Journal of Neurochemistry
Raven Press, New York

0022-3042/83/0301-0661/$03.00/0

3′,5′-Cyclic Adenosine Monophosphate- and Ca^{2+}-Calmodulin-Dependent Endogenous Protein Phosphorylation Activity in Membranes of the Bovine Chromaffin Secretory Vesicles: Identification of Two Phosphorylated Components as Tyrosine Hydroxylase and Protein Kinase Regulatory Subunit Type II

*Marek Treiman, †Wolfgang Weber, and ‡Manfred Gratzl

*Institute of Medical Physiology C, University of Copenhagen, Panum Institute, Copenhagen, Denmark; †Institut für Physiologische Chemie, Universität Hamburg, Hamburg; and ‡Department of Clinical Morphology, University of Ulm, Ulm, G.F.R.

Abstract: Membranes of the secretory vesicles from bovine adrenal medulla were investigated for the presence of the endogenous protein phosphorylation activity. Seven phosphoprotein bands in the molecular weight range of 250,000 to 30,000 were observed by means of the sodium dodecyl sulphate electrophoresis and autoradiography. On the basis of the criteria of molecular weight, selective stimulation of the phosphorylation by cyclic AMP (as compared with cyclic GMP) and immunoprecipitation by specific antibodies, band 5 (molecular weight 60,300) was found to represent the phosphorylated form of the secretory vesicle-bound tyrosine hydroxylase. The electrophoretic mobility, the stimulatory and inhibitory effects of cyclic AMP in presence of Mg^{2+} and $Zn,^{2+}$ respectively, and immunoreactivity toward antibodies showed band 6 to contain two forms of the regulatory subunits of the type II cyclic AMP-dependent protein kinase, distinguishable by their molecular weights (56,000 and 52,000, respectively). Phosphorylation of band 7 (molecular weight 29,800) was stimulated about 2 to 3 times by Ca^{2+} and calmodulin in the concentration range of both agents believed to occur in the secretory tissues under physiological conditions. **Key Words:** Chromaffin vesicles—Tyrosine hydroxylase—Type II protein kinase regulatory subunit—Cyclic AMP-dependent protein phosphorylation—Ca^{2+}-calmodulin-dependent protein phosphorylation—Catecholamine secretion. **Treiman M. et al.** 3′,5′-Cyclic adenosine monophosphate- and Ca^{2+}-calmodulin-dependent endogenous protein phosphorylation activity in membranes of the bovine chromaffin secretory vesicles: Identification of two phosphorylated components as tyrosine hydroxylase and protein kinase regulatory subunit type II. *J. Neurochem.* **40**, 661–669 (1983).

Catecholamines are stored together with other substances within the secretory vesicles of the adrenal medullary chromaffin cells (for a review, see Winkler and Westhead, 1980). The vesicular content is released into the extracellular fluid by exocytosis in response to stimulation by acetylcholine (as reviewed by Douglas, 1975). Studies with whole, perfused adrenal glands (see Douglas, 1975) as well as with isolated chromaffin cells (Kilpatrick et al., 1980; Amy and Kirshner, 1981) have shown that secretion of catecholamines requires an entry of Ca^{2+} into the cells from the external medium during the stimulation.

Work with secretory vesicles isolated from adrenal medulla (Ekerdt et al., 1981) and systems considered to be analogous (where a Ca^{2+}-dependent

Received January 25, 1982; revised July 24, 1982; accepted August 4, 1982.
Address correspondence and reprint requests to Marek Treiman, Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, P.O. Box 3333, New Haven, CT 06510, U.S.A.

Abbreviations used: kD, Kilodaltons; MOPS, 2-(N-Morpholino)propanesulphonic acid; NTA, Nitrilotriacetic acid; $^{32}P_i$, ^{32}P Orthophosphate; SDS, Sodium dodecyl sulphate; TCA Trichloroacetic acid; TES, *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid.

secretion by exocytosis also occurs) has shown that Ca^{2+} may cause exocytosis by initiating membrane fusion (Dahl and Gratzl, 1976; Gratzl et al., 1977; Gratzl and Dahl, 1978; Gratzl et al., 1980).

Protein phosphorylation systems have been postulated to participate in the events triggered by secretory stimuli in the adrenal medulla (Amy and Kirshner, 1981), in nervous tissue (Krueger et al., 1977; DeLorenzo et al., 1979; Huttner and Greengard, 1979), and in other secretory systems, including mast cells (Sieghart et al., 1978) and blood platelets (Haslam and Lynham, 1977). In some of these studies the protein phosphorylation response as well as the secretory response were shown to be dependent on Ca^{2+} (Amy and Kirshner, 1981) or Ca^{2+} and calmodulin (DeLorenzo et al., 1979).

In the present study the effects of cyclic AMP, Ca^{2+}, and calmodulin were investigated on the phosphorylation of proteins in membranes from ox adrenal medulla secretory vesicles. Our aim was to determine whether these organelles contained protein kinases that potentially could be involved in the interactions between second messengers, protein phosphorylation, and secretory events as suggested by the studies mentioned above. An attempt was also made to identify some of the phosphoproteins observed.

Preliminary reports of part of this work have been presented elsewhere (Gratzl et al., 1981*a;* Treiman and Gratzl, 1981).

MATERIALS AND METHODS

5′-[γ-^{32}P]adenosine triphosphate ([γ-^{32}P]ATP) in aqueous solution, specific activity 3000 Ci/mmol, was purchased from The Radiochemical Centre, Amersham, Bucks, U.K. All other chemicals were analytical grade.

Highly purified secretory vesicles were prepared as described recently (Gratzl et al., 1981*b*). Secretory vesicle ghosts were obtained by diluting the isolated secretory vesicles (suspended in a medium containing 2-(*N*-morpholino)propanesulphonic acid) (MOPS), 20 m*M* (pH 7.0); EDTA, 5 m*M;* and sucrose, 340 m*M* to give a final osmolality of about 420 mosmol/kg) in a 10-fold excess of MOPS, 20 m*M* (pH 7.0); EGTA 1 m*M*, and harvesting the membranes by centrifugation at 100,000 g_{max} for 60 min at 4°C. The pellet was resuspended in MOPS, 20 m*M* (pH 7.0); EGTA, 1 m*M*, and recentrifuged (twice). About 20% of total vesicle protein was sedimented as membranes, which were kept frozen at −20°C until use.

The endogenous phosphorylation activity was determined in a medium containing 20 m*M* *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) (pH 7.0); 8 m*M* $MgCl_2$ (or a concentration of $MgCl_2$ necessary to obtain free Mg^{2+} concentration of 8 m*M* in the presence of chelating agents, see below); 8 m*M* theophylline; about 1 mg/ml of secretory vesicle ghosts; and 5 m*M* [γ-^{32}P]ATP. In addition, depending on the purpose of the experiment, the following components were included in the medium, in the concentrations indicated in the text for the relevant figures: (a) cyclic AMP or cyclic GMP; (b) calmodulin, purified from bovine brain as described by Sharma and Wang (1979); (c) EGTA or nitrilotriacetic acid (NTA) to control the free concentrations of Ca^{2+} and Mg^{2+} present in the assay; (d) $CaCl_2$, to obtain the desired free Ca^{2+} concentrations in the presence of the chelating agents. The total amounts of Ca^{2+} and Mg^{2+} to yield the concentrations desired in the presence of EGTA or NTA at the ionic strength and pH employed were calculated by means of a computer programme as described by Flodgaard and Torp-Pedersen (1978), using the stability constants quoted by Sillen and Martell (1971).

The samples (40 μl) were preincubated for 5 min on ice, and for 2 min at 30°C before the reaction was started by addition of 10 μl [γ-^{32}P]ATP, the specific activity of which was varied from 10 to 45 Ci/mmol, depending upon the exposure time during autoradiography and the objective of the experiment.

Unless indicated otherwise, the reaction was stopped by addition of 50 μl of the electrophoresis sample buffer containing Tris, 40 m*M* (pH 7.8); sodium dodecyl sulphate (SDS), 4% wt/vol; β-mercaptoethanol, 4% vol/vol; glycerol, 10% vol/vol and bromphenol blue, 0.016% wt/vol. The electrophoresis was then carried out as described by Treiman et al. (1979), except that gels containing 10% wt/vol acrylamide and 0.27% wt/vol bisacrylamide were used.

For the autoradiography, the Coomassie blue-stained gels were dried and placed onto the KODAK NS-2T X-ray film. The calibration of the film response and recording of the optical densities were done as described earlier (Treiman et al., 1980). The height of the peaks was taken as proportional to the amount of $^{32}P_i$ incorporated into the individual protein bands.

Immunological methods

Antibodies against the regulatory subunits (RI and RII, respectively) of the type I and type II cyclic AMP-dependent kinases were produced in guinea pigs and rabbits, respectively, as described previously (Weber et al., 1981*a*).

Anti-tyrosine hydroxylase (tyrosine-3-monooxygenase; EC.1.14.16.2) immune serum was produced in New Zealand rabbits against the tyrosine hydroxylase fragments purified from a chymotryptic digest of bovine adrenal medulla secretory vesicles, and the γ-globulin fraction was obtained as described by Max et al. (1978). This fraction was kindly given to us by Dr. H. Rohrer, Max Planck Institut für Psychiatrie, Martinfried, G.F.R.

To identify tyrosine hydroxylase by means of the specific antibodies, the phosphorylation of the vesicle membranes was carried out in presence of cyclic AMP, 5 μ*M*, for 2 min. The reaction was terminated by adding 100 μl of the "immunoprecipitation buffer," to obtain the following final concentrations in the samples: TES, 20 m*M* (pH 7.0); Triton X-100, 0.5%; NaCl, 150 m*M;* EDTA, 5 m*M;* and Trasylol, 100 units/ml. Triton-insoluble material was pelleted at 100,000 g_{max} for 15 min in a Beckman Airfuge. To 100 μl of the supernatant, 50 μl of the γ-globulin fraction of the immune serum (dialyzed against "Immunoprecipitation buffer") was added and the mixture was incubated for 1 h at 30°C, followed by 18 h at 4°C (as described by Max et al., 1978). Control samples were incubated without antiserum. The precipitates were sedimented in the Airfuge, and the supernatants and the pellets were subjected to SDS-polyacrylamide gel electrophoresis, as described above.

For the detection of the RI and RII-proteins the phosphorylation reaction was carried out as for the identification of the tyrosine hydroxylase, using 70 μg of secretory vesicle membranes in each tube. Triton X-100, 1%, was used to stop the reaction and to extract the proteins. After pelleting of the insoluble membrane residue, the supernatants were incubated in a volume of 300 μl with 3 μl anti-R immune serum in a mixture containing (m*M*): Tris, 20 (pH 7.4); benzamidine, 20; EDTA, 4; NaCl, 130; Trasylol, 100 units/ml; bovine serum albumin, 3.5 mg/ml; 40 μl protein A sepharose gel (settled volume); and Triton X-100, 0.1%. During incubation (2 h, 4°C) the tubes were rotated. The gel was pelleted and washed 3 times with Tris, 5 m*M* (pH 7.4); NaCl, 145 m*M;* and once with Tris, 20 m*M* (pH 7.4); benzamidine 20 m*M;* and EDTA, 4 m*M*. The phosphoprotein–antibody complexes were released into solution from the gel and dissociated by treating the beads with the electrophoresis sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970).

RESULTS

General characteristics of the phosphorylated proteins

When the chromaffin secretory vesicle membranes were analyzed by means of SDS-polyacrylamide gel electrophoresis the protein pattern obtained by Coomassie blue staining consisted of many bands, resolved in the range of 200,000 to 14,000 daltons (200–14 kD). Following an incubation under phosphorylating conditions, on autoradiography several of these components showed incorporation of $^{32}P_i$. As shown in Fig. 1, the pattern of phosphorylation varied considerably depending on the conditions of the incubation. Although up to 15 bands could be distinguished on the autoradiographs in some experiments (under conditions of maximal stimulation by cyclic AMP, Ca^{2+}, and calmodulin) only seven of them were observed consistently. The molecular weights (average values and standard errors) of these components are listed in Fig. 1.

In preliminary experiments it was found that in the assay medium containing 5 μ*M* cyclic AMP the time course of phosphorylation of major components was close to linear from 10–30 s of the incubation time, leveling off between 60 and 120 s. Since strict linearity was not essential for our purposes, the 30 s time point was used for convenience in experiments concerned with activation patterns. For extensive labeling to facilitate the identification experiments (see Materials and Methods), the reaction was stopped after 2 min. The determination of the nature of the phosphorylated components and of the chemical character of labeling with $^{32}P_i$ was done as described previously (Treiman et al., 1979). In brief, after stopping the phosphorylation reaction by 10% trichloroacetic acid (TCA), the samples were treated in one of the following ways prior to SDS-electrophoresis: (a) heating on a boiling water bath in the presence of 10% TCA for 3 min; (b) exposure to 0.5 *M* cold NaOH for 5 min, (c) exposure to 0.5 *M* NaOH on boiling water bath for 3 min; (d) extracting the lipids using a mixture of diethylether-ethanol 1:1; (e) incubation with 10 μg of pronase for 30 min at 37°C. Treatments (a), (b), and (d) did not produce any change in the phosphorylation pattern of the bands. Treatments (c) and (e) resulted in a removal of the radioactivity along the whole migration range of the gel.

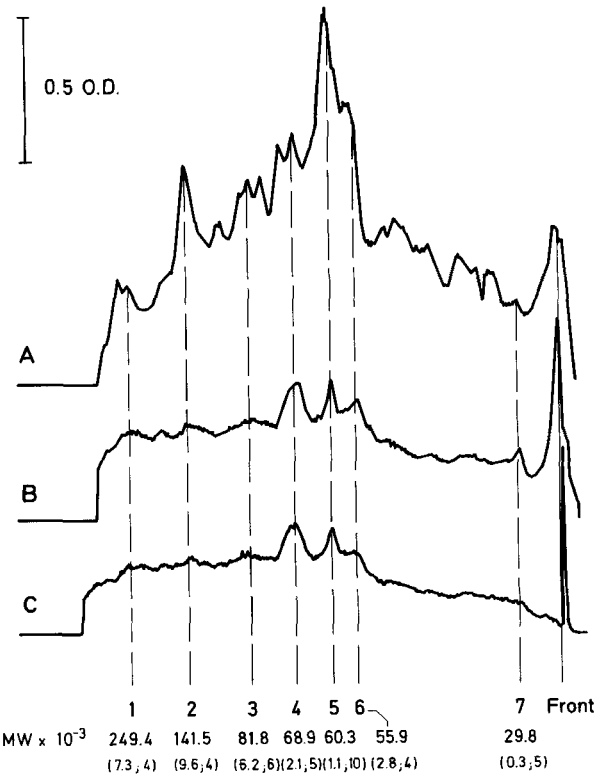

FIG. 1. The optic density (OD) tracings of the autoradiograms of the proteins in chromaffin vesicle membranes phosphorylated under various conditions: **(A)** Cyclic AMP, 5 μ*M;* **(B)** free Ca^{2+}, 100 μ*M;* calmodulin 20 μg/ml; **(C)** EGTA, 1 m*M*. The average molecular weight values are given below the respective band numbers, each with the SEM and number of determinations in the brackets. The scale (0.5 OD unit) corresponds to 340 cpm and 16.2 fmol of $^{32}P_i$ in this experiment. 35 μg of membrane protein was loaded on each gel.

Effects of cyclic AMP and cyclic GMP

The increase in the labeling of most of the phosphoproteins in the membranes from the chromaffin vesicles when 5 μ*M* cyclic AMP was included in the assay is shown in Fig. 1. Under these conditions the incorporation of $^{32}P_i$ into band 5 was greater than into any of the other components. The cyclic AMP concentration dependence of the stimulation of the phosphorylation of this band is shown in Fig. 2. The stimulatory effect of cyclic AMP could not be mimicked by cyclic GMP, even when concentra-

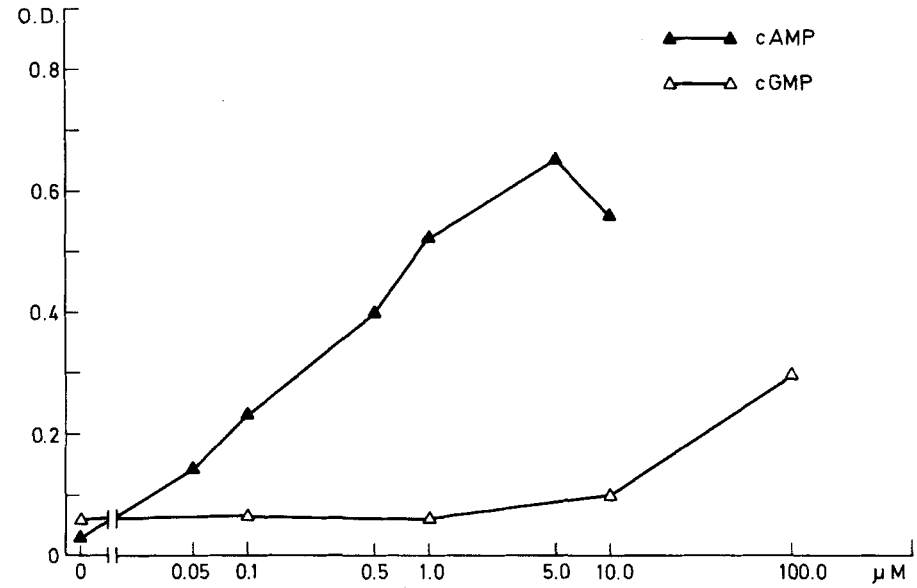

FIG. 2. Effects of cyclic AMP and cyclic GMP (as indicated by symbols in the figure) on the phosphorylation of band 5 in the chromaffin vesicle membranes. A 0.1 optic density (OD) unit corresponds to 98 cpm and 8 fmol $^{32}P_i$. 35 μg of total protein was run on the gel in this experiment. Similar stimulation of band 5 phosphorylation by cyclic AMP was seen in five experiments; the effect of cyclic GMP was seen in two experiments.

tions of cyclic GMP up to 10 times greater than those of cyclic AMP were used.

In one experiment, 1 m*M* Zn^{2+} instead of 8 m*M* Mg was used in the incubation mixture. In the absence of cyclic AMP, the phosphorylation of all the bands was almost completely abolished with the exception of the unaltered activity in band 6. In the presence of cyclic AMP 5 μM, however, band 6 phosphorylation became inhibited as well.

Immunoprecipitation of band 5

The molecular weight of band 5 in the range of 60 kD and the stimulatory effect of cyclic AMP on its phosphorylation suggested that this protein may represent a phosphorylated form of tyrosine hydroxylase (see Discussion). When the γ-globulin fraction of an immune serum containing antibodies against tyrosine hydroxylase (see Immunological methods) was incubated with the Triton X-100-solubilized protein 5, the latter could be precipitated from the solution (Fig. 3). No precipitation was observed in controls incubated without added γ-globulin.

Binding of band 6 to the anti-RII antibodies

Phosphorylated band 6 in the secretory vesicle membranes was identified as follows: The protein kinase regulatory subunits RI and RII (from rabbit muscle and bovine heart, respectively), covalently labeled with 8-azido-^{32}P-cyclic AMP in a photoaffinity reaction (Haley, 1975) served as references on autoradiographs of phosphorylated secretory vesicle membrane proteins. In Fig. 4, lane A, bands RI and RII exhibit molecular weights of 49 kD and 54

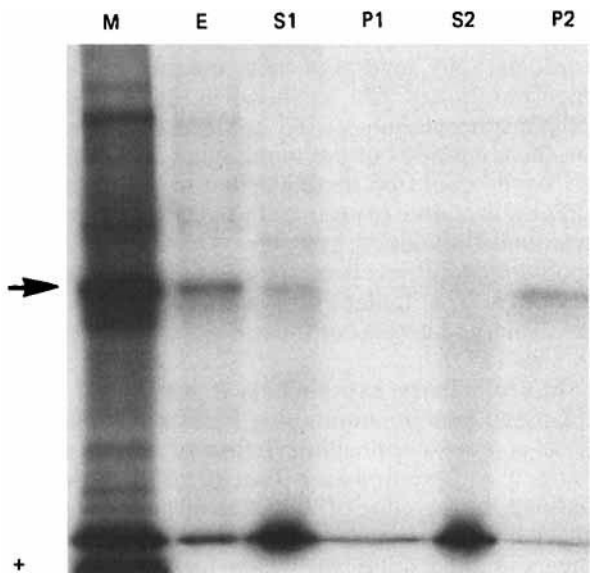

FIG. 3. Autoradiogram showing the precipitation of the phosphorylated band 5 (arrow) of the chromaffin secretory vesicle membranes by means of the antibodies against tyrosine hydroxylase. The SDS-polyacrylamide gel electrophoresis was carried out with the following material labeled with $^{32}P_i$: M, Whole membranes; E, Triton X-100, 0.5% extract of M; Sl and Pl, supernatant and pellet, respectively, obtained by centrifugation of the extract E after incubation without antibodies; S2 and P2, Supernatant and pellet, respectively obtained by centrifugation of the extract E following incubation with the γ-globulin fraction of the antiserum against tyrosine hydroxylase. The front of migration is at the bottom of the figure (+). (Representative of three experiments).

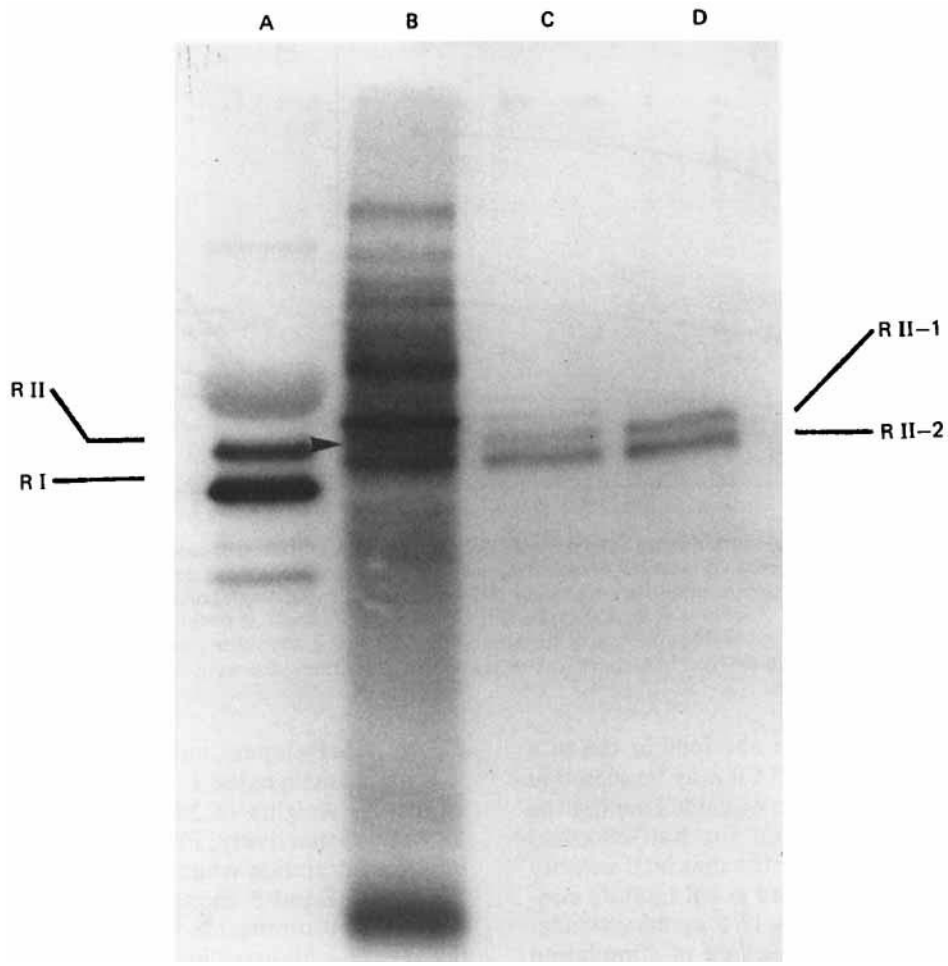

FIG. 4. Autoradiogram showing the separation of two components of band 6 (designated RII-1 and RII-2, respectively) from the phosphorylated membranes of the chromaffin secretory vesicles by means of the anti-RII antibodies. The SDS-polyacrylamide electrophoresis (toward the bottom of the figure) was carried out on the following samples: (A) The cyclic AMP-dependent protein kinase regulatory subunit standards type I and II (indicated by RI and RII, respectively), labeled with ^{32}P in a photoaffinity reaction (some unspecific labeling of serum albumin); (B) Triton X-100, 1% extract of the phosphorylated membranes (the arrowhead pointing to band 6); (C) The RII-1 and RII-2 components separated from the extract by anti-RII antibodies adsorbed to (protein A)-sepharose; (D) Material treated as for C, but incubated with antiserum at a dilution 10 times higher. Note the presence of some activity in position corresponding to band 5 (i.e., the main band in lane B) in lane C, but not in lane D.

kD, respectively. The electrophoretic mobility of the 54 kD subunit is almost identical to that of band 6 found in the Triton X-100 extract of phosphorylated secretory vesicle membranes (Fig. 4, lane B). No phosphorylated band with the electrophoretic mobility corresponding to the RI subunit could be observed in secretory vesicle membranes.

When secretory vesicle membrane proteins solubilized with Triton X-100 were incubated with the Anti-RII antibodies, the immune complexes could be adsorbed to protein A Sepharose beads, washed, and subjected to SDS electrophoresis. Two components could be separated in this way from the phosphorylated secretory vesicle membrane proteins (Fig. 4, lanes C and D): one prominent band (52 kD) and another one in the range of the reference RII (54–56 kD). Control experiments with antibodies to the RI subunit and with protein A Sepharose alone confirmed that only the two phosphoproteins mentioned above could specifically be detected by this technique.

Effects of Ca^{2+} and calmodulin

When calmodulin (20 μg/ml final concentration) was present with 100 μM free Ca^{2+} (pCa 4) in the assay medium, an increase in the phosphorylation of the band with molecular weight 29.8 kD (SEM ± 0.3 kD, n = 5; band 7 in Fig. 1) was observed. (Neither calmodulin nor Ca^{2+} when tested alone had any effect on the phosphorylation of band 7).

The dependence of the stimulation of band 7 on the concentration of calmodulin and Ca^{2+} is shown in Fig. 5. In the range of calmodulin concentrations from 10 to 40 μg/ml (corresponding to 0.60–2.40

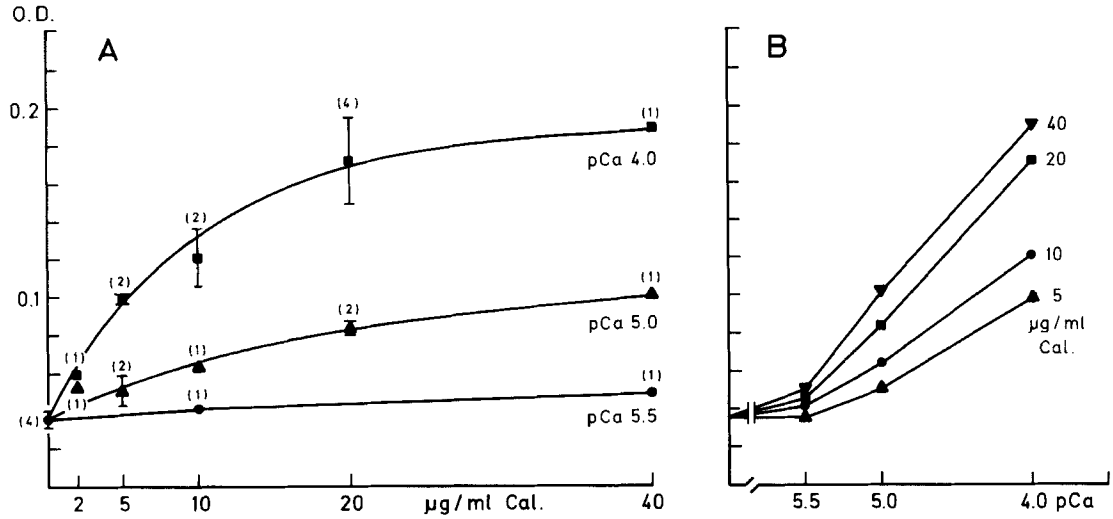

FIG. 5. (A) Dependence of the stimulation of band 7 phosphorylation on the concentration of calmodulin (Cal.) at various concentrations of free Ca^{2+} (as indicated in pCa units below each curve). The numbers in parentheses denote numbers of experiments; in each experiment, at least duplicate determinations were made. A 0.1 optic density (OD) unit corresponds to 163 cpm and 1.5 fmol $^{32}P_i$ in this figure. 35 μg of total membrane protein was run on the gel in each experiment. **(B)** Effects of varying the concentration of calmodulin (Cal.) on the dependence of the stimulation of band 7 phosphorylation on Ca^{2+} (as indicated in pCa units on the abscissa). This figure is derived from part A. The units on the ordinate are as in A.

μM), the stimulation could be obtained at the pCa 5.5 to pCa 4 (Fig. 5A). In Fig. 5A it may be seen that the concentration of free Ca^{2+} was determining the amount of calmodulin needed for half-maximal stimulation. Thus, one-half of the maximal activity displayed at pCa 4 was obtained at calmodulin concentration of 7 μg/ml, whereas 17.5 μg/ml was necessary for the corresponding degree of stimulation at pCa 5.5. Conversely, it is clear from Fig. 5B (derived from the data in Fig. 5A) that the stimulatory effect of Ca^{2+} on the phosphorylation of band 7 was dependent on the concentration of calmodulin. For example, to obtain the phosphorylation level corresponding to 0.1 OD unit, 10 times more Ca^{2+} was necessary when 5 μg/ml as compared with 40 μg/ml of calmodulin was present in the assay.

The basal level of band 7 phosphorylation (with no Ca or calmodulin added and EGTA 1 mM in the assay mixture) was slightly diminished by 50 μM trifluoperazine. In one experiment with triplicate determinations, the labeling of band 7 was 0.037 OD units ($\pm$0.007) and 0.028 ($\pm$0.003) units in the absence and presence of the drug, respectively.

DISCUSSION

The data presented in this paper demonstrate the presence of three types of phosphorylation systems in membranes of the highly purified chromaffin vesicles from bovine adrenal medulla: the cyclic AMP-dependent, the Ca^{2+}- and calmodulin-dependent, and the cyclic AMP and Ca^{2+}- and calmodulin-independent systems.

Cyclic AMP-dependent systems

Proteins designated 1, 2, 3, 5, and 6 (with average molecular weights of 249.4, 141.5, 81.8, 60.3, and 55.9 kD, respectively, Fig. 1) all showed an increase in phosphorylation when cyclic AMP was present in the assay. Band 5 showed several properties indicating that it represents the phosphorylated form of the tyrosine hydroxylase, the rate limiting enzyme in the biosynthesis of catecholamines (Levitt et al., 1965): (a) molecular weight in the range of 60 kD; (b) cyclic AMP-stimulated phosphorylation; (c) immunoreactivity toward anti-tyrosine hydroxylase antibodies. Phosphorylation of this enzyme in membranes of the chromaffin vesicles by an endogeneous protein kinase has not, to our knowledge, been reported earlier.

Endogeneous, as well as exogeneous (by protein kinase from heart) phosphorylation of the soluble tyrosine hydroxylase from bovine adrenal medulla by a soluble cyclic AMP-dependent protein kinase has been shown by Yamauchi and Fujisawa (1979*a*,*b*). The phosphorylation activation pattern of the tyrosine hydroxylase in the presence of cyclic AMP, cyclic GMP, and Ca^{2+} shown in this latter work was in agreement with the findings in the present paper. Thus, the following two possibilities may be considered: (a) The phosphorylation system of the tyrosine hydroxylase and its kinase found in the chromaffin granule membranes has, in fact, properties similar to those of its cytosolic counterpart. (b) The presence of this system in the membranes is due to a cytosolic contamination.

Several lines of evidence would argue against the

latter possibility: (a) The chromaffin granules used in the present work were of high purity. (b) The location of the tyrosine hydroxylase in the chromaffin granules has also been shown by other authors, in both subcellular fractionation (Petrack et al., 1968; Waymire et al., 1972) and immunocytochemical studies (Stephens et al., 1981). (c) The association of the protein kinase (and its regulatory subunit) responsible for the phosphorylation of the tyrosine hydroxylase with the latter enzyme appeared to be very tight. This was suggested by the fact that when RII subunits were separated from the Triton X-100 extract of the membranes by specific antibodies, part of band 5 activity accompanied the RII proteins unless incubation with antibodies was done at high dilution (see Fig. 4, lanes C and D). Indeed, this finding might be taken as indirect evidence that the type II cyclic AMP-dependent protein kinase was the one responsible for the tyrosine hydroxylase phosphorylation in this preparation.

In most mammalian tissues, two types of the cyclic AMP-dependent kinases have been found differing in the properties of their regulatory subunits (RI and RII, respectively), with the respective molecular weights 49 and 55 kD (Nimmo and Cohen, 1977). It was shown (Rosen and Erlichman, 1975) that the RII subunit from bovine cardiac muscle becomes phosphorylated in an autophosphorylation reaction stimulated by cyclic AMP. A similar stimulation by cyclic AMP of the RII autophosphorylation was demonstrated in brain by Lohmann et al. (1980). Therefore, the presence in membranes of the secretory vesicles of a phosphorylated band with apparent molecular weight of 56 kD, a phosphorylation of which was stimulated by cyclic AMP (band 6), indicated that type II protein kinase might be associated with these membranes. It has been shown that the autophosphorylation of the RII subunit of both the soluble (Walter et al., 1977) and membrane bound (Walter et al., 1978) type II cyclic AMP-dependent protein kinase in the presence of Zn^{2+} is inhibited by cyclic AMP. The phosphorylation of band 6 did show this phenomenon. Further, following extraction from the membranes by Triton X-100, this band yielded two components (designated RII−1 and RII−2, 54−56 and 52 kD, respectively) reacting with the anti-RII antibodies. Therefore, these results strongly suggest that protein 6 is composed of RII subunits. The improved resolution of these components following extraction by Triton X-100 as compared with the SDS electrophoresis of the whole membranes was probably due to the considerably reduced background of the extracts subjected to washings after incubation with antibodies.

RII subunit variants with molecular weights in the range of 50−52 kD were previously found in several tissues, including HeLa cells, kidney, lymphocytes (Weber et al., 1981*b*), and heart (Rangel-Aldao et al., 1979).

From these data we cannot conclude that type I protein kinase is absent in the chromaffin vesicle membrane. Neither can it be decided whether each of the phosphorylation sites (or classes of sites) represented by proteins 1, 2, 3, and 5 can be phosphorylated by one or more of the cyclic AMP-dependent kinases.

Ca^{2+}- and calmodulin-dependent system

The presence of a Ca^{2+}- and calmodulin-dependent protein kinase in membranes of the chromaffin granules was revealed by the effects of these agents on the phosphorylation of band 7 (29.8 kD). At calmodulin concentrations of 40 and 20 μg/ml (about 2.4 and 1.2 μM, respectively) and concentration of free Ca^{2+}, 10 μM, the phosphorylation of band 7 was stimulated 2.8 and 2.3 times, respectively. Thus, the stimulation was shown to occur in the range of the free Ca^{2+} concentrations believed to appear in nerve terminals and endocrine cells stimulated to secretion (Llinas and Heuser, 1977). The concentrations of calmodulin mentioned above fit the estimated figures for its concentration in the adrenal medullary tissue (Kuo and Coffee, 1976). Therefore, the data suggest that the stimulation of the band 7 phosphorylation by Ca^{2+} and calmodulin may be of physiological importance in connection with the involvement of the chromaffin granules in the processes of hormone synthesis, transport, storage, and release.

Burgoyne and Geisow (1981) have recently reported the stimulation by Ca^{2+} and calmodulin of a group of polypeptides (with molecular weights 59, 58, 53, and 43 kD) in membranes of the bovine chromaffin granules. (In addition, a phosphorylated band in the range of 30 kD appears on the autoradiogram in Fig. 3 of their paper. However, from this figure it is difficult to see whether or not the phosphorylation of this band was affected by the presence of Ca^{2+} and calmodulin). Burgoyne and Geisow have also observed that 59 and 43 kD proteins in their preparation were phosphorylated in a cyclic AMP-stimulated reaction.

It is not possible at present to assess the significance of the disparities between the findings of these authors and those in the present paper. The conditions of the phosphorylation assay and the method of preparing the chromaffin granules were both among the important differences in the two studies.

Cyclic AMP-, Ca^{2+}- and calmodulin-independent phosphorylation system

The phosphoprotein designated band 4 (68.9 kD) in the present work did not alter its phosphorylation in response to the various conditions tested. Phos-

phorylation of several proteins in the membranes of the chromaffin secretory vesicles in absence of any of the agents mentioned above was also observed by Burgoyne and Geisow (1981) and by Taugner et al. (1979).

In conclusion, the evidence presented in this paper supports the notion that the protein phosphorylation systems are present on the membranes of the chromaffin granules and may be involved in their physiological functions. The complexity of the phosphorylation pattern revealed in the earlier studies as well as in the present work seems to indicate that multiple functions are subserved by these systems.

Acknowledgments: We are indebted to Dr. H. Rohrer, Martinfried, G.F.R. for a gift of the γ-globulin fraction of the antiserum against tyrosine hydroxylase. We would like to thank Professor Niels A. Thorn for comments on the manuscript, Ms. Elin Engberg and Ms. Monica Elis and the Panum Institute Service Department for their excellent assistance, and Mrs. Birgit Dalgaard for typing the manuscript. This work was supported by the Danish Medical Research Council, the Nordisk Insulin Foundation, and by the Deutsche Forschungsgemeinschaft (SFB 34 and 38).

REFERENCES

Amy C. M. and Kirshner N. (1981) Phosphorylation of adrenal medulla cell proteins in conjunction with stimulation of catecholamine secretion. *J. Neurochem.* **36**, 847–854.

Burgoyne R. D. and Geisow M. J. (1981) Specific binding of ^{125}I-calmodulin to and protein phosphorylation in adrenal chromaffin granule membranes. *FEBS Lett.* **131**, 127–131.

Dahl G. and Gratzl M. (1976) Calcium-induced fusion of isolated secretory vesicles from islets of Langerhans. *Cytobiologie* **12**, 344–355.

DeLorenzo R., Freedman S. D., Yohe W. B., and Maurer S. C. (1979) Stimulation of Ca^{2+}-dependent neurotransmitter release and presynaptic nerve terminal protein phosphorylation by calmodulin and a calmodulin-like protein isolated from synaptic vesicles. *Proc. Natl. Acad. Sci. USA* **76**, 1838–1842.

Douglas W. W. (1975) Secretomotor control of adrenal medullary secretion: Synaptic, membrane and ionic events in stimulus-secretion coupling, in *Handbook of Physiology, Section 7. Endocrinology, Vol. VI. The Adrenal Gland* (Greep R. O. et al., eds.), pp. 367–388, American Physiological Society, Washington, D.C.

Ekerdt R., Dahl G., and Gratzl M. (1981) Membrane fusion of secretory vesicles and liposomes. Two different types of fusion. *Biochim. Biophys. Acta* **646**, 10–22.

Flodgaard H. and Torp-Pedersen C. (1978) A calcium ion-dependent adenosine triphosphate pyrophosphohydrolase in plasma membrane from rat liver. *Biochem. J.* **171**, 817–820.

Gratzl M. and Dahl G. (1978) Fusion of secretory vesicles isolated from rat liver. *J. Membr. Biol.*, **40**, 343–364.

Gratzl M., Dahl G., Russell J. T., and Thorn N. A. (1977) Fusion of neurohypophyseal membranes *in vitro*. *Biochim. Biophys. Acta* **470**, 45–57.

Gratzl M., Schudt C., Ekerdt R., and Dahl G. (1980) Fusion of isolated biological membranes, in *Membrane Structure and Function Vol. 3*, (Bittar E. E., ed.), pp. 59–92. John Wiley & Sons, New York.

Gratzl M., Krieger-Brauer H., Treiman M., and Ekerdt R. (1981*a*) Sidedness of acetylcholinesterase and membrane phosphorylation in secretory vesicles isolated from adrenal medulla, in *Abstracts, 7th International Biophysics Congress and 3rd Pan-American Biochemistry Congress, Mexico City, August 23–28, 1981*, p. 253.

Gratzl M., Krieger-Brauer H., and Ekerdt R. (1981*b*) Latent acetylcholinesterase in secretory vesicles isolated from adrenal medulla. *Biochim. Biophys. Acta*, **946**, 355–366.

Haley B. E. (1975) Photoaffinity labelling of adenosine 3′,5′-cyclic monophosphate binding sites of human red cell membranes. *Biochemistry* **14**, 3852–3857.

Haslam R. J. and Lynham J. A. (1977) Relationship between phosphorylation of blood platelet proteins and secretion of platelet granule constituents. I. Effects of different aggregating agents. *Biochem. Biophys. Res. Commun.* **77**, 714–722.

Huttner W. B. and Greengard P. (1979) Multiple phosphorylation sites in protein I and their differential regulation by cyclic AMP and calcium. *Proc. Natl. Acad. Sci. USA* **76**, 5402–5406.

Kilpatrick D. L., Ledbetter F. H., Carson K. A., Kirshner A. G., Slepetis R., and Kirshner N. (1980) Stability of bovine adrenal medulla cells in culture. *J. Neurochem.* **35**, 679–692.

Krueger B. K., Forn J., and Greengard P. (1977) Depolarization-induced phosphorylation of specific proteins, mediated by calcium ion influx, in rat brain synaptosomes. *J. Biol. Chem.* **252**, 2764–2773.

Kuo I. C. Y. and Coffee C. J. (1976) Purification and characterization of a troponin C-like protein from bovine adrenal medulla. *J. Biol. Chem.* **251**, 1603–1609.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Levitt M., Spector S., Sjoerdsma A., and Udenfriend S. (1965) Elucidation of the rate-limiting step in norepinephrine biosynthesis in the perfused guinea pig heart. *J. Pharmacol. Exp. Ther.* **148**, 1–8.

Llinas R. R. and Heuser J. E. (eds.) (1977) Depolarization-release coupling systems in neurons, in *Neurosciences Research Program Bulletin*, Vol. 15, pp. 574 and 591.

Lohmann S. M., Walter U., and Greengard P. (1980) Identification of endogenous substrate proteins for cAMP-dependent protein kinase in bovine brain. *J. Biol. Chem.* **255**, 9985–9992.

Max S. R., Rohrer H., Otten U., and Thoenen H. (1978) Nerve growth factor-mediated induction of tyrosine hydroxylase in rat superior cervical ganglia *in vitro*. *J. Biol. Chem.* **253**, 8013–8015.

Nimmo H. G. and Cohen P. (1977) Hormonal control of protein phosphorylation, in *Advances in Cyclic Nucleotide Research, Vol. 8* (Greengard P. and Robinson G. eds.), pp. 145–266.

Petrack B., Sheppy F., and Fetzer V. (1968) Studies on tyrosine hydroxylase from bovine adrenal medulla. *J. Biol. Chem.* **243**, 743–748.

Rangel-Aldao R., Kupiec J. W., and Rosen O. M. (1979) Resolution of the phosphorylated and dephosphorylated cAMP-binding proteins of bovine cardiac muscle by affinity labelling and two-dimensional electrophoresis. *J. Biol. Chem.* **254**, 2499–2508.

Rosen O. M. and Erlichman J. (1975) Reversible autophosphorylation of a cyclic 3′:5′-AMP-dependent protein kinase from bovine cardiac muscle. *J. Biol. Chem.* **250**, 7788–7794.

Sharma R. K. and Wang J. H. (1979) Preparation and assay of the Ca^{2+}-dependent modulator protein, in *Advances in Cyclic Nucleotide Research, Vol. 10* (Brooker G., Greengard P., and Robins G. A., eds.), pp. 187–198. Raven Press, New York.

Sieghart W., Theoharides T. C., Alper S. L., Douglas W. W., and Greengard P. (1978) Calcium-dependent protein phosphorylation during secretion by exocytosis in the mast cell. *Nature* **275**, 329–331.

Sillen L. G. and Martell A. D. (1971) Stability constants of

metal-ion complexes. Suppl. 1. The Chemical Society, London.

Stephens J. K., Masserano J. M., Vulliet P. R., and Nakane P. K. (1981) Immunocytochemical localizatin of tyrosine hydroxylase in rat adrenal medulla by the peroxidase labeled antibody method: Effects of enzyme activation on ultrastructural distribution of the enzyme. *Brain Res.* **209**, 339–354.

Taugner G., Wunderlich I., and John F. (1979) Distribution and metabolic fate of adenosine nucleotides in the membrane of storage vesicles from bovine adrenal medulla. *Naunyn Schmiedebergs Arch. Pharmacol.* **309**, 29–43.

Treiman M. and Gratzl M. (1981) Endogenous protein phosphorylation activity in bovine chromaffin granules purified on a Percoll gradient (tentative identification of a major granule membrane phosphoprotein as a subunit of tyrosine hydroxylase), in *Abstracts, 8th Meeting of the International Society for Neurochemistry, Nottingham, Sept. 7–11, 1981, p. 327.*

Treiman M., Pødenphant J., Særmark T., and Bock E. (1979) Inhibition by Ca of the adenosine 3′:5′-cyclic monophosphate-stimulated phosphorylation of proteins in membranes from ox neurohyphophyseal secretosomes. *FEBS Lett.* **97**, 147–150.

Treiman M., Worm-Petersen S., and Thorn N. A. (1980) Complex phosphorylation activity in neurosecretosomal membranes isolated from ox neurohypophyses. *Biochem. J.* **188**, 657–666.

Walter U., Uno J., Liu A. Y.-C., and Greengard P. (1977) Study of autophosphorylation of isoenzymes of cyclic AMP-dependent protein kinases. *J. Biol. Chem.* **252**, 6588–6590.

Walter U., Kanof P., Schulman H., and Greengard P. (1978) Adenosine 3′:5′-monophosphate receptor proteins in mammalian brain. *J. Biol. Chem.* **253**, 6275–6280.

Waymire J. C., Weiner N., Schneider F. H., Goldstein M., and Freedman L. S. (1972) Tyrosine hydroxylase in human adrenal and pheochromocytoma localization, kinetics, and catecholamine inhibition. *J. Clin. Invest.* **51**, 1798–1804.

Weber W., Schroeder H., and Hilz H. (1981*a*) Quantitation of heterologous protein kinase subunits RI and RII with the aid of type specific antibodies. *Biochem. Biophys. Res. Commun.* **99**, 475–483.

Weber W., Schwoch C., Schroeder H., and Hilz H. (1981*b*) Analysis of cAMP-dependent protein kinases by immunotitration: Multiple forms—multiple functions?, in *Cold Spring Harbor Conference on Cell Proliferation* Vol. 8, pp. 125–140.

Winkler H. and Westhead E. (1980) The molecular organization of adrenal chromaffin granules. *Neuroscience* **5**, 1803–1823.

Yamauchi T. and Fujisawa H. (1979*a*) *In vitro* phosphorylation of bovine adrenal tyrosine hydroxylase by adenosine 3′:5′ monophosphate-dependent protein kinase. *J. Biol. Chem.* **254**, 503–507.

Yamauchi T. and Fujisawa H. (1979*b*) Regulation of bovine adrenal tyrosine 3-monooxygenase by phosphorylation-dephosphorylation reaction, catalyzed by adenosine 3′:5′ monophosphate-dependent protein kinase and phosphoprotein phosphatase. *J. Biol. Chem.* **254**, 6408–6413.