from clavine precursors, or a high degree of specificity of, for example, the agroclavine hydrolase enzyme with respect to the unsaturation of ring D of the ergoline nucleus of its substrate. While the strain of *C. purpurea* selected for this experiment is unusual in forming only the simple amide of lysergic acid as an end product, by a mechanism which is itself of interest, it seems unlikely that the organism's biosynthetic pathway to the lysergyl nucleus would be atypical. Further, the reported formation of dihydroergotamine from given dihydrolysergic acid by *C. purpurea*[1] implies that there is no general high enzymic specificity with respect to simple analogues of normal substrates where the molecular conformation is not altered. Therefore it is suggested that the present experimental finding supports a mechanistic requirement for the Δ 8 of agroclavine and elymoclavine in lysergyl biosynthesis.

Comparative acceptance of agroclavine and festuclavine as substrates for lysergyl biosynthesis by *C. purpurea*

Fresh weight of sclerotial tissue incubated	Alkaloid substrate given	Cell-associated alkaloids after incubation % of given substrate	Alkaloids detected and quantified	
6 g	None	0	None	
6 g	Agroclavine 2 mg	60	Lysergic acid amide	63%
			Agroclavine	37%
12 g	Festuclavine 4 mg	48	Festuclavine	100%

While this may well apply to most organisms producing ergot alkaloids, the exception is *S. sorghi* which elaborates as principal alkaloid the only naturally-occurring dihydrogenated cyclic tripeptide ergot alkaloid, dihydroergosine, from a series of dihydrogenated precursors including festuclavine and dihydrolysergic acid[4]. However, since in axenic culture *S. sorghi* does not accept agroclavine into ergot alkaloid biosynthesis[4], specificity is also evident though for a mechanism which may be different from that proposed for other ergot fungi.

1 Floss, H. G., and Anderson, J. A., in: The biosynthesis of mycotoxins. Ed. P. Steyn. Academic Press, New York and London 1980.
2 Willingale, J., Atwell, S. M., and Mantle, P. G., J. gen. Microbiol. *129* (1983) 2109.
3 Quigley, F. R., and Floss, H. G., J. org. Chem. *46* (1981) 464.
4 Barrow, K. D., Mantle, P. G., and Quigley, F. R., Tetrahedron Lett. *16* (1974) 1557.
5 Banks, G. T., Szczyrbak, C. A., and Mantle, P. G., J. gen. Microbiol. *82* (1974) 345.
6 Atwell, S. M., and Mantle, P. G., Experientia *37* (1981) 1257.
7 Corbett, K., Dickerson, A. G., and Mantle, P. G., J. gen. Microbiol. *84* (1974) 39.

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Comparative analysis of phospholamban phosphorylation in crude membranes of vertebrate hearts

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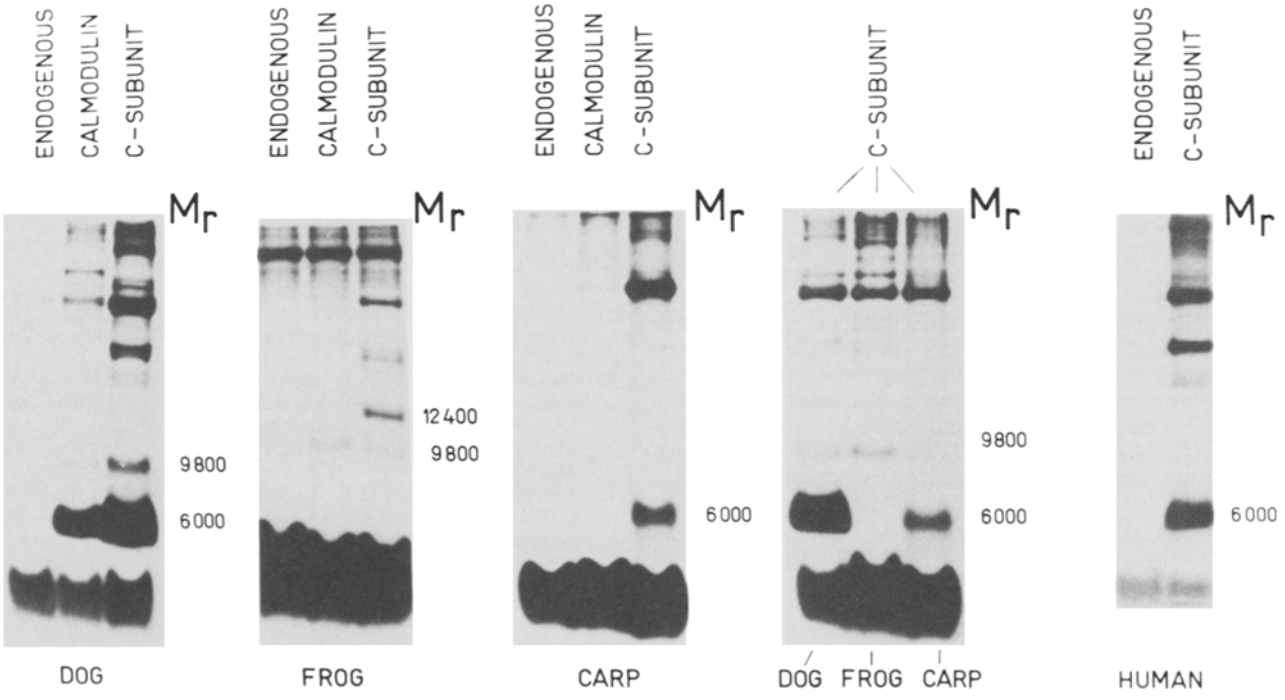
Summary. Phospholamban, a sarcoplasmic reticulum phosphoprotein, is present in the hearts of mammalian, avian, amphibian, and fish species. Phylogenetic changes are indicated by marked differences among species in cardiac phospholamban content and by the absence of Ca^{2+}/calmodulin-dependent phospholamban phosphorylation at an early developmental stage.
Key words. Cardiac muscle; phylogenesis; sarcoplasmic reticulum; phospholamban; protein kinase; Ca^{2+}-transport.

An important cellular reaction in the response of heart muscle to catecholamines is the phosphorylation of phospholamban in membranes of the sarcoplasmic reticulum[1]. Following β-adrenergic stimulation the protein is phosphorylated by a cyclic AMP-dependent protein kinase (cAMP-PK)[2]. Phospholamban phosphorylation is catalyzed also by a membrane-bound Ca^{2+}/calmodulin-dependent protein kinase (Ca-PK)[3]. The latter reaction possibly plays a role in the feedback control of heart cells in the presence of high intracellular Ca^{2+}. The present paper reports a comparative quantitative evaluation of phospholamban phosphorylation in crude membranes of various vertebrate hearts.

Materials and methods. Crude cardiac membranes were collected quantitatively by high speed centrifugation from homogenates of freshly excised and frozen hearts[4]. Human heart membranes were prepared from a small piece of ventricle tissue that had been removed 4 h post mortem. Membrane phosphorylation was carried out at 30 °C with 1 mg membrane protein per ml 40 mM histidine-HCl (pH 6.8), 0.12 M KCl, 10 mM $MgCl_2$, 15 mM NaF and 0.3 mM /γ-^{32}P/ATP (Amersham) of specific activity 25 mCi/nmole. Phosphorylated membranes were solubilized in 5% SDS, 1% β-mercaptoethanol, 0.1 mM EDTA, 50 mM Tris-H_3PO_4(pH 6.8), and separated in a SDS-urea-polyacrylamide system[5]. The concentrations of acrylamide and N, N'-methylenebisacrylamide were 12.5% and 0.83%, respectively. Following electrophoresis, gels were stained with Coomassie brilliant blue, destained, dried and autoradiographed with ORWO HS 11 film. M_r weight markers were human albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,500), cytochrome c (12,800), aprotinin (6500), and glucagon (3480). Oxalate-dependent Ca^{2+}-uptake was measured at 37 °C in 40 mM imidazol-HCl (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2ATP, 10 mM K-oxalate, 10 mM NaN_3, 2 µM $NaVO_3$, 0.2 mM EGTA, 75 µM $^{45}CaCl_2$ (Amersham) of specific activity 15 µCi/µmole and 40 to 80 µg membrane protein[4].

Results and discussion. The identification of phospholamban in crude heart membranes is facilitated by the high phospholamban content, the effective phosphorylation of the protein by cAMP-PK and Ca-PK, and the unique behavior of phospholamban in SDS-polyacrylamide gel electrophoresis. There occurs a characteristic change in electrophoretic mobility consistent with dissociation of the phosphoprotein into subunits after heat treatment of solubilized phospholamban[6]. The M_r of phospholamban oligomer is 20,000–24,000. Estimates of the M_r of dissociated subunits vary between 6000[4] and 12,000[3]. Lowest M_r values of about 6000 are observed in SDS-urea-polyacrylamide systems adapted for analysis of small polypeptide chains[5]. Such a system has been used in the present work.

The figure demonstrates the electrophoretic separation of phosphoproteins contained in crude membranes of human, dog, frog,



SDS-polyacrylamide gel electrophoresis and autoradiography of phosphorylated membranes from human, dog, frog, and carp hearts. Membranes were phosphorylated in the absence of calmodulin and catalytic subunit and in the presence of either 0.5 μM calmodulin and 0.1 mM $CaCl_2$ or 0.5 μM catalytic subunit and 1 mM EGTA. The different phosphorylation conditions are indicated by the headings 'endogeneous', 'calmodulin', and 'C-subunit'. In all experiments solubilized membranes were heated 1 min at 100 °C prior to electrophoresis. Further experimental details are given in section 'Materials and methods'.

and carp hearts. In dog membranes major parts of ^{32}P-radioactivity incorporated by either Ca-PK or cAMP-PK move with the phospholamban polypeptide of M_r 6000, when phosphorylated membranes are heated before electrophoresis (fig.). This radioactivity is associated largely with the phospholamban oligomer of M_r 22,000 when heating is omitted (experiments not shown).

Phospholamban is likewise a major phosphoprotein in crude membranes of rat and chick hearts (see also Will et al.[4]). In human heart membranes the 6000/22,000 M_r protein is phosphorylated by added catalytic subunit of cAMP-PK. However, it was not possible to demonstrate Ca^{2+}/calmodulin-dependent phosphorylation in these membranes. The Ca-PK is most probably inactivated before autopsy.

Identification of phospholamban in crude membranes of frog and carp hearts needs additional explanation. Frog heart membranes contain a protein that is phosphorylated both by Ca-PK and by cAMP-PK. The M_r of the protein is, however, 9800. This value does not change with heat treatment. A phosphoprotein of the same electrophoretic mobility is present also in dog heart membranes. As reported elsewhere[7] ^{32}P-radioactivity associated with the 6000 M_r phospholamban polypeptide is decreased and ^{32}P-radioactivity associated with the 9800 M_r protein is increased, when phosphorylated dog heart membranes are incubated with the cross-linker dimethyl 3,3′-dithiobispropionimidate. The 9800 M_r phosphoprotein should therefore be classified as one of the multimeric forms of phospholamban. The failure to entirely dissociate frog heart phospholamban may reflect structural peculiarities of the frog phosphoprotein. In addition to the protein of M_r 9800 frog heart membranes contain other low M_r substrates for cAMP-PK. These proteins are, however, not phosphorylated by Ca-PK. They are therefore not directly related to the 9800 M_r protein.

Carp heart membranes contain a protein substrate for cAMP-PK that exhibits the heat step-dependent electrophoretic mobility characteristic for phospholamban. The protein is, however, not phosphorylated upon addition of Ca^{2+} and calmodulin. This observation may indicate that carp heart sarcoplasmic reticulum does not contain Ca-PK or that carp Ca-PK is extremely labile and is inactivated during membrane preparation. Alternatively, Ca-PK may be present but it does not catalyze phosphorylation of carp phospholamban. In any event, the absence of Ca^{2+}-dependent phospholamban phosphorylation at an early phylogenetic stage conforms with the absence of this type of phosphorylation at early stages of heart ontogeny in higher vertebrates[4].

The table lists values for maximal ^{32}P-labeling of phospholamban by catalytic subunit. Membranes from various species exhibit quite different phosphorylation levels. Highest levels are

Phosphorylation of phospholamban and oxalate-supported Ca^{2+}-uptake activity in crude cardiac membranes

Species	Membrane protein (mg/g wet heart wt)	^{32}P-phospholamban (pmoles/mg membrane protein)	Ca^{2+}-uptake (nmoles Ca^{2+}/min mg membrane protein)
Carp	16.2 ± 2.0	12.2 ± 2.3	ND
Frog	9.5 ± 3.2	7.0 ± 2.2	ND
Chick	16.9 ± 1.5	19.7 ± 2.1	12.6 ± 2.1
Rat	40.2 ± 6.3	92.4 ± 10.4	11.0 ± 1.4
Dog	34.0 ± 2.1	196.1 ± 25.0	12.7 ± 2.6
Human	26.7	49.1	ND

Membranes were phosphorylated for 1 min in the presence of 0.5 μM catalytic subunit of cAMP-PK and 1 mM EGTA. SDS-polyacrylamide gel electrophoresis and Ca^{2+}-transport measurements were carried out as described under 'Materials and methods'. For quantitative evaluation of ^{32}P-phospholamban, appropriate areas were cut out from dried gels and counted by liquid scintillation spectrometry. Values represent means ± SE calculated from experimental data obtained with three different membrane preparations. ND = not determined.

observed in dog and rat heart membranes. Assuming one phosphorylation site for cAMP-PK on each subunit of phospholamban a quantity of 6.7 nmoles phospholamban subunit per g wet heart weight can be calculated for dog myocardial tissue. Calculated values for the phosphoprotein in chick, frog, and carp hearts are one to two orders of magnitude lower. Small quantities of phospholamban in frog and fish hearts correspond to the sparse reticular systems in these hearts[8,9]. A low phospholamban content in chick myocardium is, however, in contrast with the extensive network of sarcotubules present in chick heart cells[10] and with the high Ca^{2+}-transport activity of sarcoplasmic reticulum fragments in crude chick membranes (table). The low level of ^{32}P-phospholamban in chick heart membranes, as compared to dog heart membranes thus suggests possible variations among species in the relationship between the phosphoprotein and sarcoplasmic reticulum Ca^{2+}-ATPase. In dog heart membranes a one-to-one stoichiometry has been established for the two proteins[11]. The quantity of Ca^{2+}-ATPase in crude chick heart membranes has not been estimated. It appears, however, that Ca^{2+}-ATPases of mammalian and avian hearts exhibit similar specific activities[12,13]. If this holds true also for dog and chick heart enzymes the results shown in the table imply that chick heart sarcoplasmic reticulum contains far fewer phospholamban molecules than Ca^{2+}-ATPase molecules. Evidence for independent changes in maximal phospholamban phosphorylation and sarcoplasmic reticulum Ca^{2+}-transport during cardiac muscle ontogenesis has been presented in Will et al.[4].

1 Tada, M. and Katz, A., A. Rev. Physiol. *44* (1982) 401.
2 Kranias, E.G., and Solaro, R.J., Nature *298* (1982) 182.
3 Le Peuch, C.J., Haiech, J., and Demaille, J.G., Biochemistry *18* (1979) 5150.
4 Will, H., Küttner, I., Vetter, R., Will-Shahab, L., and Kemsies, C., FEBS Lett. *155* (1983) 326.
5 Swank, R.I., and Munkres, K.D., Analyt. Biochem. *39* (1971) 462.
6 Lamers, J.M.J., and Stinis, J.T., Biochim. biophys. Acta *624* (1980) 443.
7 Will, H., Levchenko, T., and Kemsies, C., in: Cellular and Molecular Aspects of the Regulation of the Heart, p. 121. Eds L. Will-Shahab, E.-G. Krause and W. Schulze. Akademieverlag, Berlin 1984.
8 Page, S., and Niedergerke, R., J. cell. Sci. *11* (1972) 179.
9 Yamamoto, T., in: Electrophysiology and Ultrastructure of the Heart, p. 1. Eds T. Sano, V. Mizuhira and K. Matsuda. Bunkodo Co, Ltd., Tokyo 1967.
10 Sommer, J.R., and Johnson, E.A., in: Handbook of Physiology, Section 2: The Cardiovascular System, p. 113. Eds R.M. Berne, N. Sperelakis and S.R. Geiger. Am. Physiol. Soc., Bethesda 1979.
11 Tada, M., Yamada, M., Kadoma, M., Inui, M., and Ohmori, F., Molec. cell. Biochem. *46* (1982) 73.
12 Levitsky, D.O., Aliev, M.K., Kuzmin, A.V., Levchenko, T., Smirnov, V.N., and Chazov, E.I., Biochim. biophys. Acta *443* (1976) 468.
13 Nakamura, J., Wang, T., Tsai, L., and Schwarz, A., J. biol. Chem. *258* (1983) 5079.

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Quantitative immunofluorescence of tyrosine hydroxylase in the adrenal medulla of spontaneously hypertensive rats[1]

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Summary. The amount of tyrosine hydroxylase protein in the adrenal medulla, which was estimated by a quantitative immunofluorescence method, was higher in spontaneously hypertensive rats than in normotensive control Wistar-Kyoto rats at 4 and 16 weeks of age before and after the development of hypertension.
Key words. Quantitative immunofluorescence; tyrosine hydroxylase; adrenal medulla; SHR.

Tyrosine hydroxylase (TH) activity was reported to be increased both in young spontaneously hypertensive rats (SHR)[2] (at 4 weeks of age) and in adult SHR (at 16 weeks of age) as compared to the normotensive control rats[3,4]. However, lower TH activity was also reported in the adrenal glands of young SHR[5]. Since enzyme protein contains an active form and an inactive form[6,7], we have tried to estimate the amount of TH protein in the chromaffin cells of the adrenal medulla of SHR by a quantitative immunofluorescence method. Cytofluorimetric quantitations of proteins using FITC-labeled antibodies[8] have been applied for this purpose. This cytofluorimetric quantitation has also been introduced for studying axonal transport of immunofluorescence materials[9]. A trial to quantitate TH based on the immunofluorescence intensity was made by taking photographs and measuring the density in the caudate nucleus of the rat[10]. In this paper, we intend to quantitate immunofluorescence intensity of TH in a single cell of the adrenal medulla in situ.

Materials and methods. Six SHR and six control Wistar-Kyoto rats (WKY) raised in our laboratory were used at 4 weeks or 16 weeks of age. 48 adrenal glands from 24 rats were obtained with perfusion (30 ml/min) of saline for 1 min and Zamboni's fixative[11] for 7 min, and were postfixed with the same fixative for additional 17 h. After washing with phosphate buffer containing 10% sucrose, 10 µm frozen sections were cut and put on a gelatin coated slide, and an immunofluorescence reaction was performed using antiserum against bovine adrenal TH. TH was purified homogeneously as judged by SDS-gel electrophoresis. No dopamine-β-hydroxylase or phenylethanolamine-N-methyltransferase activity was detected in the TH preparation. Antibodies raised in rabbits in our laboratory against the TH were examined by the immunodiffusion test of Ouchterlony[12], and the anti-TH gave a single precipitin line of identity when tested against the purified TH[13].

Immunostaining was performed as follows: Anti-TH antiserum (1:100–1:4000 in dilution with phosphate buffer saline (PBS) containing 0.3% Triton X-100) was applied on the specimens and incubated for 2 h in a moistened chamber at room temperature. After rinsing with PBS containing 1% Triton X-100, specimens were dried and fluorescein-labeled antiserum against rabbit IgG (Miles) was applied (1:250, diluted with PBS containing 0.3% Triton X-100). Following incubation at room temperature

Comparison of average fluorescence of 45–75 cells of the adrenal medulla of SHR and WKY at 4 weeks and 16 weeks of age. The measurement head delimited a square of 6.6 × 7.3 µm for a single cell but excluding the nucleus. The sensitivity is 10 times higher than figure 2

	Immunofluorescence of tyrosine hydroxylase Arbitrary units (means ± SE)	
Age (weeks)	4	16
WKY	245.8 ± 15.6 (45)	312.2 ± 18.5 (75)
SHR	311.7 ± 21.8* (45)	352.4 ± 17.3** (75)

Numbers of cells in parentheses. Difference from controls, *$p < 0.01$, **$p < 0.05$.