Vol. 99, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 31, 1981 Pages 407-413

ONTOGENETIC ASPECTS OF PHOSPHOLIPID-SENSITIVE CALCIUM-DEPENDENT PROTEIN KINASE IN GUINEA PIG TISSUES.

Bradley C. Wise,* Rolf G. G. Andersson,+ Ludmila Mackerlova,+ Robert L. Raynor,* Ingrid Solomonsson,+ and J. F. Kuo*

*Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322, USA, and +Department of Pharmacology, Linköping University, S-581 85 Linköping, Sweden

Received January 31, 1981

SUMMARY: Changes in the activity levels of phospholipid-sensitive Ca^{2+}-dependent protein kinase in various tissues from developing guinea pigs were investigated. The fetal cerebral cortex, cerebellum, midbrain, spleen and kidney contained lower levels of the enzyme activity compared to the corresponding adult tissues. Conversely, higher enzyme levels were noted for the fetal liver and retina. The activity levels in the ileum, heart and lung, on the other hand, were unaltered during development. The ontogeny-related changes in the enzyme were dissimilar to those for cAMP- and cGMP-dependent protein kinases previously reported, suggesting separate functional roles for the newly recognized Ca^{2+}-target enzyme.

A new type of Ca^{2+}-dependent protein kinase (Ca-PK) stimulated by phospholipid (such as phosphatidylserine) has been partially purified from rat brain by Takai et al. (1). We have subsequently shown that this enzyme (2) and its endogenous substrate proteins (3,4) are present ubiquitously in various mammalian or non-mammalian tissues. We have also noted that the activity levels of the enzyme vary widely (up to 300-fold) among rat tissues and that its levels are not correlated with those of cyclic AMP-dependent protein kinase (A-PK) or cyclic GMP-dependent protein kinase (G-PK) present in the same tissues (2). Phospholipid-sensitive Ca-PK is different from the previously reported calmodulin-sensitive type of Ca-PK, such as myosin light chain kinase (5-7), phosphorylase kinase (8) and glycogen synthase kinase (9), in that the former enzyme is not stimulated by calmodulin (1,2). We have observed that endogenous substrate proteins for the phospholipid-sensitive and calmodulin-sensitive enzymes are different in the rat cerebral cortex (3) and guinea pig heart (4), suggesting independent functional roles for the two Ca^{2+}-dependent protein phosphorylation systems. Most of the endogenous substrate proteins for phospholipid-sensitive Ca-PK in the cerebral cortex (3) and heart (4) are also

0006-291X/81/060407-07$01.00/0

different from those for A-PK or G-PK reported by others (for reviews see Ref. 10-12) or observed by us in parallel experiments, further suggesting that this enzyme and cyclic nucleotide-dependent protein kinases are involved in different cellular events.

In an attempt to implicate A-PK and G-PK in the physiopathology of tissues and to establish protein kinases as potential sites of bioregulation, we have previously investigated and demonstrated changes in their individual and relative activity levels in the ontogenesis of various guinea pig tissues (13), in cardiac hypertrophy in the spontaneously hypertensive rat (14) or in the rat produced by isoproterenol (15,16) and thyroxine (17), and in Morris hepatoma 3924A (18). It is conceivable that changes in phospholipid-sensitive Ca-PK may also be associated with certain physiopathologic processes or states. In the present studies, we found that activity levels of this newly identified Ca^{2+}-target enzyme were indeed altered, either increased or decreased, in certain tissues of developing guinea pigs.

EXPERIMENTAL PROCEDURES

Phosphatidylserine (bovine brain) and lysine-rich histone (type III-S) were purchased from Sigma Chemical Co. [γ-^{32}P]ATP was prepared as in (19) and protein was determined as in (20). Phospholipid-sensitive Ca-PK was assayed as in (2,3). Briefly, the standard incubation mixture (0.2 ml) contained Tris/HCl, pH 7.5, 5 µmol; $MgCl_2$, 2 µmol; lysine-rich histone, 40 µg; with or without phosphatidylserine 5 µg; with or without $CaCl_2$, 0.1 µmol; [γ-^{32}P]ATP, 1 nmol, containing about 1.1 x 10^6 cpm; appropriate amounts of enzyme proteins as indicated in individual experiments. The reaction was carried out for 5 min at 30°.

RESULTS AND DISCUSSION

The levels of soluble phospholipid-sensitive Ca-PK in the guinea pig brain were found to alter as a function of age (Fig. 1). In the cerebral cortex and cerebellum the enzyme levels, while low in the earlier fetal stage (20 days before birth), increased rapidly in the later fetal stage (5 days before birth), reached near-plateau levels in the neonate (5 days after birth), and remained around those levels thereafter. The ontogenetic changes in the enzyme levels in the midbrain were similar to those of the other parts of the brain mentioned above, except that they already reached the plateau level shortly before birth. Changes in the enzyme levels in representative peripheral tissues of the guinea pig were also examined, and the

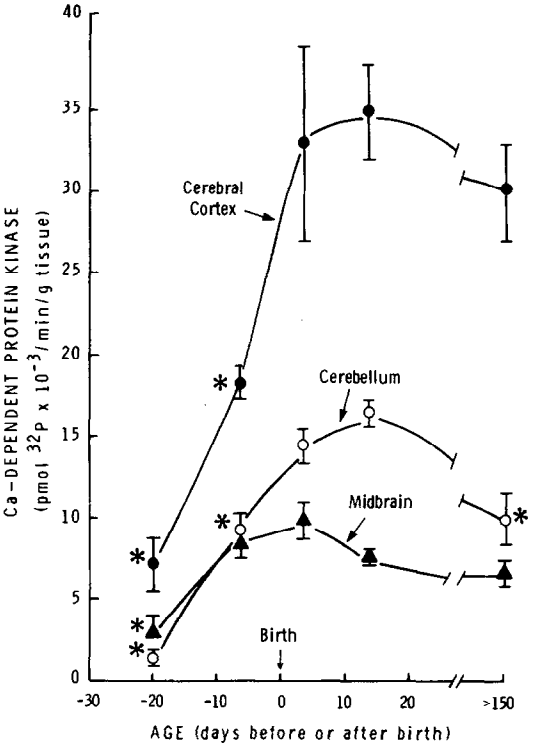

Fig. 1. Comparison of tissue levels of soluble phospholipid-sensitive Ca-PK in the cerebral cortex, cerebellum and midbrain of guinea pigs of varying ages. The tissue samples (0.2-0.6 g) were homogenized with 3 volumes of ice-cold 25 mM Tris/HCl, pH 7.5, containing 3 mM EDTA and 50 mM mercaptoethanol. The 30,000 x g supernatants (extracts) were diluted about 30-fold with the same extraction solution. The enzyme activity in the extracts (22-28 µg protein) was assayed in the absence or presence of phosphatidylserine (5 µg/0.2 ml) and $CaCl_2$ (0.5 mM), as described in Experimental Procedures. The data presented (means ± SE, 3-5 experiments) have been corrected for the basal values seen in the absence of added $CaCl_2$, which ranged 13-22% of the total activity seen in its presence, in the presence of added phospholipid. $CaCl_2$ had little or no stimulatory effect on the enzyme in the absence of added phospholipid.

*Significantly lower than the 15-day old pup ($p < 0.001$ to $p < 0.01$).

results are compared in Fig. 2. As in the brain tissue (Fig. 1), enzyme levels were lower in the fetal spleen and kidney compared to the corresponding adult tissues, whereas the opposite was noted for the liver and retina. No significant changes in the enzyme levels, however, were noted for the ileum, heart and lung.

It is possible that these changes in the enzyme levels observed during the course of tissue development may be artifacts due to the possible presence of cellular substances in extracts, which may stimulate or inhibit the enzyme activity and which also may vary during ontogeny. In order to examine this possibility, extracts from

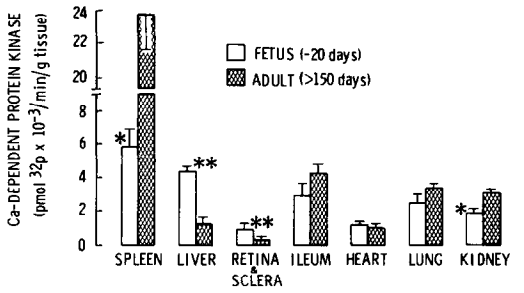

Fig. 2. Comparison of tissue levels of soluble phospholipid-sensitive Ca-PK in a variety of the fetal (20 days before birth) and adult (over 150 days old) tissues of guinea pigs. The tissue samples were homogenized and the enzyme activity in the extract (22-66 µg protein) was assayed as indicated in Fig. 1. The data have been corrected for the basal values (in the absence of added $CaCl_2$), which ranged 19-42% of the total activity (in the presence of $CaCl_2$), in the presence of phospholipid. $CaCl_2$ had little or no stimulatory effect on the enzyme in the absence of added phospholipid.

*Significantly lower than the adult ($p < 0.05$).
**Significantly higher than the adult ($p < 0.05$).

the adult and fetal cerebral cortex and heart were chromatographed on Sephadex G-200 columns, and the enzyme activity eluted in the peak fractions were compared. The results obtained, as shown in Fig. 3, were in agreement with those found using the extracts (Figs. 1 and 2). Additional experiments were conducted, in which aliquots of extracts of the cerebral cortex from the fetus (having low enzyme activity) and the adult (having high enzyme activity) were combined, and the enzyme activity in the mixed extracts was assayed. It was noted that the activity in the mixed extracts was the sum of the individual activity values seen in the adult and the fetal extracts when assayed separately (data not shown). These observations indicated that the observed changes in the enzyme activity levels, at least in the cerebral cortex, are unlikely due to changes in other substances that could influence the Ca-PK activity.

Studies dealing with the subcellular distribution of the enzyme indicated that the majority (70-80%) of the activity was associated with the particulate fraction of the fetal cerebral cortex, and that the distribution pattern of the enzyme was not altered by the development of the tissue (Table 1). In the spleen, although the majority (70%) of the enzyme was associated with the particulate fraction of

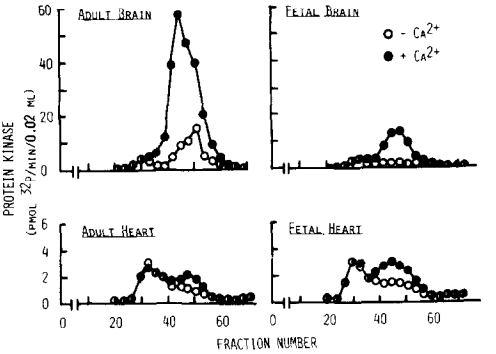

Fig. 3. Sephadex G-200 filtration of the extracts of the brain (whole) and heart of fetal (15 days before birth) and adult (about 200 days old) guinea pigs. Aliquots (0.5 ml, equivalent to 0.5 g of fresh tissue) of the individual tissues were separately loaded onto Sephadex G-200 columns (1.5 x 15 cm). The columns were previously equilibrated and the enzyme eluted with 20 mM Tris/HCl, pH 7.5, containing 50 mM mercaptoethanol and 2 mM EDTA. The fraction size was 0.4 ml. Aliquots (0.02 ml) of the fractions were assayed for phospholipid-sensitive Ca-PK in the presence or absence of phosphatidylserine and $CaCl_2$ (0.5 mM), as described in Experimental Procedures. $CaCl_2$ had little or no stimulatory effect on the enzyme in the absence of added phospholipid.

the fetal tissue, the enzyme was found to distribute nearly equally in the cytosolic and particulate fractions of the adult tissue (Table 1).

The present studies demonstrated that the levels of phospholipid-sensitive Ca-PK in various guinea pig tissues, assayable under the experimental conditions, changed differently during the course of ontogeny, i.e. either increased (brain, spleen and kidney), decreased (liver and retina) or remained the same (ileum, heart and lung). These observations are suggestive of involvements of the enzyme in a diversity of tissue functions which are related to developmental processes. We have reported earlier (13) that in the guinea pig lung and heart G-PK levels are the highest in the fetus and conversely the lowest in the adult, whereas the opposite was noted for the A-PK levels. Yet the levels of the phospholipid-sensitive Ca-PK in the same tissues were found in the present studies to be unaltered (Fig. 2), clearly supporting indirectly the notion that these three classes of protein kinases are independently involved in developmental and functional processes that are specific to the individual tissues or cell types. The ontogeny-related change in tissue level and subcellular distribution of Ca-PK in the spleen (Table 1) was intriguing, particularly in light of the heterogeneous cell types and multifunctional nature

TABLE I

Subcellular distribution of phospholipid-sensitive Ca-PK in fetal and adult guinea pig tissues.

The individual tissues (0.1-0.3 g) from the fetal (15 days before birth) and adult (about 200 days old) guinea pigs were homogenized in 5 or 10 volumes of 0.25 M sucrose dissolved in 20 mM Tris/HCl, pH 7.5, containing 50 mM mercaptoethanol. The 100,000 x g supernatant (cytosol) was used directly as the enzyme source. The pellets were suspended in the original volumes of the same homogenization solution, followed by solubilizing the particulate enzyme with 0.3% Triton X-100 and 2.5 mM EGTA for 1 hr in ice. The solubilized enzyme was recovered by centrifugation. Both the cytosolic and particulate enzyme was assayed and the data were treated as described in Fig. 1. The data shown are means ± SE of 3 experiments.

Tissue and age	Ca^{2+}-stimulated enzyme activity (pmol ^{32}P/min/g tissue)		C/P ratio
	Cytosol (C)	Particulate (P)	
Cerebral cortex			
Fetus	6,727 ± 2,336	23,000 ± 1,217	0.29
Adult	16,096 ± 1,302	42,167 ± 3,032	0.38
Spleen			
Fetus	4,114 ± 415	9,683 ± 233	0.42
Adult	16,006 ± 3,666	15,717 ± 1,446	1.01

of the tissue, both of which change during tissue maturation and immunological stimulation.

In addition to protein kinases, it would be of interest to examine whether endogenous substrate proteins specific to or common for the enzymes also would alter, qualitatively or quantitatively, during tissue development. Investigations are currently underway in our laboratory in an attempt to gain insight into possible interrelations among protein kinases and their substrate proteins.

ACKNOWLEDGEMENTS: This work was supported by Swedish Medical Research Council grants 04V-5401-01-503905401, 04X-02080-14 and 04X-04498-06, by Swedish Medical Research Council Visiting Scientist Fellowship (to JFK), and by USPHS grants HL-15696, T32-GM-07594 and T32-AM-07298.

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