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Expression of Second Messenger- and Cyclin-Dependent Protein Kinases During Postnatal Development of Rat Heart

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Abstract During early postnatal development, cardiomyocytes, which comprise about 80% of ventricular mass and volume, become phenotypically developed to facilitate their contractile functions and terminally differentiated to grow only in size but not in cell number. These changes are due to the expression of contractile proteins as well as the regulation of intracellular signal transduction proteins. In this study, the expression patterns of several protein kinases involved in various cardiac functions and cell-cycle control were analyzed by Western blotting of ventricular extracts from 1-, 10-, 20-, 50-, and 365-day-old rats. The expression level of cAMP-dependent protein kinase was slightly decreased (20%) over the first year, whereas no change was detected in cGMP-dependent protein kinase I. Calmodulin-dependent protein kinase II, which is involved in Ca^{2+} uptake into the sarcoplasmic reticulum, was increased as much as ten-fold. To the contrary, the expressions of protein kinase C-α and ι declined 77% with age. Cyclin-dependent protein kinases (CDKs) such as CDK1, CDK2, CDK4, and CDK5, which are required for cell-cycle progression, abruptly declined to almost undetectable levels after 10–20 days of age. In contrast, other CDK-related kinases, such as CDK8 or Kkialre, did not change significantly or increased up to 50% with age, respectively. Protein kinases implicated in CDK regulation such as CDK7 and Wee1 were either slightly increased in expression or did not change significantly. All of the proteins that were detected in ventricular extracts were also identified in isolated cardiac myocytes in equivalent amounts and analyzed for their relative expression in ten other adult rat tissues. J. Cell. Biochem. 69:506–521, 1998.

Key words: heart; development; CaMPK; cAPK; CDK; cGPK; Kkialre; PKC; Wee1

During the early postnatal period, the heart undergoes significant changes in various physical and biochemical characteristics [Hopkins et al., 1973; Rumyantsev, 1977; Michalak, 1987]. These changes result from the progressive expression of contractile-specific genes and the posttranslational modification and localization of proteins, which may be regulated by various hormonal, neuronal, or physical stimuli [Chien et al., 1991, 1993; Cummins, 1993; Komuro and Yazaki, 1993; Schott and Morrow, 1993; van-Bilsen and Chien, 1993]. During this postnatal period, cardiomyocytes morphologically differentiate to facilitate their contactile functions. For example, the density, size, and number of mitochondria increase to provide sufficient energy. The sarcomere becomes more distinct with ordered fashion, and T tubes and intercalated discs develop and re-orientate to cope with increased demand on efficient Ca^{2+} movement and contractile force [Zak, 1984; Goldstein and Traeger, 1985]. Biochemically, the upregulation in expression and activities of sarcoplasmic reticulum (SR) proteins [Michalak, 1987], such as the Ca^{2+}-ATPase of the SR (SERCA2), permit the increase of SR Ca^{2+} uptake and can accommodate rapid Ca^{2+} movement during each con-

Abbreviations: AP, alkaline phosphatase; cAPK, cyclic-AMP–dependent protein kinase; CaMPK, Ca^{2+}/calmodulin-dependent protein kinase; CDK, cyclin-dependent protein kinase; cGPK, cyclic-GMP–dependent protein kinase; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; KK, Kkialre; PKC, protein kinase C.

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traction of adult cardiomyocytes. The increased sensitivity of β-adrenergic signals has also been linked to the downregulation of the G_i (inhibitory) isoform of the trimeric G protein family; G_i expression declined as much as six-fold by 30 days of postnatal stage, whereas there was no change in the levels of G_s (stimulatory) [Hansen et al., 1995; Bartel et al., 1996]. An increase in cardiac troponin I expression was also implicated in the higher efficiency of β-adrenergic–stimulated relaxation of adult ventricles [Rakusan, 1984].

During development, cardiomyocytes become terminally differentiated and incapable of undergoing mitosis in response to further mitogenic stimuli. In the rat heart, the terminal differentiation occurs about 2 weeks after birth with arrest at the G_0 or G_1 phase of the cell cycle [Capasso et al., 1992]. The rate of DNA synthesis and DNA polymerase activity decline rapidly in the same period [Claycomb, 1975]. The mechanism(s) of terminal differentiation is still unknown, but it appears to result from the biochemical inhibition of mitosis and DNA synthesis rather than the permanent loss of ability to proliferate [McGill and Brooks, 1995; Tam et al., 1995]. As for a temporal cue for the terminal differentiation of cardiomyocytes, Claycom [1975, 1976] has suggested that the development of adrenergic nerves and an increase in the intracellular cyclic AMP concentration during the early postnatal period causes the inhibition of DNA synthesis. The expressions of G_q and G_{11}, phospholipase C (PLC), and protein kinase C (PKC), which are closely involved in cell growth, were also noted to be concurrently downregulated during development [Puceat et al., 1994; Rybin and Steinberg, 1994; Hansen et al., 1995]. In addition, studies with skeletal myocytes have provided clues for the underlying mechanism of the terminal differentiation through use of a group of DNA tumor viruses such as SV40, polyoma, and adenovirus, which could inhibit and reverse the terminal differentiation [Endo and Nadal-Ginard, 1989]. The cardiac myocytes of transgenic mice expressing the SV40 large T-antigen oncoprotein was shown to undergo hyperplasia while retaining the characteristics of differentiated phenotypes [Field, 1988; Katz et al., 1992]. The SV40 T antigen was found to be complexed with a group of tumor suppressor proteins such as retinoblastoma (Rb), p53, p107, and p130 [Daud et al., 1993], which are regulated by various cyclin-dependent kinases (CDKs) [Sherr, 1994]. These results implicate an important role for CDKs in the terminal differentiation of cardiac myoctes [McGill and Brooks, 1995]. In this regard, we have investigated the expression patterns of protein kinases that are involved in various cardiac functions and cell-cycle regulation during postnatal development of rat ventricles by Western blotting analysis with specific antibodies.

MATERIALS AND METHODS

Affinity-purified rabbit polyclonal or monoclonal antibodies and immunizing peptides used to raise these antibodies are listed in Table I. These antibodies were either produced in our laboratory or purchased commercially. Goat anti-rabbit IgG conjugated to alkaline phosphatase (AP) or horseradish peroxidase (HRP) was purchased from Calbiochem (San Diego, CA). Enhanced chemiluminescence (ECL) detection reagents for immunoblotting were purchased from Amersham (Buckinghamshire, England). [γ-^{32}P]ATP was obtained from DuPont (Washington, DC). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Preparation of Rat Tissue Extracts

Hearts from 1-, 10-, 20-, 50- and 365-day-old male Sprague-Dawley rats were rapidly excised after induction of anesthesia by intraperitoneal injection of pentobarbital (60 mg/kg). The ventricles of the hearts were cut, rinsed with phosphate buffered saline at 4°C, frozen in liquid nitrogen, and stored at −70°C until use. The ventricular tissues were pulverized with five strokes of a liquid nitrogen-cooled hand French press and resuspended in 10 volumes of ice-cold homogenization buffer containing 20 mM MOPS, 15 mM EGTA, 2 mM Na_2EDTA, 1 mM Na_3VO_4, 1 mM dithiothreitol, 75 mM β-glycerophosphate, 0.1 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 0.7 μg/ml pepstatin, 1 μg/ml leupeptin, and 1% Triton X-100. This was then sonicated with a Branson Probe Sonicator at 4°C with 3 × 30 s bursts. The homogenates were ultracentrifuged at 100,000 rpm (240,000*g*) for 11 min in a Beckman (Fullerton, CA) TLA-100.2 ultracentrifuge at 4°C. The supernatants were immediately frozen at −70°C until subsequent analysis. Other rat tissues, including adipose, brain, intestine, kidney, liver, lung, spleen, testis, skeletal muscle (hind leg tibial muscle), and thymus were collected from

TABLE I. List of Antibodies and Amino Acid Sequences of Synthetic Peptides Used to Raise Antibodies*

Kinase	Immunogen amino acid sequence or residues	Origin	Source
CaMPK II	NT-CTRFTDEYQLFEEL	Rat	Kinetek
	PCT-EETRVWHRRDGKWQNVHFHC	Rat	Kinetek
cAPK	NT-KKGSEQESVKEFLAKC	Human	Kinetek
CDK1	IX-PLFHDSEIDQLFRIFRALGTP-GGC	Mouse	Kinetek
	CT-CFLSKMLVYDPAKRISGKMALKHPYFDDLDNQIKKM	Mouse	Kinetek
CDK2	M2 residues 283–298 (C terminus)	Human	SC
CDK3	Y-20 residues 285–304 (C terminus)	Human	SC
CDK4	H-22 residues 282–303 (C terminus)	Human	SC
CDK5	CT-CNPVQRISAEEALQHP	Human	Kinetek
CDK7	PCT-VATKRKRAEALEQGC	Mouse	Kinetek
CDK8	NT-MDYDFKVKLSSERERC	Human	Kinetek
cGPK	CT-CDEPPPDDNSGWDIDF	Bovine	Kinetek
cGPK-1α	NT-ELEELFAKILMLKEEL	Bovine	Kinetek
Kkialre	CT-CLDNKKYYSDTKKLNYR	Human	Kinetek
	C13 residues 346–358 (C terminus)		SC
PKC-α	M7-purified PKC, monoclonal	Rabbit	UBI
PKC-ι	Residues 404–587 (C terminus)	Human	TL
Weel	X-LTVVCAAGAEPLPRNGDQWHEIRQGRLP-CGG	Human	Kinetek
	T-NTSSHRYGLRRGDQMMEDWQVNV-GGC	*S. pombe*	Kinetek

*For all antibodies, unless purchased commercially, the peptides in PBS and Freund's incomplete adjuvant were injected into rabbits, and the serum was obtained, after several boosting injections. The antibodies were purified using peptide affinity columns and titered by ELISA. The peptide sequences and their source of species are indicated in the origin column. For commercially purchased antibodies, the peptides are indicated for the corresponding kinase residues with their species origins and the companies from which the antibody was purchased: SC, Santa Cruz Biotechnology (Santa Cruz, CA); TL, Transduction Laboratory (Mississauga ON, Canada); UBI, Upstate Biotechnology Inc. (Lake Placid, NY).

50-day-old rats, and total extracts were prepared as described above.

Isolation and Fractionation of Adult Ventricular Myocytes

Isolated ventricular myocytes were prepared from 50-day-old rats as described by Rodrigues and Severson [1997]. Briefly, the excised heart was cannulated and retrogradely perfused via the aorta with oxygenated buffer A (Joklik minimal essential medium, containing 2 mM $NaHCO_3$, 1.2 mM Mg_2SO_4, and 1 mM DL-carnitine) for 5 min, followed by the same buffer containing 25 μM Ca^{2+} and 75 mU/ml of collagenase (Type II; Worthington, Freehold, NJ) for 30 min at 37°C. The softened ventricular tissue was then removed from the heart and incubated for 10 min in the same collagenase and Ca^{2+} containing buffer A with occasional agitation. Dissociated ventricular myocytes were passed through a 200 μm mesh silk screen to remove tissue debris. The isolated ventricular myocytes were then sequentially resuspended in buffer A containing 50 μM, 100 μM, 500 μM, and 1 mM Ca^{2+}. The cells were then pelleted by centrifugation for 60 s at 300 rpm and homogenized as described above, with the exception of Triton X-100. The homogenates were ultracentrifuged for preparation of cytosolic fractions. The particulate fractions were prepared by washing the pellets with the homogenization buffer; the pellets then were recentrifuged, resuspended in homogenization buffer containing 1% Triton X-100 (membrane grade), sonicated as before, and incubated for 15 min on ice followed by ultracentrifugation. This final supernatant was saved as the detergent-solubilized particulate fraction.

Gel Electrophoresis and Immunoblotting

Extracts were added to sample buffer (2% SDS, 5% glycerol, 50 mM Tris-HCl, pH 6.8, 0.1 M β-mercaptoethanol, and 0.01% bromophenol blue) and boiled at 100°C for 3 min. Protein concentrations were assayed with Bradford reagent (BioRad, Richmond, CA), and extracts were diluted with 1% SDS to yield identical protein concentrations before addition to the sample buffer. Electrophoresis was performed in 11% SDS-PAGE gels using a discontinuous buffer system as previously described [Sanghera et al., 1996]. Proteins were then electropho-

retically transferred onto nitrocellulose membranes. Membranes were then blocked with 3% skim milk powder in Tris-buffered saline (20 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 0.2 % Tween-20 [TBST]) and, after a quick rinse of the membrane with TBST, exposed to a primary antibody in TBST for 2 h with constant shaking at room temperature. Membranes were washed three times for 10 min with TBST and incubated with HRP- or AP-conjugated secondary antibody (goat antirabbit or antimouse IgG) in TBST for 30 min. After membranes were washed membranes three times for 10 min with TBST, Western blots were developed using the ECL Western blotting detection system (Amersham) or AP color development as described elsewhere [Sanghera et al., 1996].

Densitometry and Statistical Analysis

For the quantitative analysis of protein expression, the films obtained from the ECL detection system or the color-developed membranes were scanned, and the intensities of the bands were quantified using the NIH image program. Data are presented as standard error of means ± SEM, and Tukey's tests were performed with an $\alpha < 0.05$ level for significance.

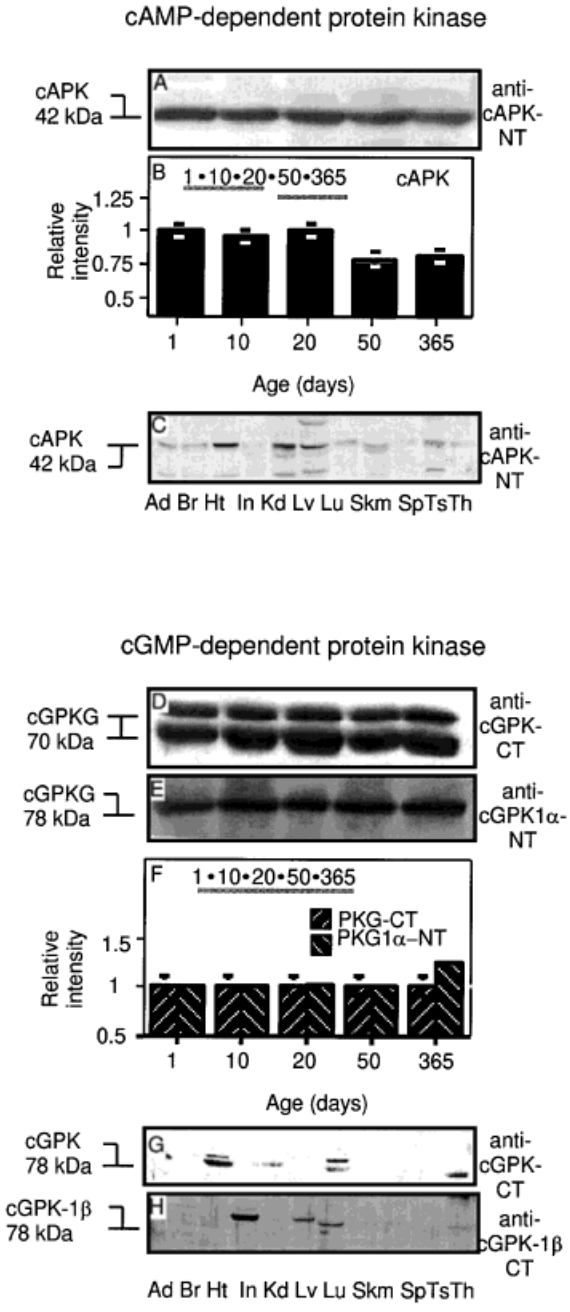
RESULTS AND DISCUSSION

Expression of Cyclic-Nucleotide-Dependent Protein Kinases: cAPK and cGPK

The cAMP-dependent (cAPK) and cGMP-dependent (cGPK) protein kinases have been implicated in the regulation of various cardiac functions. cAPK is a tetrameric holoenzyme that is activated when two cAMP molecules bind to each of two regulatory subunits (R subunits), resulting in the dissociation of two active catalytic subunits (C subunits) and relief from pseudosubstrate inhibition by the R subunits [Beebe and Corbin, 1986; Taylor et al., 1990]. R subunits are dimers and mainly occur in two forms, type I and II. C subunits are monomers with molecular masses of 40–46 kDa, and mRNA for three isoforms, C_α, C_β, and $C_{\beta 2}$ (but not C_γ) has been detected in heart based on Northern blot analysis and on cDNA analysis using PCR [Showers and Maurer, 1986; Wiemann et al., 1991]; only the C_α isoform has been detected at the protein level [Shoji et al., 1983]. Affinity-purified polyclonal antibody raised against the N terminus (NT) of the C_α subunit was used to probe ventricular extracts, and the immunoreactivity was quantified by densitometry. As shown in Figure 1A,B, a 42 kDa protein was readily detected, and it was slightly downregulated by 21% by 50 days of age. The basal level of cAMP concentration in the heart has also been reported to be decreased with age [Novak et al., 1996], and the specific activity of cAPK, which peaked at the seventh day after birth (7 nmol/min/mg protein), also declined in adult rat heart (2.5 nmol/min/mg protein) [Kuo, 1975; Haddox et al., 1979]. This indicates that cAPK is downregulated in both expression and activity during postnatal development of the heart. cAPK was detected in all of the tissues tested, but its level of expression varied among tissues (Fig. 1C). High amounts of cAPK were found in liver, kidney, and heart (Fig. 1C). The role of cAPK in cell proliferation and DNA synthesis is still controversial, as contradictory observations have been reported in different tissues, but it appears to inhibit DNA synthesis and to be involved in cell terminal differentiation during early development of the heart [Claycomb, 1976]. cAPK can also phosphorylate various proteins, including cardiac troponin C, cardiac troponin I (cTNI), cardiac L-type Ca^{2+} channel, and phospholamban, and it has been implicated in the positive inotropic and chronotropic effects of heart [Robertson et al., 1982; Hofmann and Lange, 1994; Yabana et al., 1995]. During postnatal development, the expression of troponin I (TNI) isoforms switches from cAMP-independent, slow skeletal muscle TNI to cAMP-dependent cardiac TNI. Age-specific phosphorylation of TNI and isometric tension generation is mediated by cAPK [Bartel et al., 1994]. Therefore, despite the slight downregulation of cAPK, it plays key roles in the regulation of cardiac contractility of the adult heart.

Cyclic GMP–dependent protein kinase (cGPK) is a ubiquitous homodimeric protein with a molecular mass of approximately 78–80 kDa and occurs as Iα, Iβ and II isoforms [Hofmann et al., 1992]. The presence of this kinase in heart was investigated previously, and the major isoform was proposed to be Iα [Lincoln and Keely, 1981; Mery et al., 1991; Keilbach et al., 1992]. Protein expression of cGPK during the development of heart has also been previously examined [Sandberg et al., 1989]. Our study using antibodies raised against the carboxy terminus (CT) of cGPK-Iα showed strong immunoreaction with two distinct proteins at

78 kDa and 70 kDa (Fig. 1D). When Western blotting was performed using antibody raised against the N terminus of cGPK-Iα, only the 78 kDa protein was detected (Fig. 1E). Therefore, it appears that the 78 kDa protein was indeed cGPK-Iα. The 78 kDa protein also coeluted with cGPK activity in MonoQ fractionated ventricular extracts (data not shown). The expression of cGPK-Iα did not change significantly during development (Fig. 1F), which concurs with the study by Sandberg et al. [1989]. The identity of the 70 kDa protein was not clear, but it may correspond to another isoform of cGPK which is present in noncardiac myocytes, since it was not detected in the isolated cardiomyocytes (Fig. 2). When different rat tissues were immunoblotted with the cGPK-CT antibody, both of the 70 and 78 kDa proteins were detected in heart and lung (Fig. 1G). Only the 78 kDa protein was found in brain, whereas the 70 kDa species was readily evident in kidney and thymus (Fig. 1G). When the presence of cGPK I-β and II in heart was examined with specific antibodies, the isoforms could not be detected (Fig. 1H). cGPK, in contrast to cAPK, was proposed to be involved in cell proliferation and DNA synthesis in some tissues [Millis et al., 1974; Oey et al., 1974]. In heart, the role of cGPK is not clearly understood, but the concentration of cGMP and the activity of cGPK were shown to be decreased at the early neonatal stage of heart, which were reciprocally related to those changes in cAMP and cAPK [Kuo, 1975]. Our data showing the decrease in the expression of cAPK with no changes in cGPK provide additional support for the previous suggestion that cAPK is more intimately involved than cGPK in cardiac myocytes' differentiation at the early postnatal stage [Kuo, 1975; Claycomb, 1976].



Expression of Ca^{2+}/Lipid- and Calmodulin-Dependent Protein Kinases: PKC and CaMPK II

Protein kinase C (PKC) is a family of ubiquitous phospholipid-dependent protein Ser/Thr kinases that are implicated in the regulation of many intracellular processes. Their functional

Fig. 1. Expression of cyclic nucleotide–dependent protein kinases during postnatal development of rat ventricle**.** Protein extracts (150 μg) of 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE, and Western blot analysis was performed with antibodies specific for cAPK (**A**) and cGPK (**D,E**). The immunoreactivities were plotted against the relative density as 1 arbitrary unit for 1 day ventricle, and values represent mean ± SEM from three to four separate experiments (**B,F**). Inserted numbers and underscores in the graphs show results of the Tukey multiple comparisons test. At the 0.05 significance level or above, the means of any two groups underscored by the same line are not significantly different. For tissue comparison, extracts (50 μg of total protein) of 11 adult rat tissues (adipocytes (Ad), brain (Br), heart (Ht), intestine (In), kidney (Kd), liver (Lv), lung (Lu), skeletal muscle (Skm), spleen (Sp), testis (Ts), and thymus [Th]) were subjected to 11% SDS-PAGE, and immunoblot analysis was performed with cAPK (**C**), cGPK (**G**), and cGPK-1α (**H**) antibodies.

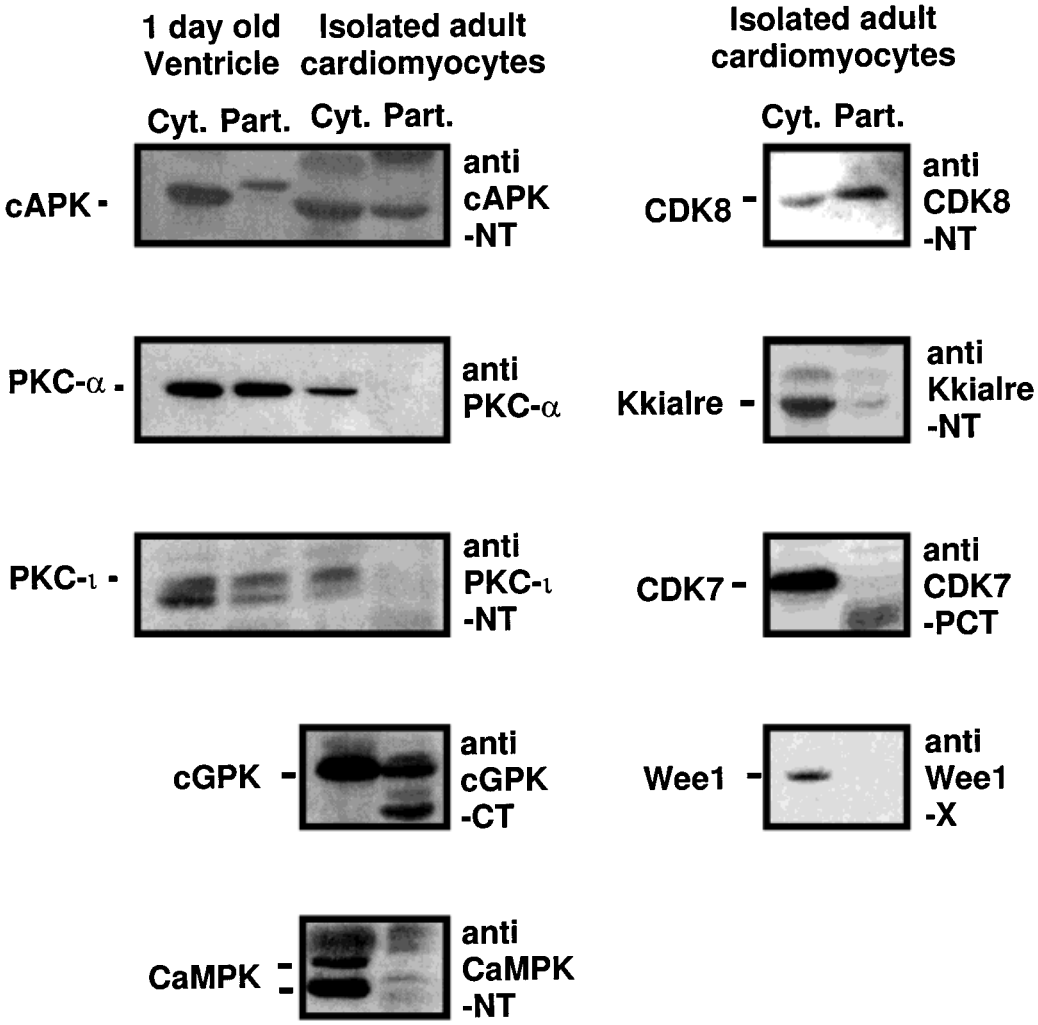


Fig. 2. Subcellular expression of second messenger– and cyclin-dependent protein kinases in isolated ventricular myocytes. Cytosolic (Cyt.) and particulate extracts (Part.) (100 mg protein) from ventricular tissue (1-day-old) or isolated adult (50-day-old) venticular myocytes were subjected to 11% SDS-PAGE and Western-blotted for immunoreactivity against antibodies for cAPK, cGPK, CaMPK II, PKC-α, PKC-ι, CDK8, Kkialre, CDK7, and Wee1.

role in heart has been investigated and reviewed previously [Steinberg et al., 1995; Sugden and Bogoyevitch, 1995; Bogoyevitch and Sugden, 1996]. Several neurotransmitters, growth factors, tumor promoters, and mechanical stresses induced PKC's activation, resulting in the increase in gene expression and protein synthesis. Others [Disatnik et al., 1994; Puceat et al., 1994; Rybin and Steinberg, 1994; Bogoyevitch et al., 1996] have also extensively investigated the expression of protein kinase C isoforms during development of heart. These studies indicated the presence of PKC-δ, PKC-ϵ and PKC-ζ isoforms, and the levels of expression of these isoforms decreased with age. The findings for the expression of PKC-α have been inconsistent and appear to depend on the antibodies that were used for the investigations. Recently, a study using four different antibodies clearly showed the expression of PKC-α in adult ventricular myocytes [Rybin and Steinberg, 1997] and also reported that a PKC antibody from Upstate Biotechnology (Lake Placid, NY) was the most sensitive antibody without nonspecific cross-reactivity. We used the same antibody to investigate the expression of PKC-α during postnatal development of heart. Only one protein of 80 kDa immunoreacted with the antibody (Fig. 3A), and the intensity of this signal declined steadily to 23% of newborn levels by 365 days (Fig. 3C). Since PKC was also reported to be associated in membrane, cytoskeleton, and nucleus, we have further fractionated the ventricular extracts into cytosolic and deter-

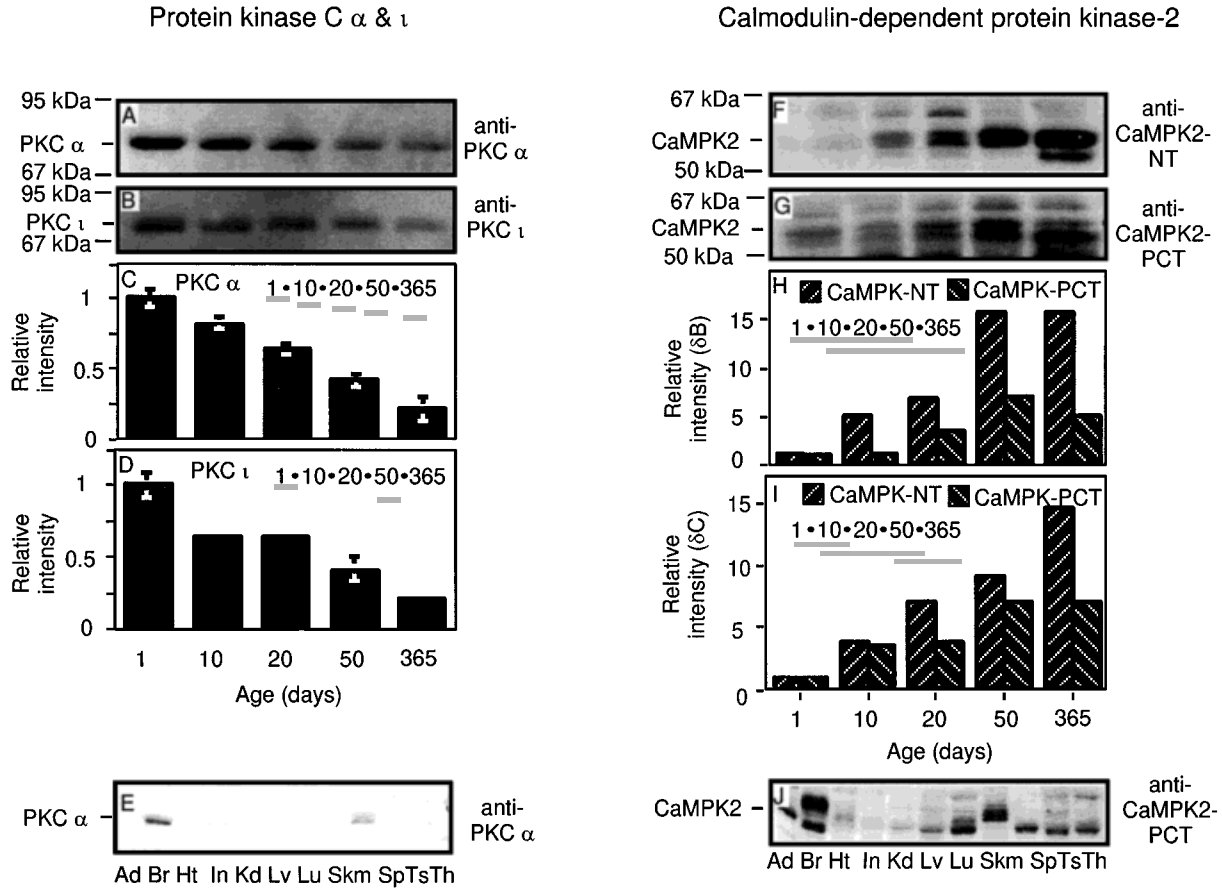


Fig. 3. Expression of PKC and CaMPK-2 during postnatal development of rat ventricle. Protein extracts (150 μg) of 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE, and Western blot analysis was performed with monoclonal antibodies specific for PKCα (**A**) and PKCι (**B**) and with affinity-purified polyclonal antibodies raised against CaMPK-2 NT (**F**) and CaMPK-2 PCT (**G**). The immunoreactivities were plotted against the relative density as 1 arbitrary unit for 1 day ventricle, and values represent mean ± SEM from three to four separate experiments (**C,D,H,I**). Inserted numbers and underscores in the graphs show results of the Tukey multiple comparisons test. At the 0.05 significance level or above, the means of any two groups underscored by the same line are not significantly different. In panels **E** and **J,** extracts (50 μg of total protein) of 11 adult rat tissues (see legend to Figure 1) were subjected to 11% SDS-PAGE, and immunoblot analysis was performed with PKCα (E) and CaMPK-2 (J) polyclonal antibodies.

gent-soluble particulate fractions. At day 1, the amount of particulate-associated PKC-α was equivalent to that of the cytosolic fraction, whereas in the adult the PKC-α was exclusively localized in the cytosolic fraction (Fig. 2). In addition to PKC-α, we also investigated the expression of a Ca^{2+}-independent atypical isoform, PKC-ι, which has not been described before in the adult heart. PKC-ι shares about 72% overall homology with PKC-ζ [Selbie et al., 1993] and is involved in the ultraviolet B-induced activated protein-1 activation [Huang et al. 1996]. The presence of PKC-ι has been detected on Western blots of cultured chick embryonic cardiomyocytes, but immunohistochemistry analysis indicated that it originated from cocultured fibroblasts [Wu et al., 1996]. We also investigated its expression in ventricular tissues during development and in freshly isolated adult ventricular cardiomyocytes. Immunoblotting with monoclonal antibody specific for PKC-ι permitted detection of a 75 kDa protein that declined in intensity to 23% of newborn levels in adult ventricle (Fig. 3B,D). PKC-ι was localized in both cytosolic and particulate fractions of 1 day heart, but it was exclusively found in the cytosolic fraction at 50 days after birth (Fig. 2). Due to the heterogeneity in the cell population of ventricular tissues, the immunoreaction with PKC-ι antibody may have resulted from noncardiomyocytes. It is, however, not clear then why PKC-ι was detected in the

adult isolated cardiomyocytes. Possible explanations for this discrepancy include contamination of the isolated cardiomyocyte sample with noncardiomyocytes or differences between the two species.

The multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaMPKII) represents a family of protein kinases activated by Ca^{2+}/calmodulin. It forms complexes with 8–12 subunits, which are composed of combinations of at least five homologous isozymes (α, β, β',γ, δ) [Braun and Schulman, 1995]. Among the five isozymes, the δ isozyme is predominantly expressed in heart and involved in the regulation of the contractility of cardiac myocytes [Witcher et al., 1992]. Recently, subtypes of the isoforms (δ_A, δ_B, and δ_C) have further been cloned [Schworer et al., 1993; Edman and Schulman, 1994]. Three immunoreacting proteins with CaMPKII have been resolved by MonoQ anion exchange column chromatography of cytosolic extracts from isolated cardiomyocytes, and each of these coeluted with calmodulin-dependent kinase activity (data not shown). The three proteins with apparent molecular masses of 58, 56 and 54 kDa on SDS-PAGE gels may represent the δ_A, δ_B, and δ_C isoforms, respectively. Immunoblotting with the CaMPKII-NT and CaMPKII-PCT antibodies permitted detection of two proteins of 56 and 54 kDa in ventricles, and the intensities were increased four- to tenfold with postnatal development (Fig. 3F,G,H,I). In the isolated ventricular myocytes, both 56 and 54 kDa proteins were also detected at the equivalent level to those in ventricles and mainly localized in the cytosolic fraction (Fig. 2). Even though the expression of CaMPKII was upregulated in the heart, the amount of CaMPKII protein was still minimal when compared to other tissues, and the molecular masses varied, indicating distinct isoforms (Fig. 3J). The highest levels of CaMPKII protein were detected in brain, followed by adipose, lung, skeletal muscle, spleen, and testis, whereas minimal amounts were found in heart, intestine, and kidney. The upregulation of CaMPK expression in heart during development may correlate with the increased demand for Ca^{2+} homeostasis and efficient control of myocardial contraction. In heart, CaMPKII was shown to be involved in the regulation of Ca^{2+} uptake into SR either by direct phosphorylation of SERCA2 [Toyofuku et al., 1994] or by phosphorylation of phospholamban (PL), which causes dissociation of the inhibitory PL from Ca^{2+}-ATPase of the SR (SERCA2) complex. In neonatal heart, the lower expression and activity of SERCA2 are responsible for the decreased SR release and uptake of Ca^{2+} [Michalak, 1987]. In addition, CaMPKII-phosphorylated PL has been detected only in the adult heart [Michalak, 1987].

The expression patterns of PKC and CaMPKII clearly show that, during development, the heart regulates the production of these kinases reciprocally. The downregulation of PKC, which closely correlates with cell proliferation, may possibly prime cardiomyocytes to respond to the terminal differentiation process. In contrast, the upregulation of CaMPK may be necessary for adult cardiomyocytes to perform cardiac functions that require efficient intracellular Ca^{2+} homeostasis.

Expression of Cyclin-Dependent Protein Kinases (CDKs)

It has been established that the progression of the cell cycle is fastidiously controlled by various cell cycle–dependent protein kinases (CDKs). The CDKs are a family of Ser/Thr proein kinases that are closely related to the gene product of cdc2 in *Schizosaccharomyces pombe* and CDC28 in *Saccharomyces cerevisiae* [Hartwell et al., 1974; Norbury and Nurse, 1992; Nasmyth, 1993]. These kinases are activated by specific regulatory subunits called cyclins, which undergo oscillating levels of expression during the cell cycle. In mammalian cells, at least eight subtypes of CDKs (CDK1–8) and their regulating partners (cyclin A–H and cyclin X) have been identified and implicated in the control of cell cycle progression [Norbury and Nurse, 1992; McGill and Brooks, 1995; Nigg, 1995]. Cardiomyocytes are mitotically active at birth and become terminally differentiated in about 2–3 weeks after birth [Rakusan, 1984; Zak, 1984]. Even though the molecular mechanism(s) for the terminal differentiation is still unknown, it has become apparent that the cardiomyocytes are biochemically inhibited and do not permanently lose their ability to undergo mitotic cell divisions. Observations of reinitiation of DNA synthesis and mitotic division of adult cardiomyocytes under certain conditions such as pressure overload [Capasso et al., 1993], anemia [Olivetti et al., 1992], phorbol ester treatment [Claycomb and Moses, 1988], and senescent heart in certain strains of rats

[Anversa et al., 1991] substantiate this theory. More clearly, the induction of hyperplasia of either the atria or the ventricle by SV40 large T-antigen oncoprotein which was expressed under the transcriptional control of the promoter regions of either atrial natriuretic factor or α-cardiac myosin heavy chain strongly supports the theory and may provide some clue to the mechanism of terminal differentiation [Field, 1988; Katz et al., 1992]. SV40 large T antigen directly associates with tumor suppressor genes such as the retinoblastoma protein (Rb) or related proteins (p53, p107 and p130) [Daud et al., 1993], and it inhibits their activities by sequestering them [McGill and Brooks, 1995]. The regulation of Rb is important for cells to progress from G1 to S phase, and it is regulated by CDKs in the normal condition [Daud et al., 1993]. Phosphorylation of Rb by CDKs prevents Rb from interacting with E2F, a transcription factor that targets many genes required during S phase, such as cyclin A and E [Nevins, 1992]. In fact, terminally differentiated myotubes could reenter cell cycle by SV40 T antigen by inducing CDK1 (also referred to as cdc2), CDK2, and their partner cyclins A and B [Okubo et al., 1994]. More recently the expression of muscle-specific gene was sufficiently inhibited by expression of cyclin D1 alone or coexpression with CDK2 and cyclin A or E during skeletal myogenesis [Guo and Walsh, 1997]. The expression of Rb and other tumor suppressors has been investigated in heart [Kim et al., 1994; Tam et al., 1995]. While p53 and p107 were detected only in neonatal rat ventricle, Rb was also present in both neonatal and adult hearts. However, Rb was hyperphosphorylated only in undifferentiated neonatal heart [Tam et al., 1995]. Correlated with the hyperphosphorylation of Rb, CDK1 and 2 were detected only in neonatal heart and not in adult heart [Tam et al., 1995]. Based on Northern and Western blots, cyclin A was expressed only in under-2-day-old hearts but not in 14 or later day hearts. Cyclin B was detected in both neonatal and adult hearts, but the level in the adult heart was markedly reduced [Yoshizumi et al., 1995]. Other cyclins, such as cyclin C, D1, D2, D3 and E, were present in both stages without detectable changes in mRNA levels in human heart. Others, however, reported that cyclin D1 and D3 protein levels also declined with age in mice hearts [Soonpaa et al., 1996]. This discrepancy may be due to assays targeting for different levels of expression or different species. In any event, the hypophosphorylation of Rb and expressional regulation of isoforms of CDKs and cyclins may well be one of the mechanisms for terminal differentiation of cardiomyocytes. Based on this hypothesis, we have further investigated the expressional regulation of a full spectrum of CDK isoforms during postnatal development of rat hearts.

The expression levels of CDK1 and CDK2 declined rapidly with aging (Fig. 4A–C), which was consistent with a previous study that showed their presence only in neonatal heart [Tam et al., 1995]. We used two different antibodies raised against the catalytic domain XI (anti-CDK1-XI) and the C terminus (anti-CDK1-CT) of CDK1, both of which immunoreacted with the same 32 kDa protein in only 1-day-old ventricular extracts (Fig. 4A,B). CDK1 was evident in Western blots of adult tissues, including adipose, brain, intestine, kidney, liver, lung, spleen, thymus, and testis, but absent in skeletal muscle of adult rat tissues (Fig. 4D). The expression patterns of CDK2, 3, 4, and 5 were also investigated and were found to be similar to that of CDK1. All of the CDKs were most evident in 1 day ventricles, and their expression abruptly dropped to undetectable levels after 20 days (Fig. 4E,F,H,I,K,L), except for CDK5, which also declined with aging. CDK3 was not detected at any stage of heart development (data not shown). Among other tissues, CDK4 and CDK5 were abundantly expressed in brain (Fig. 4J,M), CDK1 in intestine, spleen, and thymus (Fig. 4D), and CDK2 in adipose, lung, testis, and thymus (Fig. 4G). Since various CDKs show redundancy in their roles in cell-cycle control, it may possible that CDKs are selectively expressed in a tissue-specific manner. CDK1 typically associates with cyclin A and B1 and is required for G2/M transition in the cell cycle [McGill and Brooks, 1995; Nigg, 1995]. In C2C12 muscle cells, serum-induced differentiation of the cell decreased the expression and phosphotransferase activity of CDK1, even though the mRNA was transiently induced [Jahn et al., 1994]. These results implicate a crucial role of CDK1 in muscle cell differentiation. CDK2 in association with cyclin E and A is essential for G1/S transition and progression through S phase, respectively. CDK4 complexes with D-type cyclins and plays key roles in G1 progression. Rb is phosphorylated

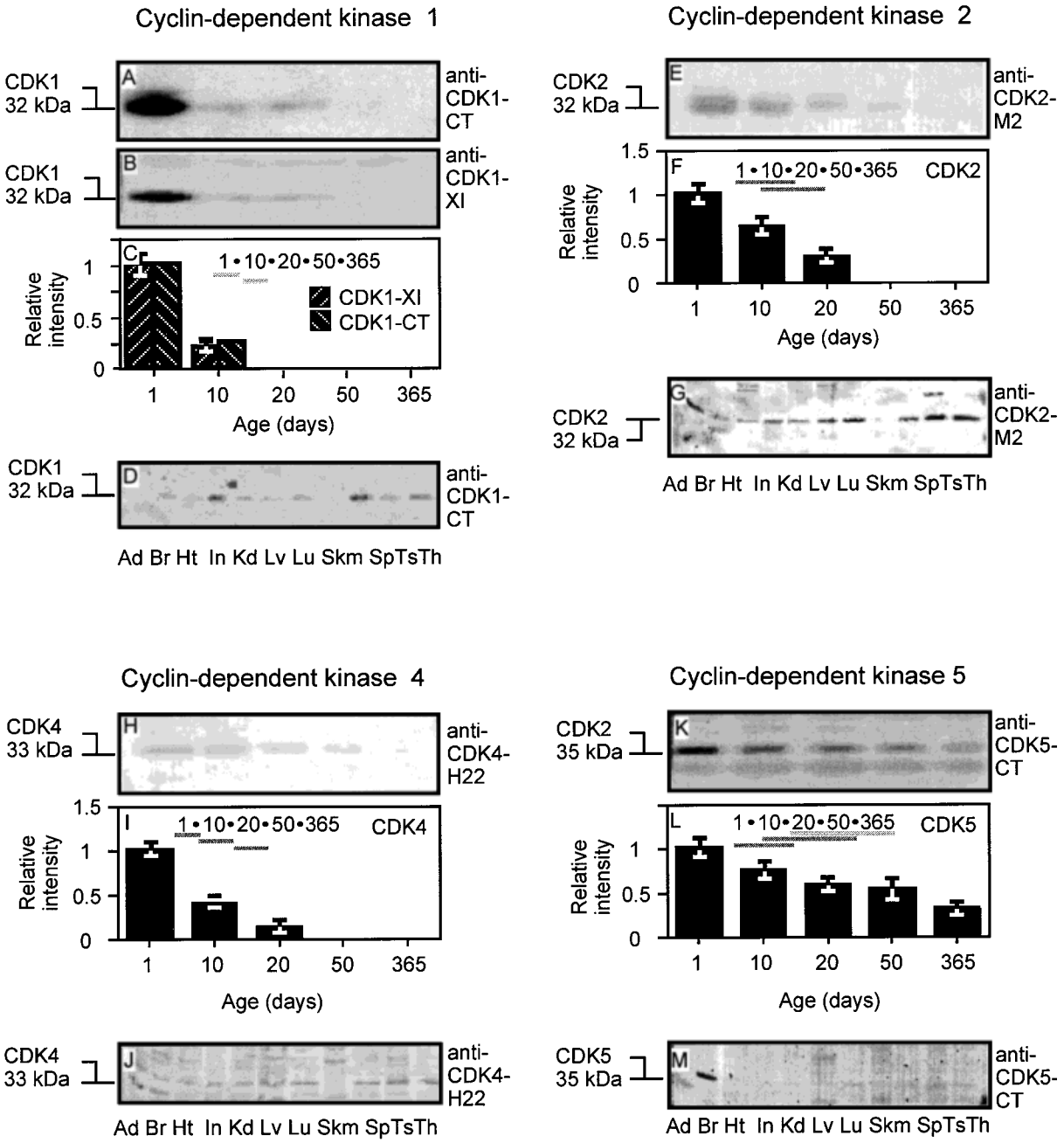


Fig. 4. Expression of cyclin-dependent kinases during postnatal development of rat ventricle. Protein extracts (150 µg) of 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE, and Western blot analysis was performed with polyclonal antibodies specific for CDK1 (**A,B**), CDK2 (**E**), CDK4 (**H**), and CDK5 (**K**). The immunoreactivities were plotted against the relative density as 1 arbitrary unit for 1 day ventricle, and values represent mean ± SEM from three to four separate experiments (**C,F,I,L**). Inserted numbers and underscores in the graphs show results of the Tukey multiple comparisons test. At the 0.05 significance level or above, the means of any two groups underscored by the same line are not significantly different. In panels **D,G,J,M,** extracts (50 µg of total protein) of 11 adult rat tissues (see legend to Fig. 1) were subjected to 11% SDS-PAGE, and immunoblot analysis was performed with CDK1 (D), CDK2 (G), CDK4 (J), and CDK5 (M) polyclonal antibodies.

predominately by CDK4/cyclin D and probably by CDK2/cyclin E and CDK1/cyclin B [Taya, 1997], and this appears to be critical in proliferation of myotubes and cardiomyocytes. The expression of CDK5, which associates with p53 and is implicated in neurofilament phosphorylation in postmitotic neurons [Lew et al., 1994; Tsai et al., 1994], was gradually diminished in the aging heart (Fig. 4L). Its precise role remains to be demonstrated [Nigg, 1995].

Unlike other CDKs, CDK8 and the CDK-related kinase, Kkialre (KK), displayed different expressional regulation. The expression of CDK8 did not change significantly during postnatal development (Fig. 5A,B). At the same time, CDK8 was readily detectable in most adult tissues and was especially abundant in skeletal muscle, brain, heart, kidney, liver, and thymus (Fig. 5C). The role of CDK8 has not been investigated as extensively as other CDKs, but it appears to be involved in basal transcription and DNA repair rather than in regulation of the cell cycle [Maldonado et al., 1996]. It complexes with cyclin C and phosphorylates the C-terminal domain of RNA polymerase II, which is thought to be involved in promoter clearance by the polymerase [Fisher, 1997]. As CDK8 was present throughout postnatal heart, this supports the putative role of CDK8 in DNA repair and basal transcription in ventricles.

The expression of KK was investigated by using two antibodies raised against the C and N termini of KK. The N-terminal antibodies recognized three proteins with molecular masses of 45, 43, and 42 kDa (Fig. 5E). The total protein levels of these three proteins increased as much as two-fold during postnatal development (Fig. 5F). The 42 kDa protein was also immunoreacted with the C-terminal antibody, and the immunoreactivity was greatly increased with age (Fig. 5D). In the isolated ventricular myocytes, KK was also detected and mainly localized in the cytosolic fraction (Fig. 2). KK was also abundantly expressed in various other tissues, including brain, kidney, liver, and skeletal muscle (Fig. 5G). The presence of this protein in the brain was previously reported based on Western blots and immunoprecipitation [Yen et al., 1995]. They observed the expression of three to four new isoforms of KK with apparent molecular masses ranging 40–52 kDa in SDS-PAGE in adult brain, whereas only one isoform, 48 kDa, was detected in fetal brain. In the heart, the three isoforms

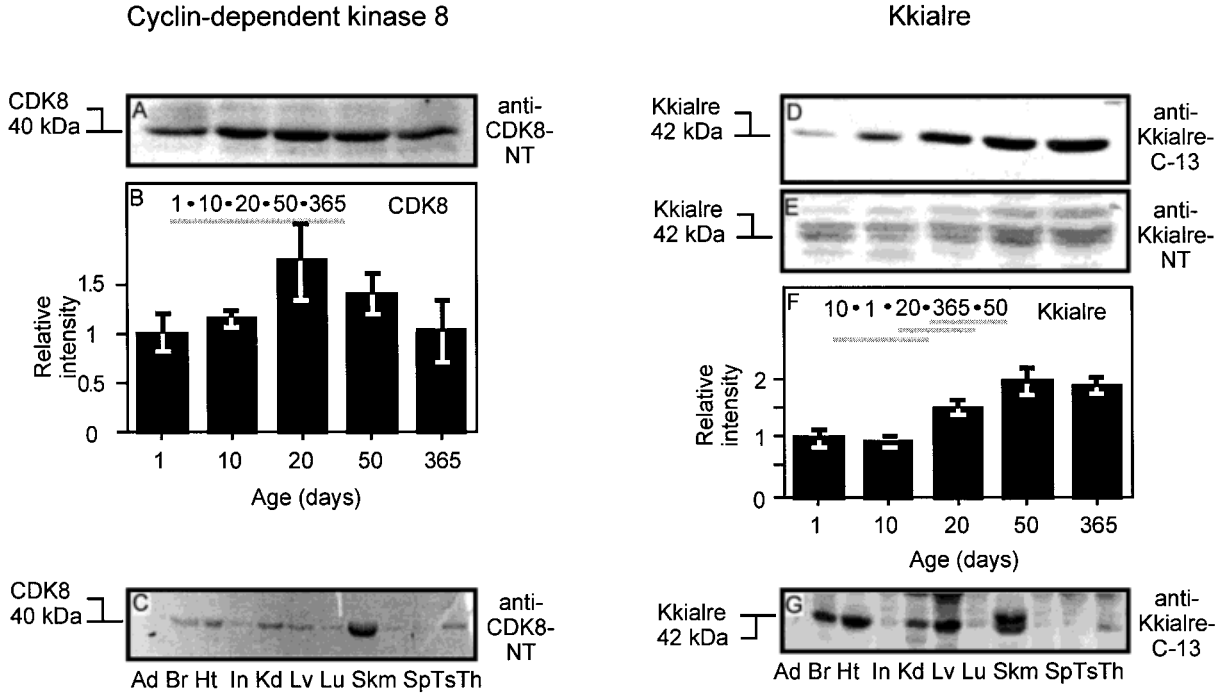
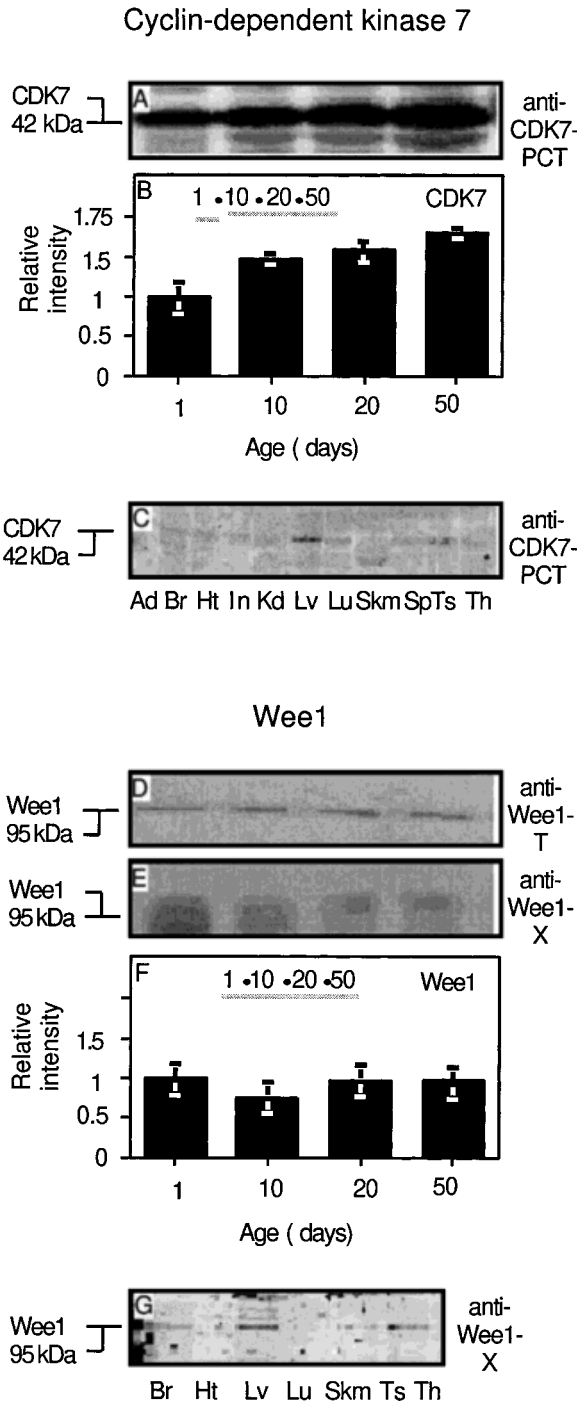


Fig. 5. Expression of CDK8 and Kkialre during postnatal development of rat ventricle. Protein extracts (150 µg) of 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE, and Western blot analysis was performed with affinity-purified polyclonal antibodies specific for CDK8 (**A**) and Kkialre (**D,E**). Panels **B,F** represent the immunoreactivities of CDK8-NT and KK-NT antibodies, respectively, which were plotted against the relative density as 1 arbitrary unit for 1 day ventricle, and values represent mean ± SEM from three to four separate experiments. Panel F represents the relative density of the three bands immunoreacted with KK-NT antibody. The bands shown in panel D correspond to the lowest band of panel E. For the quantitation of KK, the immunoreactivity of KK-NT was plotted. Inserted numbers and underscores in the graphs show results of the Tukey multiple comparisons test. At the 0.05 significance level or above, the means of any two groups underscored by the same line are not significantly different. In panels **C,G,** extracts (50 µg of total protein) of 11 adult rat tissues (see legend to Figure 1) were subjected to 11% SDS-PAGE, and immunoblot analysis was performed with CDK8 (C) and Kkialre (G) polyclonal antibodies.

also immunoreacted with the N-terminal antibody. Since among the three isoforms only the 42 kDa protein immunoreacted with the C-terminal antibody, it may indicate that these isoforms differ in the C terminus with common N termini. Nonetheless, the increase in the expression of KK isoforms during the postnatal development of the heart may indicate a specific role of KK in adult stage, maybe in the differentiated cells. In contrast to our study, Taglienti and Davis [1996] could not detect KK mRNA in the heart. The reason for this discrepancy is not clear, but it may be due to different isoforms being targeted for analysis or the KK protein level increased via reduced protein degradation rather than increased mRNA levels in the heart. The role of KK in the heart is currently under investigated in our lab.



Expression of CDK Regulating Kinases: Wee1 and CDK7

In addition to the need for cyclins for activation of CDKs, phosphorylation and dephosphorylation at specific sites are also required [McGill and Brooks, 1995; Nigg, 1995]. For instance, the phosphorylation of Thr-14 and Tyr-15 of CDK1 by Wee1 inactivates the CDK1/cyclinB complex, whereas phosphorylation of Thr-161 by CDK-activating kinase (CAK) is required for its stimulation. CAK is a member of CDKs and has been purified as a complex of 42 kDa and 37 kDa protein in HeLa cells [Fisher and Morgan, 1994; Jahn et al., 1994]. The 42 kDa catalytic subunit is 88% similar in amino acid sequence to *Xenopus* oocytes $p40^{MO15}$ [Poon et al., 1993; Solomon et al., 1993], which was renamed CDK7, and the p37 protein was identified as cyclin H [Fisher and Morgan, 1994; Makela et al. 1994]. We investigated the expression of CDK7 and Wee1 during development. Affinity-purified CDK7 proximal C-terminus (PCT) antibody immunoreacted with a 42 kDa protein in the ventricular extracts that was slightly upregulated (1.5-fold) in expression after 1 day of age and remained at a constant level afterward (Fig. 6A,B). The presence of the protein was also detected in all of the tissue

Fig. 6. Expression of CDK7 and Wee1 during postnatal development of rat ventricle. Protein extracts (150 µg) of 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE, and Western blot analysis was performed with affinity-purified polyclonal antibodies specific for CDK7 (**A**) and Wee1 (**D,E**). The immunoreactivities were plotted against the relative density as 1 arbitrary unit for 1 day ventricle, and values represent mean ± SEM from three to four separate experiments (**B,F**). Inserted numbers and underscores in the graphs show results of the Tukey multiple comparisons test. At the 0.05 significance level or above, the means of any two groups underscored by the same line are not significantly different. In panels **C,G,** extracts (50 µg of total protein) of 11 adult rat tissues (see legend to Figure 1) were subjected to 11% SDS-PAGE, and immunoblot analysis was performed with CDK7 and Wee1 polyclonal antibodies, respectively.

extracts studied in 50-day-old rats. The most abundant expression was detected in liver, whereas in the heart the expression was quite low relative to other organs (Fig. 6C).

Wee1 negatively regulates entry into mitosis by phosphorylating CDK1 at Tyr-15 [McGowan and Russell, 1993]. It was originally cloned as a 49 kDa human protein and a 68–75 kDa *Xenopus* Wee1 protein [Mueller et al., 1995]. Based on Western blotting in HeLa cells, the endogenous form, however, migrated as an approximately 94–95 kDa protein in SDS-PAGE, and the 49 kDa protein was determined to be the catalytic domain of human Wee1 [McGowan and Russell, 1995; Parker et al., 1995]. In this study, two antibodies raised against the first and last 12 residues of the *Schizosaccharomyces pombe* Wee1 (anti-Wee1-T) and kinase subdomain X of the human Wee1 (anti-Wee1-X) immunoreacted with a 95 kDa protein that exhibited no significant changes in intensity during development of the heart (Fig. 6D–F). Even though Wee1 was readily detectable in rat ventricle (Fig. 6G) and cytosolic fractions of isolated cardiomyocytes (Fig. 2), the level of expression among other tissues was very low (Fig. 6G). Higher amounts were evident in brain, liver, and testis extracts.

At present, there is little information regarding the roles of CDK7 and Wee1 in the heart. CDK7, once identified as a CDK-activating kinase (CAK), is a subunit of transcription factor IIH (TFIIH) [Drapkin et al., 1996]. TFIIH is a multisubunit complex required for transcription and for DNA nucleotide excision repair with three enzymatic activities: ATP-dependent DNA helicase, a DNA-dependent ATPase, and kinase specific for phosphorylating the C terminus of RNA polymerase II. The phosphotransferase activity is carried out by CAK, which is composed of CDK7, cyclin H, and a 36 kDa assembly factor termed Mat-1 [Tassan et al., 1995; Drapkin et al., 1996]. The steady-state mRNA level varied significantly in different cell lines and terminally differentiated tissues [Yee et al., 1995]. The upregulation in expression of CDK7 with development in the present study may be associated with the increased role of TFIIH in adult heart or cardiomyocytes rather than with the mitotic state of cells. Wee1 expression in the heart was very low compared to other tissues. Rather, the proliferation of cardiomyocytes was shown to be primarily regulated at the level of CDK expression.

In summary, the rat heart ventricle expresses various protein kinases in an age-specific manner, and they may be involved in distinct physical and biochemical characteristics during postnatal development. The expression of cyclic nucleotide–dependent protein kinases, cAPK and cGPK, was slightly decreased or did not change. In contrast, those protein kinases closely involved in cell proliferation and cell division, such as PKCs and CDKs including CDK1, 2, 4, and 5, were downregulated rapidly after 10 days of age, which may act to keep cardiomyocytes terminally differentiated. Other CDKs, such as CDK8 and Kkialre, however, did not change or rather were increased. This indicates that CDK7 and 8 may be involved in housekeeping functions, such as basal transcription and DNA repairing mechanism(s), and Kkailre may mediate terminal differentiation of cardiomyocytes.

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