in its elution from DEAE-cellulose. More recently Rubin *et al.* (3) described a neural subclass of type II which was immunologically distinct from that of other tissues. Bovine heart R^{II} has been found to exhibit a doublet upon SDS-polyacrylamide gel electrophoresis in Tris-glycine buffer (4–6). The lower band of M_r 56,000 corresponded to dephosphorylated R^{II} and could be converted into the more slowly migrating band of apparent M_r 58,000 upon phosphorylation by MgATP and catalytic (C) subunit of cAMP-dependent protein kinase (4, 5). Bovine brain R^{II} was different from bovine heart R^{II} in that only a partial shift in mobility occurred upon phosphorylation, with ^{32}P label from [γ-^{32}P]ATP appearing in both R^{II} bands of M_r 55,000 and 57,000 (7). However, R^{II} from bovine (7) or porcine (8) skeletal muscle also exhibited a doublet upon SDS-polyacrylamide gel electrophoresis under conditions similar to those described above, and the two bands were believed to represent the phosphorylated and dephosphorylated forms. Thus it appeared as if a shift in electrophoretic mobility upon phosphorylation by C subunit was characteristic of non-neural mammalian R^{II}. As will be shown in this communication, however, in neither highly purified type II holoenzyme nor crude extracts from rat heart was there a shift in mobility of R^{II} upon autophosphorylation. This finding prompted the investigation of R^{II} from hearts and other tissues of various mammalian species. From a comparison of mobility upon gel electrophoresis and Stokes radius measurements, and the effect of autophosphorylation and proteolysis, R^{II} is shown to be very tissue and species microheterogeneous. This is in apparent contrast to the catalytic subunit, which thus far appears more homogeneous (2, 4).

EXPERIMENTAL PROCEDURES

Preparation of Crude Heart Extracts—Animals were anesthetized with Nembutal and the heart removed and washed in ice-cold saline to remove excess blood. After clamping between tongs cooled in liquid nitrogen at −70 °C, the frozen tissue was ground to a fine powder and stored at −70 °C. The frozen powder was suspended at 4 °C in 4 volumes (w/v) of 10 mM potassium phosphate buffer at pH 6.8, containing 10 mM EDTA, 10 mM sodium fluoride, 20 mM benzamidine, and 4 mM 2-mercaptoethanol, and homogenized at medium

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[1] The abbreviations used are: R and C, regulatory and catalytic subunits of cAMP-dependent protein kinase, respectively; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography.

speed for 45 s with a motor-driven Teflon pestle in a glass tube homogenizer. The homogenate was centrifuged in a Beckman J-21 centrifuge at 16,000 × *g* for 15 min at 4 °C and the supernatant decanted and used for the experiment.

Preparation of Partially Purified Holoenzymes—Heart extracts were prepared as described above in either the potassium phosphate buffer containing inhibitors as above or 10 mM potassium phosphate at pH 6.8 containing 1 mM EDTA. Following centrifugation, the supernatant was filtered through glass wool before loading onto a DEAE-cellulose column at 4 °C. When comparing elution profiles of different heart species, 2 to 2.5 g of frozen heart powder were used to make each extract, and the whole supernatant (8–10 ml) was applied to a DEAE-cellulose column of 0.9 × 5.5 cm. After loading, the column was washed with 40 ml of 10 mM potassium phosphate at pH 6.8 containing 1 mM EDTA, before starting a 30-ml linear gradient from 0 to 0.45 M NaCl in the same buffer. 1.5-ml fractions were collected, and for each elution profile a 50-μl aliquot of each column fraction was taken for measurement of the conductivity using a Radiometer conductivity meter. Samples of each fraction were taken to measure [^{3}H]cAMP binding and protein kinase activities. Samples of some fractions were subjected to SDS-polyacrylamide gel electrophoresis after labeling with 8-azido[^{32}P]cAMP or autophosphorylation with [γ-^{32}P]ATP. Fractions containing optimal concentrations of either the type I or type II holoenzyme were used to measure the effect of analogs on [^{3}H]cIMP binding.

Preparation of Purified R—R^{II} subunit was purified to homogeneity from hearts and other tissues of the various species as described previously for bovine heart (9). If there was much contamination by R^I after the DEAE-cellulose step, such as was the case when tissues contained a large excess of R^I over R^{II}, the R^{II} preparation was further purified by a second DEAE-cellulose column. R^{II} was then chromatographed on a 8-(6-aminohexylamino)-cAMP-Sepharose 4B affinity column. R^{II} subunit was purified from bovine lung using a slight modification of the above procedure since the cGMP-dependent protein kinase was first eluted from the cAMP-Sepharose column by addition of 1 mM cGMP before eluting R^{II} by addition of 10 mM cAMP to the column. Dog heart R^I subunit was purified by chromatography on a N^6-(aminoethyl)-cAMP-Sepharose 4B column of the 0.12 M NaCl wash of the DEAE-cellulose. This wash was routinely used to remove R^I prior to elution of R^{II} with 0.4 M NaCl.

Autophosphorylation—Samples from fractions of DEAE-cellulose columns containing type II holoenzyme (or type I holoenzyme to confirm no phosphorylation) were incubated for 10 min at 4 °C with 5.3 mM magnesium acetate and 0.5 μM [γ-^{32}P]ATP in a total volume of 50 μl before addition of 5 μl of 10% SDS and 5 μl of 2-mercaptoethanol to stop the reaction. When C subunit was added, samples were incubated as above with 5 mM magnesium acetate, 10 μM [γ-^{32}P]ATP, 0.5 μM cAMP, and 2 nM C subunit. On some occasions in order to follow the time course of ^{32}P incorporation, a larger reaction volume was used, and aliquots were pipetted onto phosphocellulose papers and washed in 75 mM phosphoric acid as described in the assay for protein kinase activity (10). An aliquot was also saved for SDS-polyacrylamide gel electrophoresis. To samples containing purified heart R^{II} were added 4 mM magnesium acetate, 20 μM [γ-^{32}P]ATP, 1 μM cAMP, and C subunit (~10 nM) in 10 mM potassium phosphate buffer at pH 6.8 containing 1 mM EDTA. After incubation at 20 °C for up to 60 min, aliquots were taken for gel electrophoresis and to phosphocellulose papers to determine ^{32}P incorporation.

Gel Electrophoresis—Samples were labeled with 8-azido[^{32}P]cAMP as described by Rubin *et al.* (3). Five μl of 10 μM 8-azido[^{32}P]cAMP were added to 45 μl of sample at 4 °C. After incubation in the dark at 4 °C for 30 min (or less in some cases), samples were irradiated with UV light for 15 min at 4 °C before addition of 5 μl of 10% SDS and 5 μl of 2-mercaptoethanol to stop the reaction. Samples were sometimes stored at −20 °C before boiling for 5 min prior to gel electrophoresis. Slab gel electrophoresis was performed with a 3% acrylamide stacking gel and usually a 7.5 or 8.5% acrylamide running gel in 0.025 M Tris buffer at pH 8.3 containing 0.192 M glycine and 0.1% SDS (11). Gels were stained with Coomassie Blue G-250 from Bio-Rad. Following destaining in 7% acetic acid, 45% methanol, autoradiographs from gels with samples labeled with ^{32}P from 8-azido[^{32}P]cAMP or [γ-^{32}P]ATP were prepared by exposure to x-ray film (Kodak X-Omat XRP-1).

Gel Filtration—Samples (50 μl) were injected onto a Bio-Rad HPLC TSK-250 gel filtration column (300 × 7.5 mm) equilibrated with 50 mM potassium phosphate at pH 6.8 containing 1 mM EDTA. Chromatography was performed under 40–60 kp.s.i. using a Beckman 344 HPLC system with a model 165 detector and an Altex C-R1B recorder-integrator. The flow rate was 0.7 ml/min. The standard proteins used to calculate Stokes radii (12) were myoglobin (1.9 nm), ovalbumin (2.8 nm), phosphorylase *b* (4.9 nm), aldolase (5.0 nm), catalase (5.2 nm), β-galactosidase (6.9 nm), monomeric bovine serum albumin (3.6 nm), and dimeric bovine serum albumin (4.75 nm). The myoglobin and ovalbumin were in a kit from Bio-Rad, and the other standards were from Sigma.

[^{3}H]cIMP Binding—Aliquots (20 μl) of DEAE-cellulose column fractions were incubated for 5 min at 4 °C with 12 nM [^{3}H]cIMP in 14 mM potassium phosphate buffer at pH 6.8 containing 0.2 mg/ml of histone in a total volume of 80 μl, before separation of bound [^{3}H]cIMP by Millipore filtration as described previously (13). For fractions containing the type I holoenzyme, 6 mM magnesium acetate and 0.3 mM ATP were also present. Concentrations of analogs used were 19 nM for 8-NH_2-cAMP, 8-aminobutylamino-cAMP, 8-SH-cAMP, and cAMP, and 100 nM for 8-benzyl-S-cAMP.

[^{3}H]cAMP Dissociation from R^{II}—0.9 μM [^{3}H]cAMP was incubated with R^{II} (2 μg/ml) for 60 min at 30 °C in a total volume of 500 μl before the addition of 5 μl of 10 mM cAMP to initiate exchange. 40-μl aliquots were taken to assay [^{3}H]cAMP binding to R^{II} immediately before addition of the cold cAMP and at various times thereafter by the Millipore technique as described earlier (14).

Reassociation of R^{II} with C Subunit—Purified R^{II} subunit preparations of bovine, rabbit, dog, and rat heart were diluted to 0.25 nM with 10 mM potassium phosphate at pH 6.8 containing 1 mM EDTA and 1 mg/ml of bovine serum albumin. Undiluted R molar concentration was determined using molecular weights determined by SDS gel electrophoresis and protein concentration determined by the method of Bradford (15), using bovine serum albumin as the standard. Bovine heart C subunit was diluted immediately before use with the above buffer to a final concentration of 1.2 nM. Increasing concentrations of R^{II} were incubated with constant C in a reaction volume of 70 μl at 25 °C for 30 min. A 25-μl aliquot from the incubation mixture was added to each of two tubes containing 25 μl of protein kinase assay mix, one with and one without 100 μM cAMP. Final concentrations were 65 μM heptapeptide substrate, 20 mM potassium phosphate at pH 6.8, 10 mM magnesium acetate, and 100 μM [γ-^{32}P]ATP. Following incubation for 12 min at 25 °C, 40-μl aliquots were precipitated onto phosphocellulose papers in 75 mM phosphoric acid as described by Roskoski (10).

Assays—[^{3}H]cAMP binding activity was assayed using Millipore filtration as described previously (16). Protein kinase activity was measured using precipitation on phosphocellulose papers (Whatman P81) in 75 mM phosphoric acid as described by Roskoski (10). The substrate, 65 μM synthetic heptapeptide, was incubated for 5 min at 30 °C in 20 mM Tris-HCl buffer at pH 7.4 containing 10 mM magnesium acetate and 0.1 mM [γ-^{32}P]ATP in the presence or absence of 3 μM cAMP as described previously (13). Protein-bound phosphate was determined chemically by the method of Buss and Stull (17). C subunit was purified to homogeneity from bovine heart as described in Ref. 18.

Materials—[^{3}H]cAMP was purchased from New England Nuclear, and [^{3}H]cIMP and 8-azido[^{32}P]cAMP were from ICN Corp. Cyclic nucleotide analogs were obtained from Sigma or ICN, except for 8-SH-cAMP which was a gift from Dr. Jon Miller and Dr. Robert Suva of the Biomedical Research Laboratory, Life Sciences Division, SRI International, Menlo Park, CA. Trypsin and benzamidine were from Sigma and *Staphylococcus* V8 protease was from Miles Laboratories, Inc. The heptapeptide substrate, Leu-Arg-Arg-Ala-Ser-Leu-Gly, was from Peninsula Laboratories. DEAE-cellulose was DE-22 from Whatman. The 8-(6-aminohexylamino)-cAMP-Sepharose 4B and N^6-(aminoethyl)-cAMP-Sepharose 4B were purchased from P-L Biochemicals. Bovine heart, brain, and lung were obtained fresh from a local slaughterhouse. Porcine heart and rabbit skeletal muscle were the frozen preparations from Pel-Freeze. Rat tissues were obtained fresh from decapitated rats. Frozen rabbit hearts and dog hearts were donated by Dr. Balwant Khatra and Dr. Kurt Steiner, respectively, of this department. Guinea pig hearts were removed and frozen before use. *Erythrocebus patas* hearts were donated in the frozen form by the Delta Regional Primate Center at Tulane University. Equine heart was the fresh tissue from Ellington Agricultural Center, Nashville, TN.

RESULTS

Studies of R^I and R^{II} in Crude Extracts of Heart—The electrophoretic technique using Tris glycine buffer clearly

separated R^I from R^{II} forms with 7.5 or 8.5% acrylamide slab gels giving the best separation, although a 12% acrylamide gel also gave adequate separation. Using purified R^{II} from dog, rabbit, and bovine heart it was found that each of the upper and lower bands of dog heart R^{II}, which represented the autophosphorylated and dephosphorylated forms, migrated further than the corresponding band of bovine heart R^{II}. Rabbit heart R^{II} migrated further than bovine and dog heart R^{II} but was clearly different from R^I (data not shown). Since such differences in M_r of R^{II} between three different species of heart could possibly result from changes in R^{II} occurring during purification procedures, the electrophoretic mobility of the various heart R^{II} forms was investigated in crude extracts using 8-azido[^{32}P]cAMP to specifically label R^I and R^{II}. The homogenization buffer used to make the heart extracts contained NaF, benzamidine, EDTA, and potassium phosphate to reduce or eliminate protein kinase, protein phosphatase, and protease activities. In addition the incubation with 8-azido[^{32}P]cAMP was performed immediately after preparation of the heart extract and on occasions a shorter incubation time than 30 min was used to minimize possible posthomogenization changes in R^{II}. For the radioautograph shown in Fig. 1, a 7.5% acrylamide gel was used to separate R^I and R^{II} in crude extracts of four heart species. The bovine heart R^{II} sample used in Fig. 1 (*lane 5*) was a fraction obtained by DEAE-cellulose chromatography which contained R^{II} together with some breakdown fragments of R^{II} which electrophoresed close to the dye front. Although the radioautograph shown in Fig. 1 was overexposed for the bovine heart type II sample, analysis of a film exposed to the gel for a shorter time indicated two bands with apparent M_r values of 56,000 and 58,000. R^I which had an apparent M_r of 49,000 was the lowest main band present and exhibited varying intensities of radioactive label in each of the crude heart extracts. The intensity of R^I and R^{II} bands reflected qualitatively the relative amounts of R^I and R^{II} found in tissues by DEAE-cellulose chromatography but was not a reliable quantitative measure of the proportions of R^I and R^{II} since although saturating concentrations of 8-azido[^{32}P]cAMP were used (19), the binding time was shorter than that required for complete equilibration (20) in order to reduce possible posthomogenization changes in R^{II}. Specificity of 8-azido[^{32}P]cAMP binding to R^I and R^{II} was confirmed by the inclusion of cAMP to inhibit specific binding of the analog (data not shown). Dog and rat heart R^{II} had a M_r of 56,000 which was higher than that of 52,000 for guinea pig and rabbit heart R^{II} (Fig. 1). Rat heart and dog heart R^{II} appeared similar upon electrophoresis of the crude extracts, since only the R^{II} band of M_r 56,000 apparently due to R^{II} totally saturated with phosphate at the autophosphorylation site, was present in the crude extract of dog heart (Fig. 1, *lanes 2* and *9*). As will be seen below dephosphorylated dog heart R^{II} clearly migrated further than rat heart R^{II}. If the dog heart crude extract was prepared in 10 mM potassium phosphate buffer at pH 6.8 containing 1 mM EDTA instead of the buffer containing inhibitors, the faster migrating R^{II} band of M_r 54,000 appeared (data not shown). This finding indicated that the inhibitors contained in the buffer were indeed having some effect against protein phosphatase activities.

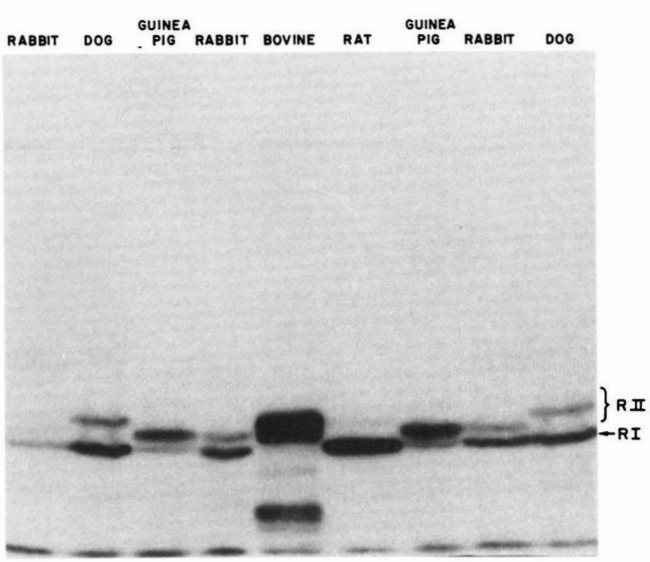

FIG. 1. **SDS gel electrophoretic separation of heart R^I and R^{II} forms.** Radioautograph of crude extracts of dog, rat, guinea pig, rabbit, and bovine hearts labeled with 8-azido[^{32}P]cAMP as described under "Experimental Procedures" and electrophoresed on a 7.5% polyacrylamide slab gel.

To determine whether the differences in electrophoretic mobility of R^{II} from bovine, rat, dog, rabbit, and guinea pig hearts were reflected in other properties of R^{II}, heart extracts were chromatographed on DEAE-cellulose columns. Using identical conditions for each heart extract, the type II holoenzymes eluted at very similar points on the salt gradient, the type II peak coinciding with a salt concentration of approximately 0.22 mM in each case. The presence of R^I and R^{II} where expected in the elution profile was confirmed by gel electrophoresis of column fractions which had been labeled with 8-azido[^{32}P]cAMP. From the peaks of [^{3}H]cAMP binding and protein kinase activity, it was estimated that dog and rabbit heart contained approximately 50% type I and 50% type II R, whereas rat heart was more than 80% type I and guinea pig heart more than 80% type II. This was in agreement with previous studies for rabbit and guinea pig hearts (2, 21). From these proportions, it can be seen that 8-azido[^{32}P]cAMP binding under the nonequilibrium conditions used here underestimated the high proportion of R^{II} relative to R^I in guinea pig heart as well as in rabbit and dog hearts. This was probably because 8-azido-cAMP tends to bind more efficiently to R^I than to R^{II} (19, 20).

Previous studies with the type II holoenzyme from bovine heart and the type I holoenzyme from rabbit skeletal muscle or rat heart (13, 22) had demonstrated pronounced differences between the type I and type II enzymes in the analogs of cAMP which were most efficacious in stimulating [^{3}H]cIMP binding. Thus 8-SH-cAMP and 8-benzyl-S-cAMP were the most effective with the bovine heart type II holoenzyme, whereas 8-NH_2-cAMP and 8-aminobutylamino-cAMP were most effective with rat heart or rabbit skeletal muscle type I (13, 22). These analogs were tested for their ability to stimulate [^{3}H]cIMP binding in crude extracts as well as in eluate fractions from DEAE-cellulose column chromatography of extracts from dog, rabbit, guinea pig, and rat hearts (data not shown). In all cases with the type I holoenzyme the two amino analogs caused a more than 2-fold greater stimulation of [^{3}H]cIMP binding than did the two thio analogs, whereas the two thio analogs were considerably more effective than the two amino analogs with the type II-containing heart fractions. Some variation was found in the magnitude of the fold stimulation of [^{3}H]cIMP binding in the different heart species which probably resulted from differences in the proportions of R and C subunits in the fractions, together with unknown factors present in the extract. As might be expected, stimulation of [^{3}H]cIMP binding by analogs in crude heart extracts reflected the relative amounts of the type I and type II holoenzymes. In extracts from guinea pig, dog, and rabbit

hearts where the proportion of the type II isozyme was 50% or greater, stimulation by the thio analogs was 2- to 3-fold greater than by the amino analogs. On the other hand, with rat heart, which has less than 20% type II, stimulation by the thio analogs was less than by the amino analogs. Thus there did not appear to be any major differences between the different heart type II holoenzymes with respect to stimulation of [^{3}H]cIMP binding by cAMP analogs.

Characterization of Purified Heart R^{II}—To further examine the differences found between R^{II} in crude extracts of various species of heart, R^{II} purified from each heart species was compared on the basis of electrophoretic mobility in the Tris glycine SDS gel system and whether or not autophosphorylation affected this mobility. The properties that distinguish purified R^{II} from four different heart species are demonstrated in Fig. 2. Dog heart R^{II} had a lower apparent M_r than bovine or rat heart R^{II}, while rabbit heart R^{II} had the lowest apparent M_r (Fig. 2, *left*). Rat and rabbit heart R^{II} were not present as doublets and upon phosphorylation with [γ-^{32}P]ATP and C subunit, the single bands did not shift mobility (Fig. 2, *left*), although ^{32}P had been incorporated into the single protein bands (Fig. 2, *right*). On the other hand, dog and bovine heart R^{II} were present as doublets and showed increased protein staining of the upper bands of the R^{II} doublets upon autophosphorylation (Fig. 2, *left*). Only the upper bands of dog and bovine heart R^{II} contained ^{32}P label (Fig. 2, *right*) while the lower bands of the doublets had not been phosphorylated. The small differences between the phosphorylated bands of dog and bovine heart R^{II} (Fig. 2, *right*) and between their dephosphorylated bands (Fig. 2, *left*) were consistently found in other SDS-polyacrylamide gel separations.

In addition to the purified R^{II} preparations shown in Fig. 2, R^{II} purified from guinea pig heart which like rabbit heart had M_r 52,000, did not show a shift in mobility upon autophosphorylation (data not shown). Autophosphorylation of R^{II} from dog, rat, guinea pig, and rabbit hearts was also studied in type II holoenzyme-containing fractions eluted from DEAE-cellulose columns. Results of endogenous phosphorylation or phosphorylation with added C subunit were consistent with phosphorylation studies using purified R^{II} preparations. Thus by comparing bands of 8-azido[^{32}P]cAMP-labeled R^{II} with R^{II} bands phosphorylated by [γ-^{32}P]ATP, it was found that rat, rabbit, and guinea pig heart R^{II} values did not detectably change electrophoretic mobility upon phosphorylation, whereas a shift in mobility was found with dog heart R^{II} (data not shown). It should be mentioned that in contrast to the crude extracts which were prepared in buffer containing inhibitors of protease, protein kinase, and protein phosphatase activities, the buffer used in the procedure for purifying R^{II} was 10 mM potassium phosphate at pH 6.8 containing 1 mM EDTA. Thus the retention of differences in electrophoretic mobility after the purification procedure strongly suggests that the microheterogeneity of R^{II} does not simply reflect proteolysis or changes in phosphorylation.

Gel Filtration of R^{II}—In addition to showing differences in electrophoretic mobility on SDS gels, at least one R^{II} behaved differently on HPLC gel filtration (Fig. 3). Porcine, bovine, and dog heart R^{II} had Stokes radii between 6.0 and 6.1 nm, and rabbit muscle R^{II} likewise had a Stokes radius of 6.1 nm. In contrast rat liver R^{II} had a Stokes radius of 7.3 nm. When porcine heart R^{II} and rat liver R^{II} were chromatographed together the two peaks were clearly distinct. The first small peak to elute from the HPLC column of rat liver R^{II} was probably due to multimeric forms of R^{II} which would elute close to the void volume. SDS gel electrophoresis did not detect differences between the mobility of the protein in the void volume from that in the main peak of the rat liver R^{II} profile (data not shown). Rat liver R^{II} had similar electrophoretic mobility as dephosphorylated bovine heart R^{II} but did not shift mobility upon autophosphorylation (Fig. 4). Dog heart R^{I}, cGMP-dependent protein kinase, and C subunit eluted from the HPLC column with Stokes radii lower than R^{II} (Fig. 3). The finding that R^{II} from rat liver differs markedly in Stokes radius from R^{II} of rabbit skeletal muscle and dephospho-R^{II} of bovine and porcine hearts distinguishes from each other these proteins which comigrate on SDS gel electrophoresis. Since differences in Stokes radii reflect differences in the shape of a molecule, two R^{II} may have the same apparent M_r on SDS gels but their amino acid sequence presumably differs resulting in different shapes. Alternatively, it is possible that changes in folding may reflect other groups bound to amino acids in the peptide chain.

Partial Proteolysis of R^{II}—Selective cleavage of R^{II} by specific proteases was used to investigate what part of the R^{II} molecule might be responsible for differences in electropho-

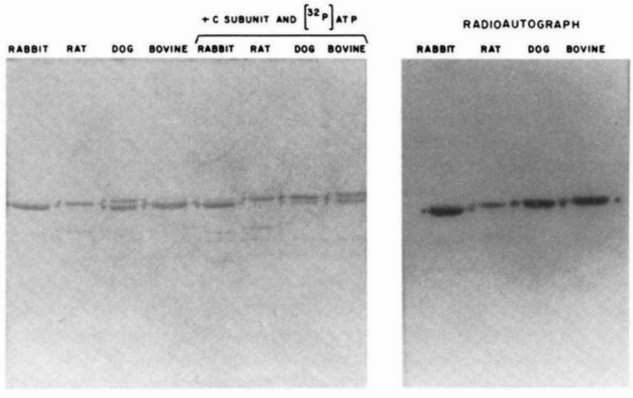

FIG. 2. **Comparison of R^{II} from hearts of different species.** Purified R^{II} from rabbit, rat, dog, and bovine hearts was electrophoresed on an 8% polyacrylamide gel before and after autophosphorylation. *Left*, protein stain of various heart R^{II}. *Right*, radioautograph of phosphorylated heart R^{II} (*lanes 5–8* on *left*).

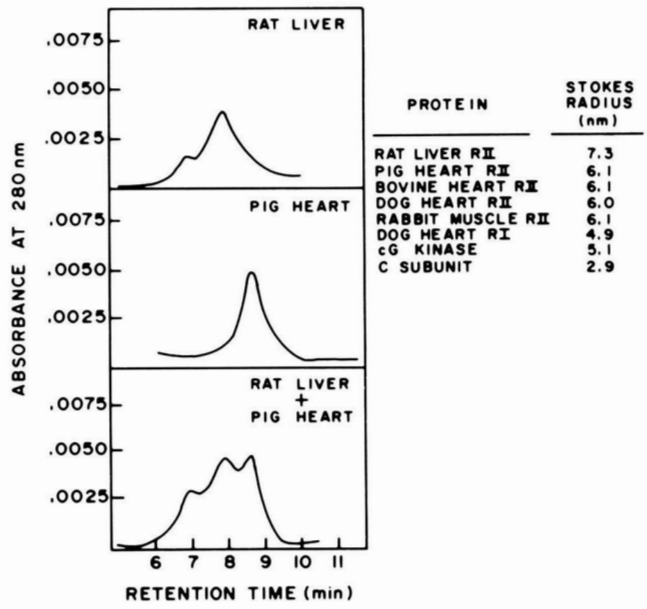

FIG. 3. **HPLC gel filtration of R^{II} and other proteins.** Rat liver and pig heart R^{II} were each diluted to 0.07 mg/ml before injection. The mixture (*lower*) also contained 0.07 mg/ml of each. Chromatography and calculation of Stokes radii were done as described under "Experimental Procedures."

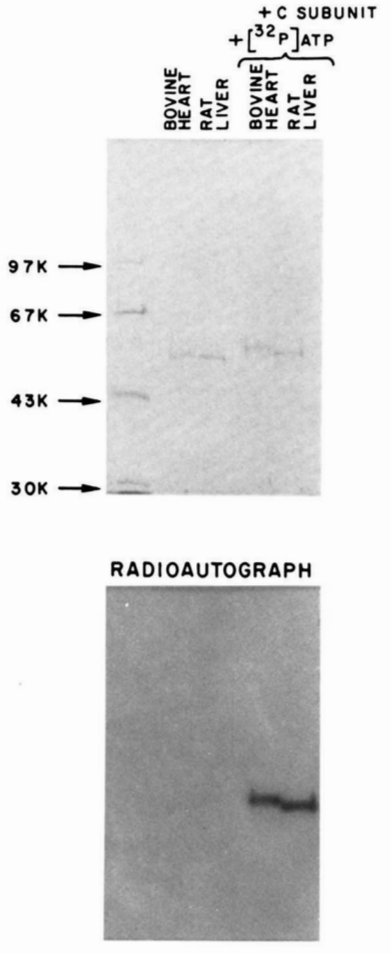

FIG. 4. **Comparison of rat liver and bovine heart R^{II}.** Autophosphorylation and SDS slab gel electrophoresis (8% gels) were performed as described under "Experimental Procedures." Rat liver R^{II} (0.6 μg loaded) incorporated 0.30 mol of ^{32}P per mol of subunit, and bovine heart R^{II} (0.8 μg loaded) incorporated 0.22 mol of ^{32}P per mol of subunit.

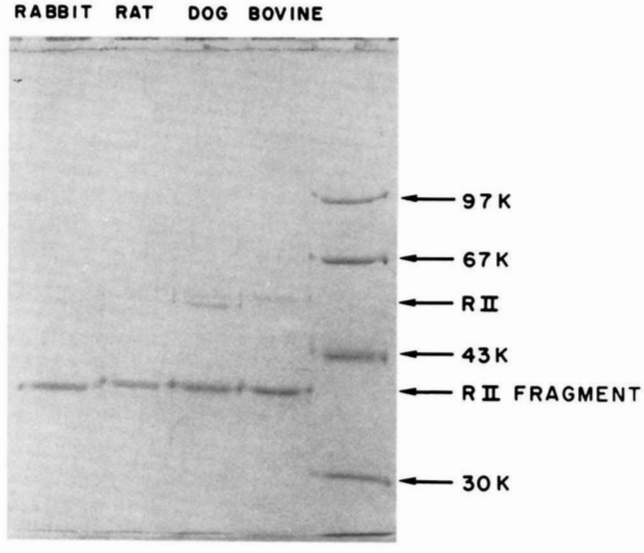

FIG. 5. **Comparison of tryptic fragments of R^{II} from hearts of different species.** Heart samples (2 μg) were treated with trypsin (0.2 ng) for 30 min at 30 °C in a total volume of 55 μl before addition of SDS to stop the reaction. A 10% polyacrylamide gel was used.

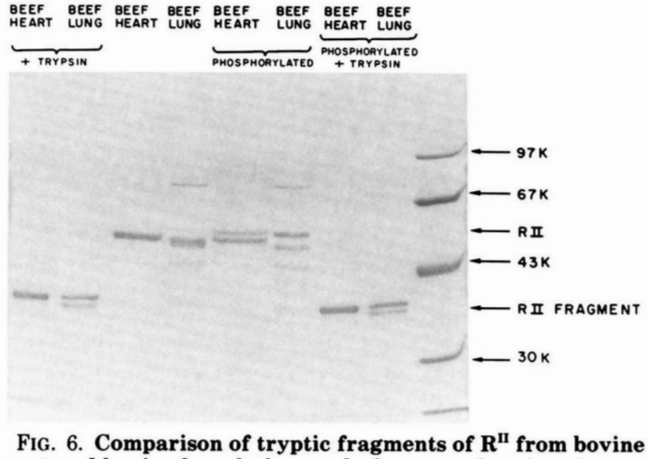

FIG. 6. **Comparison of tryptic fragments of R^{II} from bovine heart and bovine lung before and after autophosphorylation.** Bovine heart (8 μg) and bovine lung (6 μg) R^{II} preparations were either untreated or phosphorylated with C subunit and MgATP for 20 min at 4 °C. Trypsin (5 ng) was added where indicated, and samples were incubated at 30 °C for 1 h in a total volume of 50 μl before the addition of SDS and 2-mercaptoethanol. A 10% polyacrylamide slab gel was used.

retic mobility in the R^{II} forms. When R^{II} from various species of heart which represented four forms was incubated with a low concentration of trypsin, differences in electrophoretic mobility were retained in the large fragment with apparent M_r of approximately 35,000–40,000 (Fig. 5). The smaller fragment of apparent M_r 10,000–20,000 generated by mild trypsin treatment migrated at the dye front in 7.5% polyacrylamide gels (Fig. 5) but could be visualized on 12% polyacrylamide gels. Trypsin at low concentrations cleaves primarily near the autophosphorylation site in R^{II} to generate the large C-terminal fragment and a smaller N-terminal fragment (9, 23). Thus relative differences in mobility of the R^{II} were retained in the C-terminal fragment of the molecule. The C-terminal fragment generated by trypsin cleavage of bovine heart R^{II} contains the autophosphorylation site. It was, therefore, not unexpected that the radioactivity coincided with the larger R^{II} fragment when R^{II} of any of the four forms was autophosphorylated with [γ-^{32}P]ATP prior to electrophoresis (data not shown). However, it can be seen in Fig. 5 that mild trypsinization abolished the difference between phosphorylated and dephosphorylated forms of R^{II} which shifted mobility upon autophosphorylation. Thus for both dog and bovine heart R^{II} only one protein band represented the tryptic fragments, whereas doublets were present in the unproteolyzed R^{II} samples, and these are faintly visible on the gel in Fig. 5 above the more densely staining fragment bands. Further support that the C-terminal region of R^{II} retained differences between the R^{II} came from studies with bovine lung R^{II} which contained a mixture of approximately equal proportions of R^{II} of M_r 56,000 and 52,000 (Fig. 6). When bovine lung R^{II} underwent mild trypsinization, two distinct fragments were formed corresponding to proteolysis of the two R^{II} forms (Fig. 6). The slower migrating fragment of bovine lung R^{II} comigrated with the M_r 39,000 fragment generated by trypsinization of bovine heart R^{II}. Upon autophosphorylation the upper band of bovine lung R^{II} with M_r 56,000 underwent a shift in mobility but the band of M_r 52,000 did not shift (Fig. 6). Mild trypsinization abolished the effect of autophosphorylation upon mobility of the upper band of bovine lung R^{II} (Fig. 6). Likewise, it can clearly be seen in Fig. 6 that a mixture of phosphorylated and dephosphorylated bovine heart R^{II} generated only a single fragment upon trypsin treatment. When [γ-^{32}P]ATP was used to phosphorylate bovine lung R^{II}, both protein bands were radioactively labeled in untreated R^{II} as well as in the trypsin-

generated fragments confirming that the two fragments from bovine lung R^{II} did not represent phosphorylated and dephosphorylated forms of a fragment (data not shown). It appeared that R^{II} dimers contained R^{II} subunits of the same form in the bovine lung R^{II} preparation since two bands were separated by gel electrophoresis in the absence of SDS under nondenaturing conditions (data not shown). If R^{II} had formed a hybrid dimer or if a mixture of hybrid and homogeneous dimers had been present in the preparation, one or three bands, respectively, would have been expected.

Staphylococcus V8 protease cleaves bovine heart R^{II} mainly in the hinge region at a site seven amino acids further toward the C-terminal end of the peptide chain than does trypsin, with the autophosphorylation site on serine 95 falling between the two proteolytic cleavage sites (24). Thus, in contrast to trypsin, the smaller N-terminal fragment of M_r 17,000 will contain the autophosphorylation site rather than the larger C-terminal fragment after *Staphylococcus* V8 proteolysis. When R^{II} from rabbit heart or bovine heart which had been autophosphorylated with [γ-^{32}P]ATP was incubated with *Staphylococcus* V8 protease, the dense ^{32}P-labeled bands corresponding to the N-terminal fragments were clearly of different apparent M_r (Fig. 7). A 15% acrylamide gel was used in Fig. 7 so that fragments of M_r 10,000–20,000 would not run off the gel as occurred with 7.5 or 8.5% acrylamide gels. The C-terminal fragment can faintly be seen in the autoradiograph in Fig. 7 since there may also have been some cleavage on the side of the autophosphorylation site opposite to that where most of the cleavage occurred. These experiments clearly

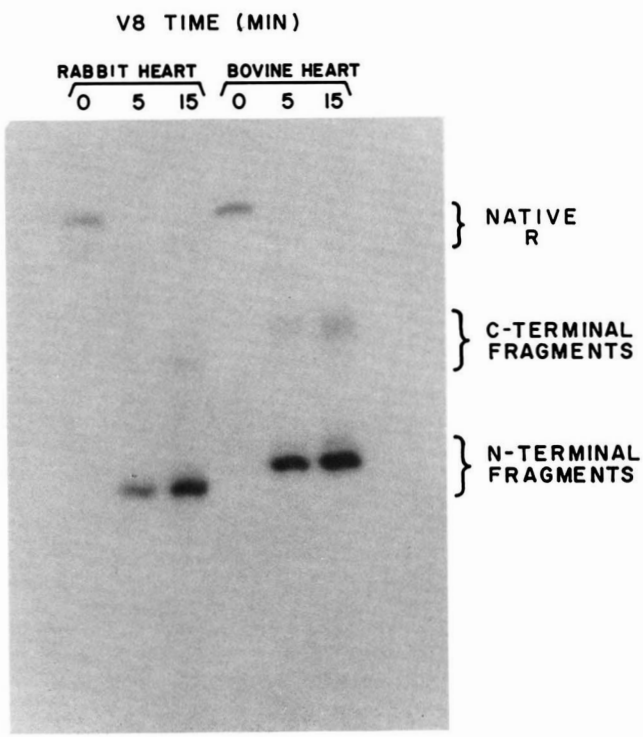

FIG. 7. **Effect of *Staphylococcus* V8 protease on rabbit and bovine heart R^{II} subunits.** Rabbit (7 μg) and bovine (6 μg) heart R^{II} were phosphorylated with 60 μM [γ-^{32}P]ATP, 10 mM magnesium acetate, and 80 nM C subunit in a volume of 15 μl prior to the addition of 200 μl of 10% trichloroacetic acid and centrifugation. The pellet was resuspended in 150 μl of 125 mM Tris (pH 7.8), 10% glycerol, 1 mM EDTA, and 0.1% SDS. 50-μl aliquots were incubated at 30 °C for 5 or 15 min with 10 μl of 125 μg/ml of *Staphylococcus* V8 protease and then boiled for 5 min. Samples were electrophoresed on 15% acrylamide slab gels in Tris glycine buffer followed by autoradiography as described under "Experimental Procedures."

demonstrated that both the N-terminal and C-terminal regions of R^{II} differ in the different subclasses and suggested that it was extremely unlikely that rabbit heart R^{II} was generated artefactually by a proteolytic clip at the N or C terminus. These studies thereby provide evidence that the R^{II} subclasses were not derived by proteolysis but were more likely to represent different gene products.

Behavior of R^{II} in the Holoenzyme—In order to test whether differences in the R^{II} molecule affected the behavior of the type II holoenzyme the ability of the different R^{II} to associate with C subunit and bind cAMP was investigated. It has already been discussed that analog stimulation of [^{3}H]cIMP binding by the different forms of heart type II holoenzyme did not show marked differences. This suggested that the R^{II} behaved similarly in their interactions with cAMP and C subunit. R^{II} purified from rabbit, dog, bovine, and rat hearts also exhibited no significant differences in their curves of dissociation of bound [^{3}H]cAMP upon the addition of excess unlabeled cAMP (Fig. 8, *top*). In each case the curves were biphasic suggesting that like bovine heart R^{II} (14), each R^{II} possessed two different intrachain binding sites for cAMP. Approximately 50% of the [^{3}H]cAMP was bound in Site 1 and 50% in Site 2 for all four heart R^{II} preparations. [^{3}H]cAMP dissociated from Sites 1 and 2 with rates ($t_{1/2}$) of approximately 23 and 1.5 min, respectively, at 30 °C (Fig. 8, *top*), which agrees approximately with rates found previously for bovine heart R^{II} (25). Activation of protein kinase activity by increasing concentrations of cAMP also followed a very similar curve for the four different holoenzymes containing the R^{II} forms represented by bovine, rabbit, dog, and rat heart (Fig. 8, *middle*). Using equal concentrations of catalytic activity for each, half-maximal activation was achieved with cAMP concentrations between 135 and 205 nM. In a previous study using more points on the curve for cAMP concentrations between 0 and 100 nM than were used in Fig. 8, *middle*, activation was found to be cooperative with a Hill coefficient of 1.62 (13). This more highly purified preparation of rat heart R^{II} (13) did not seem to behave very differently from the DEAE-cellulose fraction used in Fig. 8, *middle*, and it appears likely that all R^{II} forms may demonstrate positive cooperativity of activation of protein kinase by cAMP. The ability of different R^{II} forms to inhibit C subunit activity also exhibited similar concentration curves (Fig. 8, *bottom*). Purified heart R^{II} from four different mammals incubated with increasing concentrations of C subunit appeared to reassociate with C subunit to inhibit its activity at similar ratios of R^{II} and C (Fig. 8, *bottom*), suggesting that the affinities of R^{II} for C subunit were similar. Thus on various tests of the behavior of R^{II} and C in the holoenzyme, there did not appear to be any marked differences between the different R^{II} forms represented by four species of heart.

Rates of autophosphorylation, which may also reflect differences in the interaction between R and C subunits, also did not differ greatly between different heart R^{II} subclasses. Under the conditions used phosphorylation rates were very rapid for all of the R^{II} tested with phosphorylation complete within 5 min. Bovine and rabbit heart R^{II} preparations which prior to phosphorylation contained 1.76 and 1.95 mol of phosphate/mol of R^{II} monomer, respectively, were phosphorylated to a maximum of about 0.8 mol of phosphate/mol of R^{II} monomer. This is consistent with reports that R^{II} isolated from bovine heart may contain between 1 and 3 mol of phosphate/mol of R^{II} monomer, much of which may be located at sites other than the autophosphorylation site (26–28). Other R^{II} preparations from tissues listed in Table I also rapidly incorporated between 0.3 and 1 mol of phosphate/mol

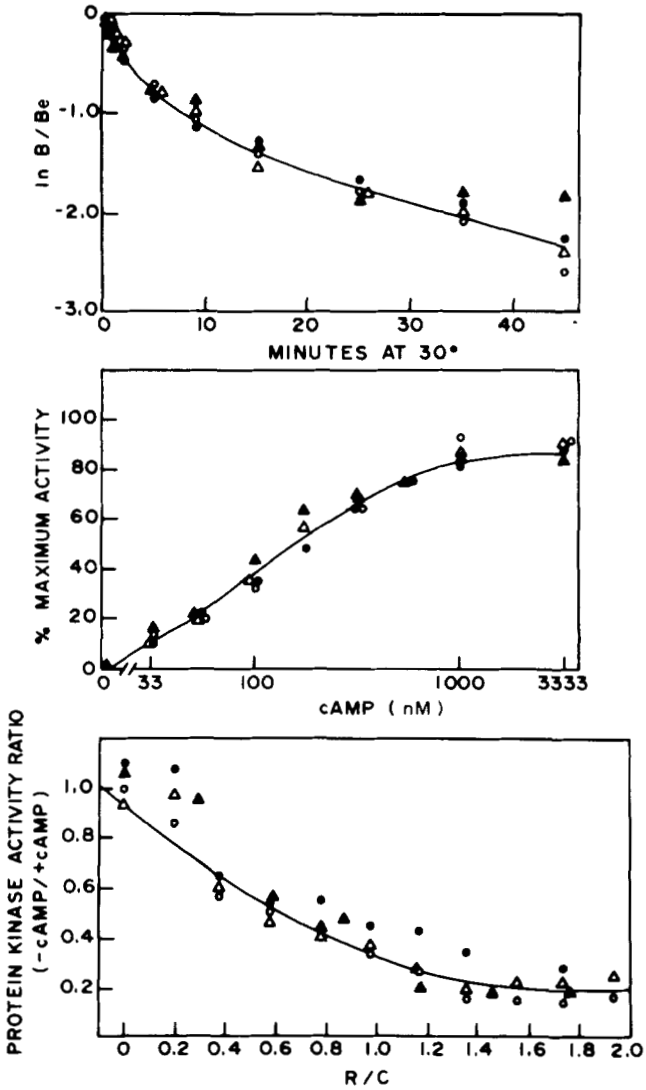

FIG. 8. **Comparison of cAMP binding and activation properties of R^{II} from four different heart species.** *Top*, [^{3}H]cAMP dissociation from R^{II} was measured as described under "Experimental Procedures." Rat heart R^{II} was a DEAE-cellulose column preparation while rabbit, bovine, and dog were purified R^{II}. *Middle*, activation of type II holoenzyme by increasing concentrations of cAMP was measured using the protein kinase assay described under "Experimental Procedures." DEAE-cellulose column fractions containing the type II holoenzyme were used as enzyme sources (catalyzed incorporation of 28–38 pmol of ^{32}P per min in assay). *Bottom*, inhibition of C subunit activity by reassociation with R subunit was performed as described under "Experimental Procedures." Inhibition of C by R^{II} is plotted as a function of protein kinase activity ratio *versus* the ratio of R to C in the initial recombination mixture. O, bovine heart; ●, rabbit heart; ▲, rat heart; △, dog heart.

of R^{II} monomer when incubated with [γ-^{32}P]ATP and C subunit.

Classification of Different Forms of Type II R Subunit—From the studies described in the present paper and other unpublished data from our laboratory R^{II} from various tissues and species has been resolved into five forms based on differences in electrophoretic mobility and the effect of autophosphorylation (Table I). However, some R^{II} which appear identical with respect to these criteria may in the future prove to be different. Indeed one such case has already been described in the present study in which rat liver R^{II} was shown to have a different Stokes radius from rabbit skeletal muscle R^{II} with

TABLE I

Distribution of subclasses of type II R subunit

Apparent M_r by SDS gels	Shifts to higher M_r form on SDS gels after phosphorylation	Tissue
Type IIA		
56,000	Yes	Bovine heart
		Porcine heart
54,000	Yes	Dog heart
		Equine heart
Type IIB		
52,000	No	Rabbit heart
		Guinea pig heart
		Bovine brain
56,000	No	Rat heart
		Rabbit skeletal muscle
		Rat liver
51,000	No	Rat brain
		Rat adipose tissue
		Monkey (*E. patas*) heart
Equal mixture of 52,000 and 56,000	52,000 No 56,000 Yes	Bovine lung

which it comigrated on SDS gels (Fig. 3), making a total of at least six different forms of R^{II}. It is possible to make an operational classification to separate those R^{II} which shift mobility upon autophosphorylation (type IIA) and those which do not (type IIB). However, this classification can only be operational until more is understood about the reason for the shift in mobility for some forms of R^{II} but not others. As is discussed below there does seem to be some support from evolutionary relationships for such an operational classification of R^{II}. Perhaps other electrophoretic techniques may in the future detect changes upon autophosphorylation in the forms of R^{II} which do not change electrophoretic mobility in the Tris glycine, SDS gel electrophoresis system commonly used. Interestingly, in addition to the different R^{II} forms found in different species of heart, different forms of R^{II} were found in different tissues of the same animal (Table I). Thus rabbit skeletal muscle R^{II} had M_r 56,000 while rabbit heart R^{II} had M_r 52,000, but neither R^{II} shifted electrophoretic mobility upon autophosphorylation. More than one R^{II} was also found in some tissues, as was found for bovine lung which contained approximately equal amounts of two different R^{II} forms. One form of bovine lung R^{II} had M_r 52,000 and was type IIB which did not shift upon autophosphorylation while the other R^{II} was type IIA with M_r 56,000 like that found in bovine heart (Table I). Even though one form of R^{II} usually predominated in a tissue, minor forms of 20% or less of the total R^{II} could sometimes be detected. The bovine brain was found to contain a small amount (~15%) of a form which appeared identical to that of bovine heart. Rat adipose tissue contained a minor form of R^{II} (~15%) similar to that of rat liver or rat heart. *E. patas* heart also contained a minor form (~20%) of R^{II} of M_r ~56,000.

R^I and cGMP-dependent Protein Kinase—A comprehensive study of the possible existence of different forms of type I R subunit and cGMP-dependent protein kinase was not carried out. However, a few species were examined. The R^I was purified to apparent homogeneity from dog heart, monkey heart, and rabbit skeletal muscle, and the pure proteins were subjected to SDS gel electrophoresis as was done for R^{II}. The dog heart R^I consistently migrated at slightly lower mobility (M_r 50,000) than the other two which were not detectably

different from each other in mobility (M_r 49,000). The cGMP-dependent protein kinase was oftentimes a slight contaminant of R^{II} preparations and could be detected as a band of M_r 80,000 on SDS gels if the R^{II} samples were heavily overloaded. The pure bovine lung cGMP-dependent protein kinase migrated identically to this M_r 80,000 band of R^{II} preparations from bovine heart and lung, monkey heart, rabbit skeletal muscle, rat heart and liver, and dog heart. Thus, at least by the single criterion of SDS gel electrophoretic mobility the cGMP-dependent protein kinase exhibited less microheterogeneity than R^I or R^{II}.

DISCUSSION

The present study describes the existence of differences in R^{II} both in different tissues of the same animal and in the same tissue in different species. Different R^{II} were described on the basis of electrophoretic mobility of R^{II} both in crude extracts and in purified preparations. At least one R^{II} also differed in Stokes radius from another form with which it appeared to be identical in electrophoretic mobility. The data obtained with crude extracts in the presence of inhibitors of proteases, protein kinases, and protein phosphatases together with experiments of mild proteolysis strongly suggested that the different R^{II} did not simply reflect the effects of proteolysis or other processes but were more likely to be the result of differences in gene expression. Differences in R^{II} were present in both N-terminal and C-terminal fragments of the molecule. Efforts are underway at present to determine the amino acid sequence of such fragments from different heart R^{II} in an attempt to locate possible sequence differences. It seemed unlikely that differences in apparent M_r were due to differences in phosphorylation of amino acid residues at sites other than the autophosphorylation site of R^{II} since they were retained under conditions when phosphatases should be active, *i.e.* in crude extracts of heart tissues in the absence of the inhibitors of proteases, protein kinases, and protein phosphatases. While it was not clear what caused the shift in electrophoretic mobility upon autophosphorylation of R^{II} in various bovine, dog, and porcine tissues, it could be stated that the N-terminal M_r 10,000–20,000 portion is required for the shift even though it does not contain the autophosphorylation site. Sequencing in the immediate vicinity of the autophosphorylation site in R^{II} from different species of heart may be useful to reveal possible differences in this region of the molecule between bovine, dog, and pig and the other species.

Differences in antibody specificity were not investigated in the present study. Previous work had distinguished a neural R^{II} subclass on the basis of different antibody reactivity (3, 29, 30). Bovine heart, muscle, kidney, and liver R^{II} values were all equally ineffective at displacing bovine brain R^{II} from antibodies prepared against bovine brain R^{II} (7, 30). Rat and hamster brain R^{II} values were more effective than non-neural R^{II}, however, at displacing bovine brain R^{II} from its antibody (7). Bovine skeletal muscle and heart R^{II} values have also recently been found to differ from bovine brain R^{II} in their two-dimensional tryptic peptide maps and in the main phosphorylated peptide (31), but the bovine heart and bovine skeletal muscle enzymes behaved identically by the same criteria (31). Previous studies have reported the presence of two bands of R^{II} with apparent M_r values of approximately 55,000 and 57,000 by labeling crude extracts of bovine brain with 8-azido[^{32}P]cAMP (3, 7), but it is not clear whether these bands directly correspond to those R^{II} forms represented by bovine brain and heart, respectively (Table I), or whether they represent phospho and dephospho forms of R^{II}. Phosphorylation of a bovine brain R^{II} preparation containing predominantly one band of M_r 55,000 did result in the shift of part of the band to form a second band of M_r 57,000, but phosphate was incorporated into both bands (7). Such a finding could, therefore, be consistent with the data presented here suggesting the presence of two different bovine brain R^{II} with only one form demonstrating a shift in electrophoretic mobility upon phosphorylation. Interestingly α-chymotrypsin was able to digest the autophosphorylated bovine brain R^{II} of M_r 55,000 but not that of M_r 57,000 when bovine brain R^{II} was in complex with anti-heart R^{II} antibodies (7). This finding would be consistent with the anti-heart R^{II} antibodies protecting an R^{II} represented by bovine heart (autophosphorylated M_r 57,000) but not an R^{II} of M_r 52,000 (Table I) from α-chymotrypsin attack, supporting the notion that two R^{II} exist in bovine brain. It is possible that the supposed distinction between neural and non-neural R^{II} may in fact reflect a more widespread feature of R^{II} and that instead of a neural and non-neural subclass there are many forms, each not necessarily confined to either neural or non-neural tissues. Further antibody studies like those described above of protection against protease action may be useful to discover differences in secondary and tertiary structure between different R^{II} forms.

It is interesting that despite the observed differences, R^{II} forms which were tested appeared to have similar functional properties based on those activities studied.[2] Perhaps the differences in electrophoretic mobility reflect changes in the R^{II} molecule which do not greatly affect its functional activity. Such changes which do not alter functional capabilities would presumably not be unfavorable in terms of evolution and would allow the considerable genetic drift which has presumably occurred. Even though it is realized that amino acid sequences of the different R^{II} are needed to confirm the evolutionary relationships of various R^{II}, it is of interest that the four species containing type IIA represented the ungulates and carnivores, a branch of the evolutionary tree which separated about 80 million years ago (32) from the lagomorphs, rodents, and primates which contained type IIB.

It is difficult to compare other preparations of R^{II} presented in the literature with the R^{II} forms described here. Sometimes, as in the case of porcine skeletal muscle R^{II} (8), a shift in mobility upon phosphorylation was reported, but R^{II} preparations should be electrophoresed together with known R^{II} forms to classify them according to apparent M_r. Apparent M_r values of R^{II} from the same species and tissue but reported by different groups of workers frequently vary by as much as 2,000 which is as small as the difference between different forms. Despite the problems in making comparisons between different studies, Weber *et al.* (29) recently summarized variants of R^I and R^{II} which have been found in mammalian tissues. Some variants may be proteolytic breakdown products of the native forms of R, and they may prove to be interesting in studying the control of R turnover if they can be shown to exist in the intact cell as well as in extracts. However, other variants, like the R^{II} forms reported in the present paper seemed to be discrete entities and not formed by proteolysis of another R^{II} entity. Although greater variation is becoming apparent as R^I and R^{II} are prepared from more mammalian tissues, the type I and type II holoenzymes described so far all conform to the properties originally used to classify the two forms (2). Thus the type I holoenzyme elutes from a DEAE-cellulose column with salt concentrations less than 0.1 M while the type II isozyme requires salt concentrations

[2] The adipose tissue R^{II} has been found to have different kinetic properties and is the subject of a separate publication.

greater than 0.1 M (2). The type II holoenzyme undergoes autophosphorylation while the type I isozyme does not (4, 33). The type I holoenzyme binds ATP with high affinity while the type II holoenzyme has a low affinity ATP-binding site (4). In crude extracts the type II holoenzyme dissociates into its subunits slowly in the presence of histone or high salt (0.5 M), but reassociation of the subunits in dilute buffer is rapid (1, 2). In contrast the type I holoenzyme rapidly dissociates in the presence of histone or high salt, while reassociation is slow (1, 2). To the list of differences between types I and II should be added the more recent finding that they exhibit different cAMP analog specificity (13, 22). Thus even though a particular R^{II} preparation may migrate close to R^{I} on SDS gels, it can readily be recognized as type II by the properties described above. In a few cases that have been reported in which the type I or type II holoenzymes did not elute where expected from a DEAE-cellulose column, the presence of type I or type II in the cAMP-binding and protein kinase activity peaks of the elution profile have been determined by the combination of SDS gel electrophoresis and the known characteristics of the two types. Thus mouse adipose tissue R^{I} which eluted from a DEAE-cellulose column together with R^{II} at approximately 0.2 M NaCl migrated with apparent M_r 49,000 on SDS gel electrophoresis (34). However, it could not be ruled out that the M_r 49,000 species was really a type II form since the adipose tissue R^{II} studied here was found to be only slightly larger (M_r 51,000) than the usual R^{I} M_r of 49,000. The type II holoenzyme from rat adipose tissue did not elute from a DEAE-cellulose column at the same salt concentration as type II from rat heart, but both type II isozymes behaved similarly with respect to dissociation by salt or histone (1). Likewise in the present studies type II has been confirmed in all cases by DEAE-cellulose chromatography and autophosphorylation. It has been suggested by Weber *et al.* (29) that immunotitration with antibodies against rabbit skeletal muscle R^{I} and bovine heart R^{II} might be a rapid and reliable means for classification and quantification of R^{I} and R^{II} from tissue extracts. However, in the light of the present study and those comparing brain and muscle R^{II} (7, 31), care should be taken to assess the effectiveness of antibodies prepared against bovine heart R^{II} with other R^{II} since the microheterogeneity displayed by R^{II} on SDS gel electrophoresis may be reflected in different antigenic reactivity.

The data presently available suggests that phosphorylation at the autophosphorylation site but not other sites may result in the large shift in electrophoretic mobility of bovine heart R^{II}, but it has not been demonstrated that phosphorylation specifically of serine 95 is associated with the mobility change. There have also been few studies of the effects of phosphorylation at other sites on R^{II}. Changes in the properties of R^{II} which generally would act to facilitate activation of the bovine heart type II holoenzyme by cAMP have been associated with autophosphorylation (4, 33, 35–37). Presumably these changes resulted only from phosphorylation at serine 95 rather than at other phosphorylation sites such as serines 44 and 47 and serines 74 and 76 which are phosphorylated *in vitro* by glycogen synthase kinase 3 and glycogen synthase kinase 5 (casein kinase II), respectively (27). Hemmings *et al.* (27) reported that as isolated bovine heart R^{II} contained 1.5–1.8 mol of phosphate/mol of subunit, but the majority of this phosphate was associated with serines 74 and 76, and only a small amount with the autophosphorylation site. This finding may thereby explain why purified R^{II} can be phosphorylated up to 1 mol of phosphate/mol of subunit by C subunit, although there may already be almost 2 mol of phosphate/mol of R^{II} monomer. Possible correlations of phosphorylation of particular serine residues in R^{II} with changes in behavior must be investigated before ascribing a possible regulatory role to R^{II} phosphorylation by C subunit or other protein kinases. It should also be pointed out that the phosphate incorporated by autophosphorylation of each R^{II} was present in the carboxyl-terminal tryptic fragment, which would not be expected for the phosphorylation of sites other than the autophosphorylation site.

The present study strikes a note of caution when investigating type II protein kinases since not all of them will behave like the much studied bovine heart enzyme. The question has been raised as to whether or not the previously observed differences between neural and non-neural protein kinases may in fact reflect a more widespread pattern of microheterogeneity of R^{II}. Further studies of R^{II} forms may prove to be exciting from an evolutionary point of view as well as probing the question of functional differences between these subclasses. It is interesting that R^{II} apparently displays more microheterogeneity among species than do C subunit and cGMP-dependent protein kinase.

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