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Rapid purification and identification of calcyphosine, a Ca^{2+}-binding protein phosphorylated by protein kinase A

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A method is presented for the rapid purification of dog thyroid calcyphosine, a protein previously identified as a major substrate for cyclic AMP-dependent protein kinase in dog thyroid slices stimulated by thyrotropin [Lecocq, Lamy and Dumont (1979) Eur. J. Biochem. **102**, 147–152]. The protein was previously identified as a spot on two-dimensional gels and is now purified in its native form by a procedure involving three chromatographic steps. Homogeneous calcyphosine identified by SDS/PAGE, immunoblotting and peptide sequencing can be obtained within 7 h. As for calmodulin, Ca^{2+}-dependent conformational changes can be shown by Ca^{2+}-dependent hydrophobic interaction chromatography using phenyl-Sepharose. Unlike calmodulin, calcyphosine is a substrate for protein kinase A.

INTRODUCTION

Thyroid function is regulated by thyrotropin via adenylate cyclase activation and by various neurotransmitters acting through both adenylate cyclase and the phospholipase C cascade. Calcyphosine was initially identified as a major phosphorylated substrate for cyclic AMP-dependent protein kinase after stimulation of dog thyroid cells by thyrotropin (Lecocq et al., 1979). In fact, calcyphosine is subjected to multiple regulation in these cells. It is rapidly phosphorylated during full functional activation of the cell via the cyclic AMP cascade, but is not phosphorylated in response to acetylcholine which activates the Ca^{2+}–phosphatidylinositol cascade. This agent has some similar effects to thyrotropin but mostly they are opposite.

Calcyphosine synthesis is enhanced by thyrotropin and cyclic AMP analogues that trigger cell proliferation and maintain expression of the differentiated thyrocyte phenotype. Its synthesis is decreased by epidermal growth factor and phorbol 12-myristate 13-acetate which also trigger cell proliferation but repress expression of differentiation (Lecocq et al., 1990).

Polyclonal antibodies against calcyphosine (Lecocq et al., 1990) have been used as a tool to isolate cDNA encoding calcyphosine (Lefort et al., 1989) (EMBL accession number X14047). An open reading frame of 189 codons encodes a polypeptide of calculated molecular mass 21104 Da which is comparable with the apparent molecular mass of 24000 Da previously estimated by SDS/PAGE. The primary structure of the encoded polypeptide contains a serine residue (Ser-40) which could be phosphorylated by cyclic AMP-dependent protein kinase. The primary structure reveals significant similarities to most of the Ca^{2+}-binding proteins containing EF hand domains, particularly calmodulin. Its ability to bind Ca^{2+} has been demonstrated using ^{45}Ca (Lefort et al., 1989).

In order to elucidate the role of calcyphosine in the thyroid and other tissues, it is necessary to obtain large amounts of it. We report here a particularly rapid method which allows purification of 90 μg starting from 4 g of dog thyroid tissue.

MATERIALS AND METHODS

Materials

Mes, Tris, leupeptin, endoproteinase Asp N, the catalytic subunit of protein kinase A and pepstatin were obtained from Boehringer-Mannheim. PEFABLOC was from Pentapharm (Basle, Switzerland). ExcelGel, phenyl-Sepharose CL-4B and DEAE-Sephacel were purchased from Pharmacia (Uppsala, Sweden). DEAE-MemSep 1010 HP cartridge and Millex-VV unit (0.1 μm) were from Millipore (Milford, MA, U.S.A.), and phenyl-TSK columns were from Bio-Rad (Richmond, CA, U.S.A.). Dithiothreitol (DTT) and Coomassie Brilliant Blue G were obtained from Serva (Heidelberg, Germany). EGTA was from Sigma (St Louis, MO, U.S.A.). Strataclean resin was from Stratagen (La Jolla, CA, U.S.A.). [^{35}S]Methionine, [γ-^{32}P]ATP and rainbow molecular-mass standards were obtained from Amersham International (Amersham, Bucks, U.K.). Insta-Gel II was purchased from Packard (Meriden, CT, U.S.A.). H.p.l.c.-grade water was produced on Milli Q apparatus (Millipore; Milford, MA, U.S.A.). All other reagents were of analytical grade and purchased from UCB (Leuven, Belgium). Polyclonal antibodies against calcyphosine were prepared as described by Lecocq et al. (1990).

Collection and incubation of tissue

Dog thyroids were resected from animals used for physiological experiments and immersed in Krebs–Ringer bicarbonate buffer. The two lobes were cut into small pieces of about 150 mg and immediately transferred to liquid nitrogen. For labelling of proteins with [^{35}S]methionine, thyroid slices about 0.2 mm thick were prepared at room temperature and incubated (500 mg of wet tissue per flask) at 37 °C for 4 h, under 95 % O_2/5 % CO_2 in 5 ml of Krebs–Ringer bicarbonate buffer containing [^{35}S]methionine (200 μM; specific radioactivity 1 Ci/μmol), 8 mM glucose and 1 g/l BSA.

Abbreviation used: DTT, dithiothreitol.

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Tissue homogenization and centrifugation

Frozen tissue (1–4 g) was ground in a mortar cooled with liquid nitrogen. All subsequent manipulations were performed at 4 °C. The resulting powder was homogenized in 8 ml of the starting buffer used for anion-exchange chromatography (0 M NaCl) using a glass grinder. The homogenate was centrifuged at 48000 *g* (20000 rev./min) in an SS34 Sorvall rotor for 30 min; the supernatant, referred to as the soluble fraction, was used as the starting material.

Electrophoresis procedure

SDS/PAGE and silver staining of the gel was carried out on ready-to-use gel (ExcelGel) according to the instructions provided by the manufacturer. The maximum loading volume was 5 μl of sample in solution in chromatographic eluent plus 35 μl of sample buffer, otherwise separation was disturbed. When required, protein was concentrated using Strataclean as follows. The resin suspension (10 μl) was added to up to 1 ml of sample solution and mixed by vortexing. After 1 min the solution was centrifuged for 1 min in a Microfuge (Beckman; Palo Alto, CA, U.S.A.) and the supernatant was discarded. Then 35 μl of sample buffer was added to the pellet and the mixture boiled for 3 min before the resin-containing solution was loaded on the ExelGel. A 100-fold concentration could be achieved by this technique.

Protein determination

Protein assays were performed as previously described (Minamide and Bambourg, 1990) with BSA as standard. This method can be used for 20 μl protein samples with protein concentration as low as 20 μg/ml using the multiple application technique; no interference from any reagent used in the purification was seen.

Radioactivity counting and autoradiography

Portions of up to 100 μl of the various protein solutions to be counted were dissolved in 10 ml of Insta-Gel II and counted in a liquid-scintillation analyser (Packard Tri-Carb, model 1600A; Meriden, CT, U.S.A.) for a number of counts at least equal to ten times the background. Subsequent addition of an internal standard confirmed that, under our conditions, the efficiency was the same for all samples. Labelled proteins separated by SDS/PAGE were detected by fluorography or autoradiography (Lecocq et al., 1990).

Conventional anion-exchange chromatography

All procedures were performed at 4 °C. The column was 3.5 cm × 1.6 cm (internal diameter); DEAE-Sephacel conditioned with Cl^- as counterion was equilibrated with the starting buffer (20 mM sodium acetate, pH 5.5, 1 mM EDTA, 1 mM DTT) containing 50 mg/l PEFABLOC, 0.5 mg/l leupeptin and 0.7 mg/l pepstatin. The starting sample was loaded on to the column at a flow rate of 1 ml/min; this was followed by a 45 min wash at 1 ml/min with the starting buffer plus 0.05 M NaCl. The sample was eluted with starting buffer containing 0.2 M NaCl, and 4 ml fractions were collected.

Anion-exchange h.p.l.c.

The starting buffer was the same as that used for conventional anion-exchange chromatography. The DEAE-MemSep cartridge conditioned with Cl^- as counterion was equilibrated with the starting buffer plus 0.05 M NaCl. The pooled fractions from the preceding chromatography corresponding to a maximum of 1 g of tissue were diluted five times with starting buffer and then filtered through a Millex-VV unit. The filtered solution kept on ice was loaded on to the DEAE-MemSep cartridge at a flow rate of 5 ml/min by means of a peristaltic pump and thereafter the cartridge was connected to a Waters h.p.l.c. model 650 supplied with a 486 variable-wavelength detector set at 280 nm linked to a C-R6A integrator from Shimadzu. H.p.l.c. was performed at room temperature. The flow rate was 10 ml/min, elution was isocratic from 0 to 2.5 min with starting buffer plus 0.05 M NaCl followed by a linear gradient of NaCl in starting buffer from 0.05 to 0.21 M in 3.8 min.

Hydrophobic interaction chromatography

All procedures were performed at room temperature, and h.p.l.c. was conducted on the equipment described for anion-exchange h.p.l.c. The phenyl-TSK column was equilibrated with binding buffer (50 mM Tris/HCl, pH 7.5, 1 mM $CaCl_2$). The pooled fractions from anion-exchange h.p.l.c. were made up to 1 mM $CaCl_2$ by addition of a 10 mM $CaCl_2$ stock solution. This solution was loaded at 1 ml/min on to a Phenyl-TSK column using a Pharmacia Super Loop connected between the h.p.l.c. pumps and the columns. The Super Loop was then disconnected and the column directly connected to the h.p.l.c. apparatus. The flow rate was 1 ml/min, elution was isocratic from 0 to 10 min with binding buffer followed by isocratic elution for 10 min in 50 mM Tris/HCl, pH 7.5, containing 1 mM EGTA.

Hydrophobic interaction chromatography was also performed on a conventional column using freshly homogenized tissue (0.2 g in 2 ml of binding buffer containing protease inhibitors). A column 1 cm in diameter was filled with 2 ml of phenyl-Sepharose CL-4B and equilibrated at 2 ml/min with 20 ml of binding buffer. All subsequent steps were performed at 1 ml/min. The supernatant was loaded on to the column followed by 45 ml of binding buffer to wash the column. Bound proteins were eluted in 50 mM Tris/HCl, pH 7.5, containing 1 mM EGTA.

Microsequence determination

About 200 pmol of purified calcyphosine was separated on an SDS/12.5% polyacrylamide gel and then electroblotted on a poly(vinylidene difluoride) membrane. *In situ* digestion with endoproteinase Lys C and subsequent separation of the peptides by reversed-phase h.p.l.c. were performed as described by Rasmussen et al. (1991). Sequencing of the peptides was carried out on a 477A pulsed liquid-phase sequenator (Applied Biosystems).

In vitro phosphorylation of calcyphosine

Calcyphosine (about 2 μg) was phosphorylated in 70 μl of buffer [50 mM Mes, pH 6.9, 10 mM $MgCl_2$, 0.5 mM EDTA, 150 mM [γ-^{32}P]ATP (specific radioactivity 45 μCi/μmol), 1 mM DTT and 1 mg/ml BSA]. The reaction was started by the addition of 1 munit of the catalytic subunit of protein kinase A and incubation was for 10 min at 30 °C. The reaction was stopped by extraction of the proteins with Strataclean resin and boiling for 3 min in 34 μl of sample buffer.

RESULTS

Relative abundance of calcyphosine in dog thyroid

Total protein was loaded on to a conventional hydrophobic interaction column in the presence of Ca^{2+}. A washing step was performed to eliminate most of the unbound proteins followed by an elution step in the presence of 1 mM EGTA. During the elution step, 1 ml fractions were collected. Proteins from each fraction were extracted with Strataclean resin and separated on

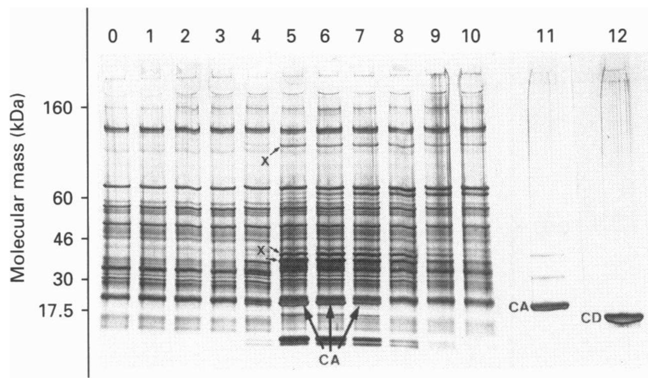

Figure 1 SDS/ExcelGel electrophoresis and silver staining of fractions obtained during conventional hydrophobic interaction chromatography

Lane 0, protein that did not bind to the column in the presence of Ca^{2+}; lanes 1–10, additional proteins specifically eluted when Ca^{2+} was chelated by EDTA; lane 11, purified calcyphosine; lane 12, purified calmodulin. CA and CD indicate the location of calcyphosine and calmodulin respectively. × indicates some of the unknown proteins eluted in the presence of EGTA.

an ExcelGel. The result obtained by silver staining of the gel is presented in Figure 1. Lane 0 shows proteins eluted during the washing step before the addition of 1 mM EGTA. Calcyphosine was eluted only in the presence of EGTA, mainly in fractions 5, 6 and 7, at an estimated molecular mass of 24 kDa, i.e. the same as purified calcyphosine (lane 11). Only faint bands can be seen at the level of migration of calmodulin. This result demonstrates that, when homogenized in 1 mM $CaCl_2$, dog thyroid calcyphosine is much more abundant than calmodulin in the soluble fraction of the homogenate; in fact, it is the most abundant protein that interacts with a hydrophobic column in a Ca^{2+}-dependent way.

Purification

Homogenization and h.p.l.c. procedures were as described in the Materials and methods section. It is convenient to start with 4 g wet weight of tissue. We determined the limiting step according to column capacity to be the anion-exchange h.p.l.c. At this step, loading material in excess of 1 g of tissue impairs the resolution. Nevertheless, as one run on the DEAE-MemSep cartridge takes just a few minutes, it can be repeated as many as four times and still allows about 90 μg of pure calcyphosine to be obtained in less than 7 h.

Conventional anion-exchange chromatography

Dog thyroids were homogenized in the starting buffer for anion-exchange chromatography and centrifuged as described in the Materials and methods section. The supernatant was loaded on to a DEAE-Sephacel column. The column was then washed with buffer containing 0.05 M NaCl. Proteins were eluted in buffer containing 0.2 M NaCl, and 4 ml fractions were collected. Fractions 2–4, which contain the bulk of the eluted protein (as assessed by measurement of A_{280}) were pooled for subsequent purification by anion-exchange h.p.l.c.

MemSep anion-exchange chromatography

A quarter of the pooled fractions obtained by conventional anion-exchange chromatography was five times diluted with cold starting buffer (0 M NaCl) to lower the NaCl concentration to 0.05 M, the final volume being 15 ml. The solution was kept cold

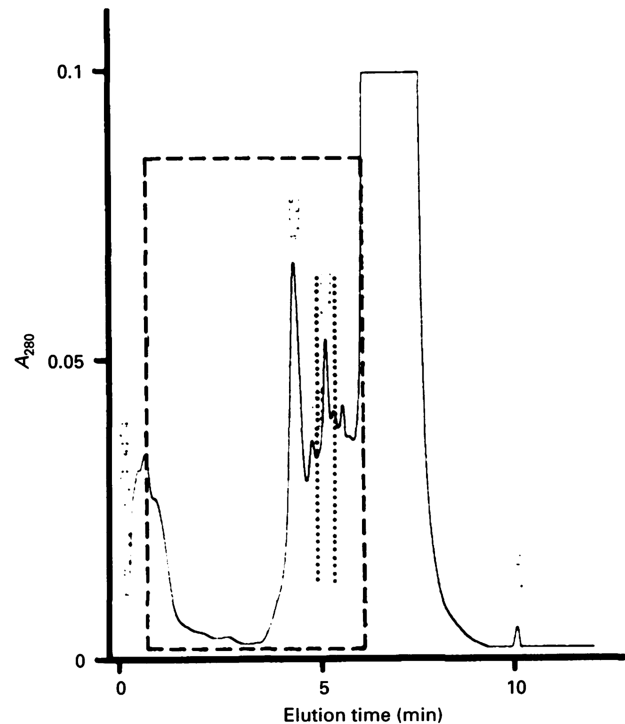

Figure 2 H.p.l.c. anion-exchange chromatography (DEAE-MemSep cartridge)

The pooled fractions from the DEAE-Sephacel column were diluted to 0.05 M NaCl with starting buffer at pH 5.5 and loaded by means of a peristaltic pump on to a DEAE-MemSep cartridge at 5 ml/min. The cartridge was then connected to an h.p.l.c. column. Flow rate was 10 ml/min. Isocratic elution was performed at 0.05 M NaCl from 0 to 2.5 min followed by a linear gradient of 0.05 to 0.21 M NaCl from 2.5 to 6.3 min. A_{280} was monitored. Fractions of volume 2 ml were collected. Calcyphosine was immunodetected in fractions 9 and 10 delimited by the two vertical dotted lines on the Figure (retention time of about 5 min). The rectangle represents the area reproduced in Figure 3 for chromatography performed at different pH values.

during loading on to the DEAE-MemSep cartridge, but h.p.l.c. was carried out at room temperature. The elution profile is shown in Figure 2. Calcyphosine was eluted in fractions 9 and 10 as shown by immunodetection. Fractions 9 and 10 obtained from the four runs were therefore pooled for subsequent purification. A pH of 5.5 was selected for anion-exchange chromatography after optimization of the pH using the Waters Auto-Blend method. The pH range 8.3–5.0 had been investigated (Figure 3). It is obvious from the Figure that lowering the pH gradually leads to cleaner profiles with loss of material eluted before calcyphosine and the appearance of sharper peaks in the area of the protein of interest. The best profile is obtained at pH 5.5 (Figure 2). At pH 5.0, calcyphosine no longer binds to the column and is eluted before the NaCl gradient.

Hydrophobic interaction chromatography

This separation takes advantage of the fact that, like other proteins that bind Ca^{2+}, particularly calmodulin (Dedman and Kaetzel, 1983), calcyphosine exposes hydrophobic regions in the presence of Ca^{2+} and these regions are hidden when Ca^{2+} is chelated. The pooled fractions from the MemSep anion-exchange chromatography were made 1 mM in Ca^{2+} and then loaded on to the Phenyl-TSK column. The elution profile obtained is shown in Figure 4. Some material is detected at the beginning of the chromatogram and corresponds to material that does not bind to

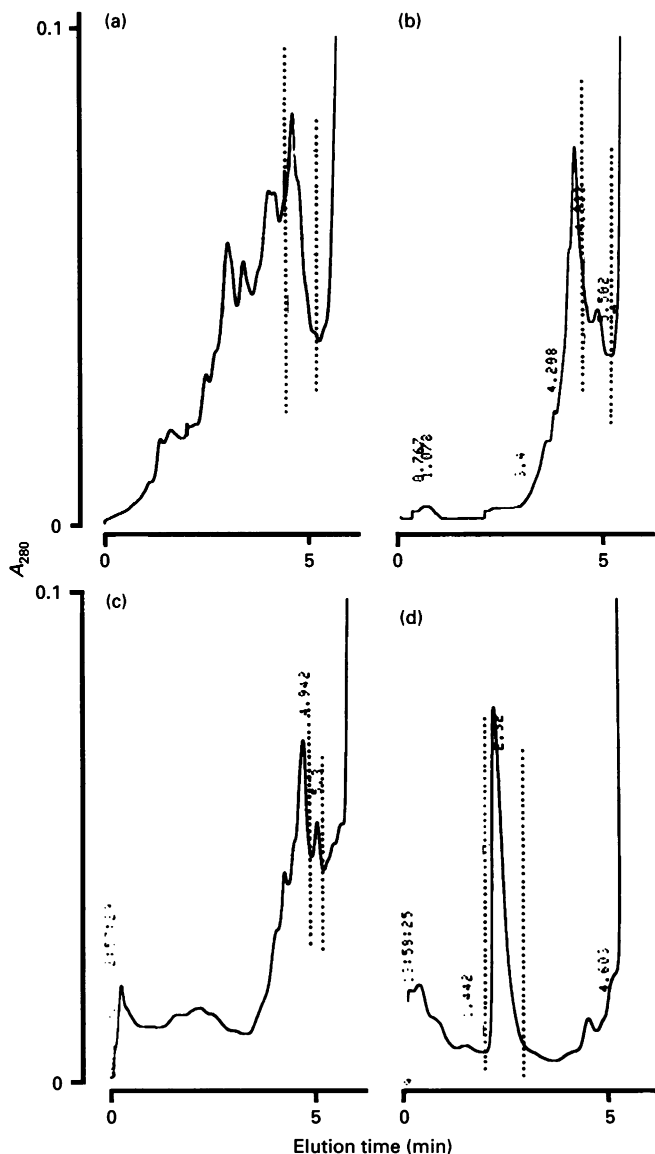

Figure 3 H.p.l.c. anion-exchange chromatography at different pH values

Chromatography was performed as described in Figure 2 except that the pH was changed. Parts of the chromatograms corresponding to the area delimited by the rectangle in Figure 2 are presented. (**a**) pH 8.3; (**b**) pH 6.5; (**c**) pH 6.1; (**d**) pH 5.0. The vertical dotted lines delimit the regions where calcyphosine was immunodetected.

the column. Thereafter the A_{280} returns to the baseline until 1 mM Ca^{2+} is replaced by 1 mM EGTA; a single peak of bound material is eluted. Calcyphosine was detected in this peak using a monospecific antibody against this protein (Lecocq et al., 1990). Moreover a microsequence analysis of a peptide generated by endoproteinase Lys C digestion gave L-R-A-Q-X-L-S-R-G-A-L-G-I-Q-G-L-A-R-F-F-R-R-L-D-R-D-R-S-R-S-L-D-S-R, which corresponds to the sequence of calcyphosine from 11 to 44 amino acid residues, as deduced from cDNA cloning (Lefort et al., 1989). No derivatization of cysteine was performed before sequence analysis, which explains the unidentified phenylthiohydantoin amino acid designated X. Calcyphosine can be shown to be a substrate of the catalytic subunit of protein kinase A. Figure 5 (lane 5) shows incorporation of ^{32}P into the 24 kDa protein.

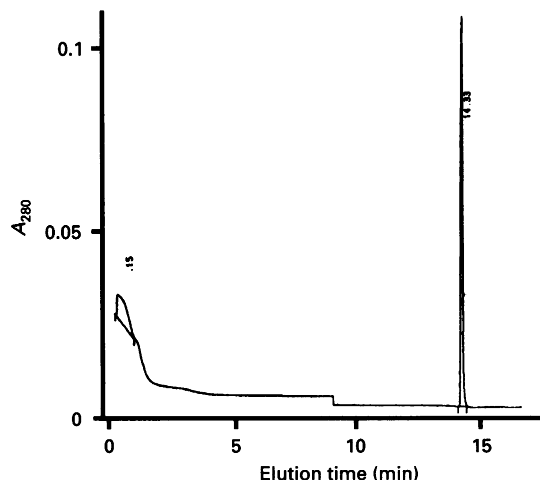

Figure 4 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography on a phenyl-TSK column was performed on pooled fractions 9 and 10 from anion-exchange h.p.l.c. at pH 5.5. The Ca^{2+} concentration was made 1 mM, and the sample loaded on to the column by means of a Super Loop (50 ml) at a flow rate of 1 ml/min. The Super Loop was then disconnected and the chromatogram developed. The flow rate was 1 ml/min; 0–10 min with buffer containing 1 mM Ca^{2+}; 10–20 min with buffer containing 1 mM EGTA. The A_{280} was monitored. The broad peak at the front of the chromatogram corresponds to protein that did not bind to the column in the presence of Ca^{2+}. Elution with 1 mM EGTA produced a single peak with a retention time of 14.33 min which corresponds to pure calcyphosine. This peak was collected manually.

Estimation of purity

At each step of the purification procedure a sample was taken, concentrated with Strataclean resin as described in the Materials and methods section and subjected to SDS/PAGE. The results obtained by silver staining of the gel are shown in Figure 5. As expected, lanes 1 and 2, corresponding to the centrifuged homogenate and the fractions from the conventional anion-exchange chromatography respectively, were heavily overloaded, lane 3 corresponding to the fractions pooled after anion-exchange h.p.l.c. had many definite bands and lane 4 (EGTA eluate from hydrophobic interaction chromatography) showed a silver-stained band of molecular mass estimated to be 24 kDa in agreement with previous estimates (Lecocq et al., 1979). In addition there were two very faint bands which cannot be seen on the photograph and a diffuse band at a molecular mass of 67 kDa which corresponds to a contaminant very often seen on silver staining of SDS/polyacrylamide gels (Ochs, 1983). We therefore concluded that, at this final stage, the purity of calcyphosine was nearly 100 %

Estimation of yield and intracellular concentration

In an initial preparation ^{35}S-labelled calcyphosine was purified from thyroid slices incubated with [^{35}S]methionine as described in the Materials and methods section. In a second preparation calcyphosine was purified from 1 g of tissue that had not been incubated with radioactivity but to which 125000 c.p.m. of ^{35}S-labelled calcyphosine was added. At each step radioactivity and protein concentration were determined in a sample. This allowed estimation of the yield, the amount of protein recovered and the amount of calcyphosine at each step. A typical purification scheme is shown in Table 1. It can be calculated that 1 g wet weight of tissue contains approx. 66 μg of calcyphosine. Using a molecular mass of 21104 for calcyphosine calculated from its primary structure (Lefort et al., 1989) and taking into account

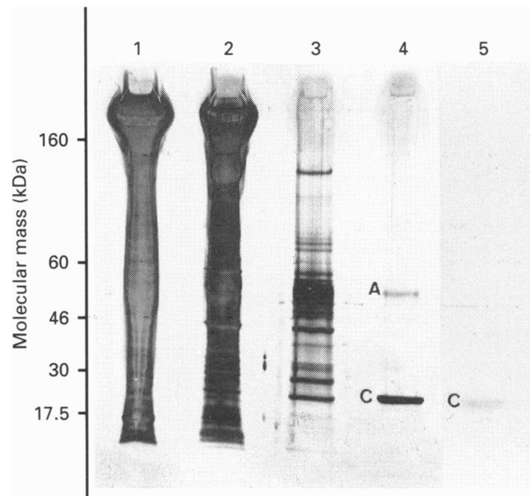

Figure 5 SDS/ExcelGel electrophoresis with silver staining at various purification steps and autoradiography of phosphorylated calcyphosine

At each step, a sample was subjected to SDS/ExelGel electrophoresis and silver stained. Lane 1, material loaded on a conventional anion-exchange column; lane 2, material recovered by elution of the column with 0.2 M NaCl; lane 3, pooled fractions 9 and 10 from chromatography on a DEAE-MemSep cartridge; lane 4, the single peak from hydrophobic interaction chromatography eluted at a retention time of 14.33 min; lane 5, autoradiography of phosphorylated calcyphosine separated on ExcelGel: a single band at a molecular mass corresponding to calcyphosine is observed. Apparent molecular masses were estimated using rainbow standards. C indicates calcyphosine at an apparent molecular mass of 24 kDa and A an artifact that is explained in the text.

Table 1 Protein recovery

The protein recovered at each step is calculated for 1 g wet weight of tissue. The purity represents the proportion by weight of calcyphosine to the total amount of protein for each step. The yield is expressed as the amount of calcyphosine recovered at each step assuming 100% in the homogenate. The data are representative of four independent purifications.

Purification step	Protein recovered (mg)	Purity (%)	Yield (%)
Homogenate	122	0.05	100
Centrifugation (soluble fraction)	73	0.07	84
Conventional anion-exchange chromatography	16	0.29	70
H.p.l.c. anion-exchange chromatography	0.269	10	41
Hydrophobic interaction chromatography	0.023	≈ 100	35

the characteristics of thyroid tissue (i.e. 1 g of thyroid tissue contains approx. 1 mg of DNA; one cell contains 1 pg of DNA; the volume of a thyrocyte is about 10 μm^3 of which 50% is associated with the cytosol), we estimated the cytosolic concentration of calcyphosine to be of the order of 30 μM.

DISCUSSION

In this work we have developed a chromatographic procedure for purification of calcyphosine to homogeneity from dog thyroid in less than 7 h with a yield of 35%. We can be very confident about the criterion of purity used, i.e. SDS/PAGE, as this criterion is not used in the chromatographic steps. No denaturing conditions are used in the separation and the protein retains its ability to be phosphorylated by protein kinase A. Hydrophobic interaction chromatography shows that the protein retains the ability to alter its structure in the presence of Ca^{2+}.

The identity of calcyphosine has been demonstrated. The purified protein migrates on two-dimensional electrophoresis like the protein identified by this procedure, the regulation of which is of great interest: phosphorylation in response to thyrotropin and the cyclic AMP cascade, Ca^{2+} binding, synthesis increased by the differentiating cyclic AMP cascade (thyrotropin, forskolin) and decreased by phorbol ester and epidermal growth factor dedifferentiating treatment. It is also recognized by an antiserum against the protein spot on the two-dimensional gels. This protein is identical with calcyphosine, as shown by analysis of a microsequence of the purified protein which fits perfectly the sequence deduced from cDNA cloning.

Binding of calcyphosine to a hydrophobic matrix in the presence of Ca^{2+} and its release in the presence of EGTA suggests that, like calmodulin, it exposes hydrophobic domains in the presence of Ca^{2+}, which should allow binding to target proteins. This suggests that calcyphosine may be the initial step in a pathway of Ca^{2+} action that is an alternative to calmodulin. It is tempting to speculate that the hydrophobic properties of Ca^{2+}–calcyphosine are important for the activation of Ca^{2+}–calcyphosine-dependent proteins or structures. The concentration of calmodulin in cells and organelles varies widely: for example, its concentration in photoreceptor outer segments is only 4 μM (Nagao et al., 1987) whereas it exceeds 1 mM in intestinal microvilli (Mooseker, 1985). Assuming a cytosolic localization for calcyphosine in dog thyrocyte, we calculate that its concentration is 30 μM, consistent with the observation that it was the major protein released by EGTA when a crude thyroid extract was loaded on to a phenyl-Sepharose column.

Immunohistochemical studies reveal that calcyphosine is located in the cytosol and is present in numerous tissues including various endocrine glands (thyroid, pancreas, adrenal, pituitary gland) and epithelia (respiratory, digestive, genitals) (P. Halleux and J. J. Vanderhaegen, unpublished work). The role of calcyphosine is, as yet, unknown. As it is found in several tissues, it is presumably not involved in the specialized iodine metabolism of the thyrocyte. All of the cells in which calcyphosine is found are epithelial and of the secretory type which leads us to propose that it may play a role in the regulation of ion transport.

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