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Insulin-Regulated Protein Kinases During Postnatal Development of Rat Heart

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Abstract The control of glucose uptake and glycogen metabolism by insulin in target organs is in part mediated through the regulation of protein-serine/threonine kinases. In this study, the expression and phosphotransferase activity levels of some of these kinases in rat heart ventricle were measured to investigate whether they might mediate the shift in the energy dependency of the developing heart from glycogen to fatty acids. Following tail-vein injection of overnight fasted adult rats with 2 U of insulin per kg body weight, protein kinase B (PKB), the 70-kDa ribosomal S6 kinase (S6K), and casein kinase 2 (CK2) were activated (30–600%), whereas the MAP/extracellular regulated kinases (ERK)1 and ERK2 were not stimulated under these conditions. When the expression levels of the insulin-activated kinases were probed with specific antibodies in ventricular extracts from 1-, 10-, 20-, 50-, and 365-day-old rats, phosphatidylinositol 3-kinase (PI3K), PKB, S6K, and CK2 were downregulated (40–60%) with age. By contrast, ventricular glycogen synthase kinase-3β (GSK3β) protein levels were maintained during postnatal development. Similar findings were obtained when the expression of these kinases was investigated in freshly isolated ventricular myocytes, where they were detected predominantly in the cytosolic fraction of the myocytes. Compared to other adult rat tissues such as brain and liver, the levels of PI3K, PKB, S6K, and GSK3β were relatively low in the heart. Even though CK2 protein and activity levels were reduced by ~60% in 365 day as compared to 1-day-old rats, expression of CK2 in the adult heart was as high as detected in any of the other rat tissues. The high basal activities of CK2 in early neonatal heart may be associated with the proliferating state of myocytes. J. Cell. Biochem. 71:328–339, 1998.

Key words: insulin; heart; development; PI 3-kinase; protein kinase B; S6 kinase; casein kinase 2

During postnatal development of the heart, the myocardial glycogen content and the glucose and lactate metabolism decline as the dependence on fatty acids for energy source increases [Riva and Hearse, 1991; Shelley, 1961; Warshaw, 1970; Warshaw and Terry, 1970; Veerkamp et al., 1985; Hue et al., 1994]. Under normal conditions, the adult heart preferentially utilizes fatty acids while glycolysis is inhibited (glucose-sparing effect). The lower fatty acid metabolism in neonatal heart may reflect the delayed maturation of enzymes associated with mitochondrial fatty acid transport and metabolism enzymes, such as palmitoyl-CoA transferase, acetyl-CoA synthetase, and palmitoyl-carnitine transferase [Warshaw, 1972; Warshaw and Terry, 1970]. Concurrently, enzyme activities involved in glycolysis also decrease during postnatal development of the heart [Veerkamp et al., 1985]. The glucose-sparing effect of the adult heart has been proposed to result from the increase of citrate, which can inhibit 6-phosphofructo-1 kinase (PFK-1) and 6-phosphofructo-2 kinase (PFK-2) [Hue et al., 1994]. PFK-1 catalyzes the "so-called" first committed step of glycolysis, while PFK-2 is responsible for synthesis of fructose-2,6-bisphosphate, which is a positive effector of PFK-1 [Hue et al., 1988; Garland et al., 1964]. The activities of enzymes involved in citric acid cycle are also low in the neonatal heart [Warshaw, 1972; Veerkamp et al., 1985]. The high glycogen con-

Abbreviations used: AP, alkaline phosphatase; CDK, cyclin-dependent protein kinase; ERK, extracellular-regulated kinase; Grb2, growth factor receptor binding protein-2; GSK-3, glycogen synthase kinase 3; IRS-I, insulin receptor substrate-I; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; S6K, 70-kDa ribosomal protein S6 kinase; PDK, phosphatidylinositol 3,4-bisphosphate-dependent kinase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKI, peptide inhibitor of cAMP-dependent protein kinase; Rsk, 90-kDa ribosomal S6 kinase; SH2, Src homology 2.

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tent, the greater dependence on glycolysis, and the possible adaptive effects of fetal hypoxia on glucose metabolism may contribute to the greater tolerance for hypoxia and ischemia by neonatal heart [Dawes et al., 1995; Lopaschuk and Stanley, 1997]. In fact, during mild to moderate myocardial ischemia, increased translocation of glucose transporters (Glut-1 and Glut-4) and enhanced glycolysis provide ATP to protect the ischemic heart [Dawes et al., 1995]. Therefore, the metabolic changes that take place during postnatal development may underlie the increased susceptibility to ischemia of the aging heart.

The metabolism of glycogen in the heart can be influenced by several extracellular factors, including energy demand, diet, and hormones such as adrenaline and insulin [Hue et al., 1994]. Upon binding and activating its tyrosine kinase receptor, insulin stimulates multiple signaling pathways to increase glucose uptake and the synthesis of glycogen, lipid, and protein [Denton and Tavare, 1995]. The activated insulin receptor phosphorylates insulin receptor substrate-I (IRS-I) and, in turn, this tyrosine phosphorylated protein serves to recruit diverse signaling cascades. One such signaling pathway leading to enhanced protein synthesis involves the sequential activation of phosphatidylinositol 3-kinase (PI3K), phosphatidylinositol 3,4-bisphosphate-dependent kinase (PDK), protein kinase B (PKB), FRAP, and the 70-kDa ribosomal protein S6 kinase (S6K). The interaction between tyrosine-phosphorylated IRS-I and PI3K is mediated by the Src homology 2 (SH2) domain of PI3K. However, the intervening steps between PKB, FRAP, and S6K are unclear. Another insulin signaling pathway has been proposed to lead to activation of the 85-kDa ribosomal S6 kinase (RSK). This is initiated by the binding of growth factor receptor binding protein 2 (Grb2) via its SH2 domain to tyrosine-phosphorylated IRS-I. Grb2 is an adapter protein that is bound to the guanine nucleotide exchange factor SOS, which activates the 21-kDa G protein Ras. Activated Ras recruits Raf1 to the plasma membrane, and in a protein kinase cascade, Raf1, mitogen-activated kinase kinases (MEK) 1 and 2, extracellular-regulated kinases (ERK) 1 and 2, and RSK 1 and 2 become sequentially activated. Insulin signaling may also involve the activation of protein kinase C, either through pertussis toxin-sensitive trimeric G protein- or PI3K-dependent pathways [Farese, 1996]. The G protein-mediated pathway appears to be involved in the activation of phosphatidylinositol-specific phospholipases C and/or D, whereas the PI3K-dependent pathway, may be involved in the activation of phosphatidylcholine-specific phospholipases C and/or D. Both pathways result in the production of diacylglycerol, which activates PKC, but the downstream actions of PKC in the context of insulin signaling are poorly defined. Likewise, insulin can induce activation of casein kinases (CK) 1 and 2 through obscure mechanisms [Allende and Allende, 1995].

The insulin enhancement of glycogen synthesis stems from the activation of glycogen synthase. This enzyme is activated by protein phosphatase-1 *via* dephosphorylation at multiple serine residues, including those that are phosphorylated by glycogen synthase kinase-3 (GSK3). Insulin treatment of various cells causes the activation of protein phosphatase-1 and the inhibition of GSK3, which is directly phosphorylated and inhibited by PKB [Cross et al., 1995; Burgering and Coffer, 1995]. Insulin also increases glucose uptake into cells by translocating glucose transporters, mainly Glut-4, from an intracellular location to the plasma membrane [Denton and Tavare, 1995]. Insulin-stimulated Glut-4 redistribution is suppressed by Wortmannin and LY294002, specific PI3K inhibitors, but not by rapamycin [Fingard et al., 1993]. This implies that the signaling pathway to Glut-4 translocation requires PI3K but not S6K. By mechanisms that remain to be clarified, insulin also decrease fatty acid oxidation by inactivating 5′-AMP-activated protein kinase (AMPK) [Makinde et al., 1997]. AMPK negatively regulates acetyl CoA carboxylase which catalyzes a formation of malonyl CoA from acetyl CoA. The elevation in the level of malonyl CoA by insulin results in the inhibition of carnitine-palmitoyl transferase 1 which, ultimately, reduces fatty acid oxidation [Stanley et al., 1997]. Therefore, the increase in the activity and expression of mitochondrial fatty acid transport and metabolism enzymes, and the decrease in circulating insulin levels during postnatal development contribute to the age-dependent characteristics of glucose metabolism in the heart.

This study focused on the expression and activities of the insulin-regulated protein kinases that may influence glucose metabolism during the postnatal development of rat ventricles. Tail vein injection of insulin into fasted, adult rats was shown to cause the rapid activa-

tion of PKB, S6K, and CK2, without stimulation of ERK1 or ERK2, as measured in ventricular extracts. During the postnatal development of rat heart, the insulin-activated kinases PI3K, PKB, S6K, and CK2 underwent gradual decreases in protein expression by up to 60% in 365-day-old rats as compared to 1-day-old rats, but the level of GSK-3β was not changed.

EXPERIMENTAL PROCEDURES

Materials

Affinity-purified rabbit polyclonal antibodies for CK2, PKB, p70 S6K, and GSK3-β were raised against the synthetic peptides LKPVKKKKIKREIKILENLR-GGC (CK2-III), CSHSFFDELRDDPNNK (GSK3β11), FHVETPEEREEVTC (PKB-PH), CRRPHFPQFSYSASSTA (PKB-CT), CFPMISKRPEHLRMNL (S6K-CT), and AGVFDIDLDQPEDAGSEDELEEGGQLNESC (S6K-NT). Monoclonal mouse antibody for PI3K (#05–217) was purchased from Upstate Biotechnology Incorporated (Lake Placid, NY). Goat anti-rabbit IgG and goat anti-mouse IgG conjugated to alkaline phosphatase (AP) or to horseradish peroxidase were bought from Calbiochem (San Diego, CA). Enhanced chemiluminescence (ECL) detection reagents for immunoblotting were purchased from Amersham (Arlington Heights, IL). Whatman P81 phosphocellulose filter paper was from VWR (Mississauga, ON) [γ-^{32}P]ATP was obtained from DuPont (Washington, DC). Other reagents were purchased from Sigma-Aldrich (St. Louis, MI), unless otherwise stated.

Preparation of Rat Tissue Extracts and Insulin Treatment

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). For insulin treatment, 50-day-old rats were fasted overnight and insulin (2 U/kg) was injected into the tail vein 2 and 5 min before excision of the hearts. Control rats were injected with saline instead of insulin. The ventricles of the hearts were cut, rinsed with phosphate buffer 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 phenylmethane sulfonyfluoride, 1 μg/ml aprotinin, 0.7 μg/ml pepstatin, and 1 μg/ml leupeptin, and sonicated with a Branson Probe Sonicator at 4°C with 3 × 30 sec bursts. The homogenates were ultracentrifuged at 100,000 rpm (240,000*g*) for 11 min in a Beckman TLA-100.2 ultracentrifuge at 4°C. The supernatants (cytosols) were used for the assays. Other rat tissues including adipose, brain, intestine, kidney, liver, spleen, testis, skeletal muscle (hind leg tibial muscle), and thymus were also collected from 50-day-old rats and homogenized as described above.

Isolation and Fractionation of Adult Ventricular Myocytes

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 mmol/l $NaHCO_3$, 1.2 mmol/L MgSO4 and 1 mmol/L DL-carnitine) for 5 min, followed by the same buffer containing 25 μM Ca^{2+} and 75 mU/ml of collagenase (Type II, Worthington Biochemical Corp., 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 re-suspended in buffer A containing 50 μm, 100 μm, 500 μm and 1 mM Ca^{2+}. The cells were then pelleted by centrifugation for 60 sec at 300 rpm (45*g*) and homogenized as previously described. The homogenates were ultracentrifuged for preparation of cytosolic fractions. The particulate fractions were prepared by washing the pellets with the homogenization buffer and recentrifuging. Pellets obtained were 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 SDS-PAGE sample buffer (2.5% SDS, 10 % glycerol, 50 mM Tris-

HCl, pH 6.8, 0.5 mM β-mercaptoethanol, and 0.01% bromophenol blue) and boiled at 100°C for 3 min. Protein concentrations were assayed with Bradford reagent (Bio-Rad, Richmond, CA) and the extracts were diluted with 1% SDS to yield identical protein concentrations before adding to the sample buffer. Electrophoresis was performed in 11% SDS-PAGE gels as described [Sanghera et al., 1996]. Proteins were then electrophoretically 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 quickly rinsing 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 horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit or anti-mouse IgG) in TBST for 30 min. After washing membranes three times for 10 min with TBST, Western blots were developed using ECL Western blotting detection system (Amersham) or alkaline phosphatase color development as described elsewhere [Sanghera et al., 1996].

Anion Exchange Chromatography, Immunoprecipitations, and Kinase Assays

Resource Q (Pharmacia, Gaithersburg, MD) column chromatography and casein and myelin basic protein (MBP) phosphotransferase assay was performed as described elsewhere [Hei et al., 1993]. Briefly, crude cytosolic extracts (2 mg protein) were subjected to fast protein liquid chromatography (Pharmacia) after loading onto a 1-ml Resource Q HR5/5 column that was previously equilibrated with 10 mM MOPS (pH 7.2), 5 mM EGTA, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM dithiothreitol, and 25 mM β-glycerophosphate. After washing off unbound proteins with 1 volume of the same buffer, proteins were eluted with a 10-ml gradient of 0–0.8 M NaCl at a flow rate of 1 mg/ml and 0.5 ml fractions were collected. The phosphotransferase activities towards casein and MBP were assayed by incubation with 50 μM [γ-^{32}P]ATP (2,000 cpm/pmol) in 20 μl of reaction medium A (20 mM MOPS pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 0.5 mM PKI, 1 mM sodium orthovanadate, and 1.25 mg/ml of casein or MBP) and 5 μl of Resource Q fractions for 10 min at 30°C. The assay was terminated by spotting a 15-μl aliquot of the reaction mixture onto a 1.5 cm^2 piece of p81 phosphocellulose paper. After washing the paper 10 times with 1% phosphoric acid, the radioactivity was determined by liquid scintillation counting.

PKB, S6K, and ERK1 were specifically immunoprecipitated by incubation of 500 μg of crude extracts in 3% NETF (3% Nonidet P-40, 100 mM NaCl, 5 mmol/L EDTA, 50 mM Tris-HCl, pH 7.2, and 50 mM NaF), 40 μl of Protein A-Sepharose (previously blocked with 0.1% BSA and equilibrated with 3% NETF, 1:1 volume ratio) and 5 μg of the appropriate antibodies for 3 h with constant rotation at 4°C. The Protein A-Sepharose beads were pelleted by centrifugation and washed twice with 6% NETF (containing 6% Nonidet P-40) and once with 0% NETF. The beads were then washed with reaction buffer (12.5 mM MOPS, 12.5 mM β-glycerophosphate, 0.5 mM EGTA, 7.5 mM $MgCl_2$, 50 (M NaF) and used for enzyme assays of S6K (with S6 peptide [AKRRRLSSLRA]), PKB (with MBP), and ERK1 (with MBP). The immunoprecipitated proteins were incubated at 30°C for 20 min in presence of 50 μm [γ-^{32}P]ATP (2,000 cpm/pmol) in 30 μl of reaction medium A. The reactions were terminated by addition of 5 × SDS-PAGE sample buffer. The phosphorylated MBP and S6 peptides were resolved by SDS-PAGE (11% and 20%, respectively) and electrophoretically transferred to nitrocellulose membranes. The phosphorylated MBP and S6 peptides were visualized by autoradiography and the radioactivity was quantitated by scintillation counting of the excised bands.

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 by the NIH image program. Data are presented with standard error of means (± SEM), and analysis of variance (two-way ANOVA), and Tukey's test were performed with an $\alpha < 0.05$ level of significance.

RESULTS

In Vivo Insulin Activation of Protein-Serine Kinases in Rat Heart

To investigate the protein-serine kinases that might be relevant to insulin's actions in the

adult rat heart, overnight fasted, 50-day-old rats were tail vein injected with a maximal physiological dose of insulin (i.e., 2 U/kg), and their hearts were subsequently excised for the preparation of ventricular extracts. Previous studies in our laboratory have determined that optimal insulin activation of PKB and S6K in skeletal muscle occurs within 5 min of insulin injection into rats (S. Bhanot, S. Pelech, J. McNeill, data not shown). Similar results were also obtained for the rat heart ventricles, where PKB phosphotransferase activity toward MBP was elevated four-fold by 2 min postinsulin injection (Fig. 1A,C) and S6K activity toward an S6 C-terminal sequence peptide was stimulated two-fold after 5 min of insulin treatment (Fig. 1D,F). This reflected increases in the specific enzyme activities of these kinases, since the total amounts of PKB and S6K proteins were unchanged in these immunoprecipitation experiments as shown by Western blotting (Fig. 1B,E).

Casein kinase 2 (CK2) has also been reported previously to be modestly activated following insulin treatment in several mammalian cell lines and rat tissues, although these observations have been in dispute [Hei et al., 1993; Maeda et al., 1991]. To test whether insulin activated CK2 in rat heart, ventricular extracts were subjected to anion-exchange chromatography. Phosphotransferase activity toward casein was eluted from a Resource Q column with approx. 0.5 M NaCl (i.e., fractions 18 and 19 in Fig. 2), and this could be attributed to CK2 based on its sensitivity to heparin inhibition and its coelution with immunoreactivity to a CK2-specific antibody (Fig. 2 inset). Insulin evoked a modest 40% measurable increase in the casein phosphotransferase activity of CK2 2 to 5 min after injection into the rats (Fig. 2).

Another family of protein-serine kinases that has been commonly linked to insulin signal transduction include the 42- and 44-kDa MAP kinases Erk2 and Erk1, respectively. However, recently their roles in insulin's physiological actions have been challenged by contradictory observations [Denton and Tavare, 1995]. MAP kinase activation in response to insulin was evaluated both after Resource Q fractionation (Fig. 3A) and immunoprecipitation with an Erk1-specific antibody (Fig. 3B,C). In the frac-

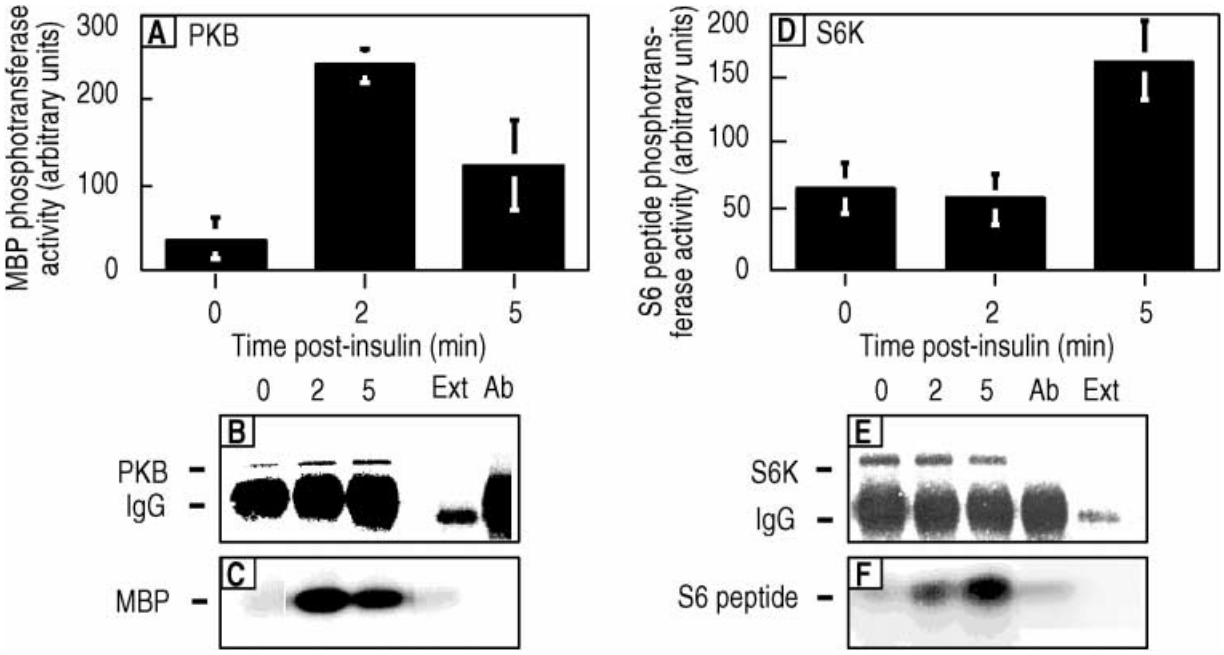

Fig. 1. Activation of protein kinase B (PKB) and p70 S6 kinase (S6K) by insulin in rat ventricle. Total protein extracts (500 µg) from control (saline injected) and 2 and 5 min insulin (2 U/kg) treated adult rat ventricles were immunoprecipitated with either PKB-PH antibody (A–C) or S6K-CT antibody (D–F), and subsequently assayed for phosphotransferase activity toward myelin basic protein (MBP) or an S6 peptide (RRLSSLRA). **C,F**: Representative autoradiograms with MBP or the S6 peptide, respectively. **A,D**: The mean quantitation of the results from four separate experiments where the autoradiograms of MBP and S6 peptide were scanned and expressed as arbitrary units (mean ± SEM). **B,E**: Western blots of the immunoprecipitates that were probed with PKB-CT antibody and S6K-NT antibody, respectively. To confirm the specificity of the immunoprecipitation experiments, controls were performed in the absence of the antibodies (Ext) or ventricular extracts (Ab).

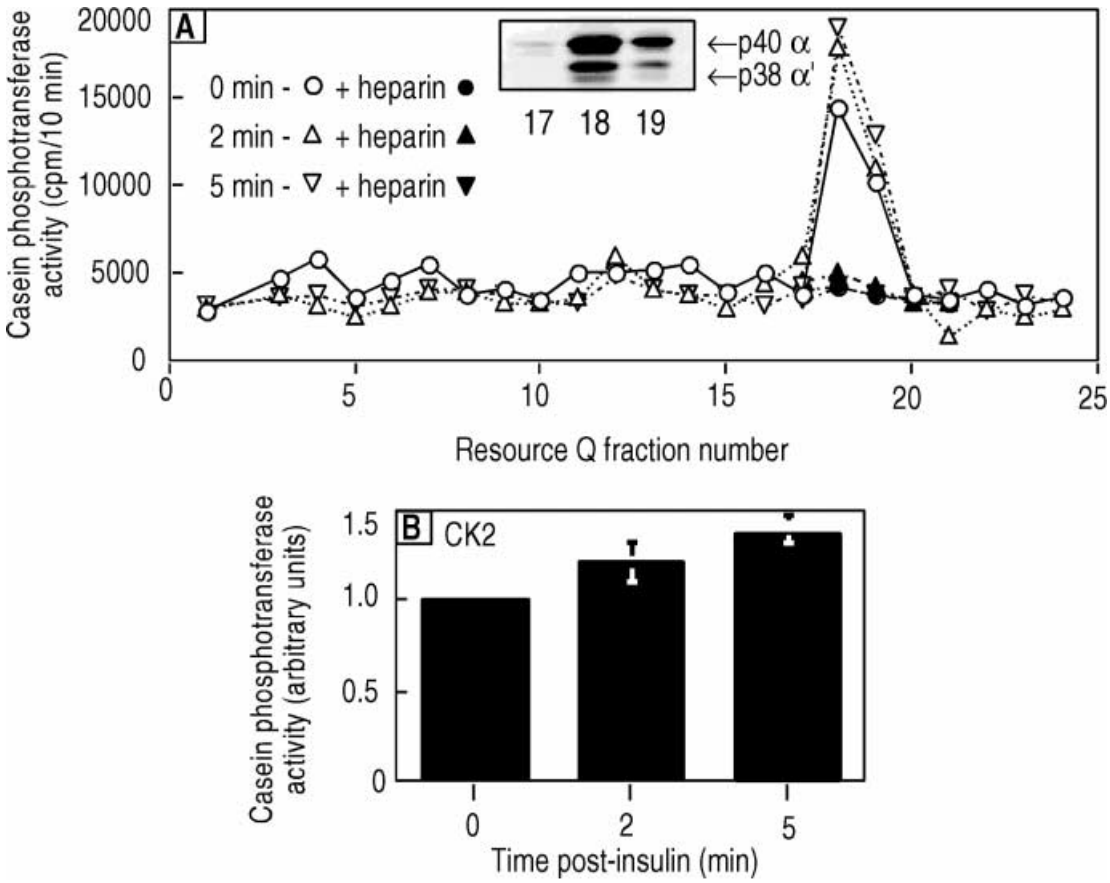

Fig. 2. Activation of casein kinase 2 (CK2) by insulin in rat ventricle. Extracts (2 mg) from control (○,●) and 2 (△,▲), and 5 min (▽,▼) insulin-treated adult rat ventricles were applied to Resource Q column that was eluted with a linear 0–0.8 M NaCl gradient. **A**: Fractions were assayed for phosphotransferase activity with casein in the absence (open symbols) and presence of 10 μg/ml heparin (closed symbols). Western blotting with CK2-III antibody confirmed that CK2 eluted in fractions 18 and 19 (inset). **B**: The mean results (±SEM) from three separate experiments for measurement of CK2.

tions (12–14) in which Erk1 and Erk2 eluted from the Resource Q column (Fig. 3A), there was no detectable increase in the MBP phosphotransferase activity as a consequence of insulin exposure for 2 or 5 min. Measurement of the kinase activity of Erk1 after its specific immunoprecipitation from unfractionated ventricular extracts of control and insulin treated rats also failed to show insulin activation of Erk1 (Fig. 3B,C). Likewise, similar negative results have been obtained for Erk1 and Erk2 activation in the skeletal muscles of rats injected with 2 U of insulin/kg for 1 to 15 min (S. Bhanot and S. Pelech, unpublished observations). Furthermore, these findings are consistent with a previous report that failed to detect activation of MAP kinase by insulin in cardiomyocytes [Lefebvre et al., 1996].

Developmental Regulation of the PI3K/PKB/S6K Pathway in Rat Ventricles

Phosphatidylinositol 3-kinase (PI3K) has been implicated as an important enzyme for insulin signaling, as this kinase can directly associate with the insulin receptor and IRS-1, and is involved in glucose transport through regulation of Glut-4 translocation [Tanti et al., 1996; Katagiri et al., 1997]. PI3K expression in the rat heart ventricle was probed in Western blots (Fig. 4A) with a monoclonal antibody raised against the p85 regulatory subunit of PI3K. Densitometric analysis revealed that expression levels of the p85 subunit of PI3K declined by 50% during the first year of postnatal development (Fig. 4B). The difference in PI3K levels between 1 and 50 days, however, was not

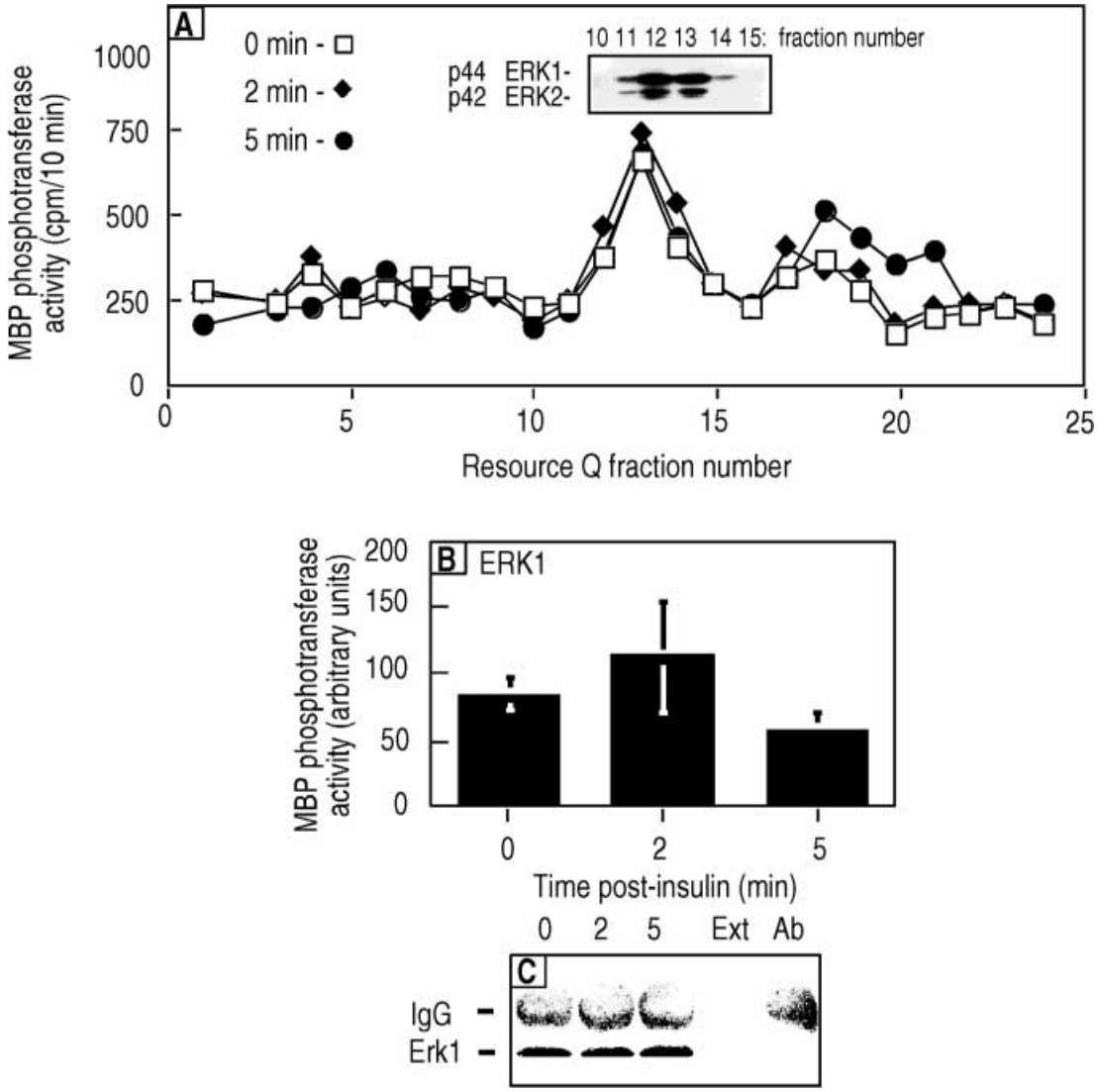

Fig. 3. Lack of activation of MAP kinases Erk1 and Erk2 by insulin in rat ventricle. **A:** Cytosolic ventricular extracts (2 mg protein) from control (□) and 2 (◆), and 5 min (●) 2 U/kg insulin-treated 50-day-old rats were applied to Resource Q column, which was eluted with a linear 0–0.8 M NaCl gradient, and the fractions were assayed for MBP phosphotransferase activity. Data is representative of two separate experiments. **B,C:** Ventricular extracts (500 μg protein) from control, 2 and 5 min insulin-treated rats were immunoprecipitated with Erk1-CT antibody. B shows phosphotransferase activities of Erk1 toward MBP (values represent mean ± SEM from four experiments). The Western blot with Erk1-CT antibody in C demonstrates that similar amounts of Erk1 were immunoprecipitated from the ventricular extracts. To confirm the specificity of the immunoprecipitation experiments, controls were performed in the absence of the antibody (Ext) or ventricular extract (Ab).

statistically significant. The adult heart was a minor organ for p85 PI3K expression, as much higher levels were detectable in adipose, brain, liver, lung, spleen, and thymus (Fig. 4C). Within adult rat cardiomyocytes, PI3K was principally detected in the cytosolic fraction (Fig. 5), where the enzyme is thought to reside in an inactive form.

PDK is a recently described kinase that is activated by lipid products of the PI3K reaction and which itself phosphorylates and stimulates PKB [Alessi et al., 1997]. Without specific antibody reagents for PDK, the developmental regulation of PKB in heart was examined next. Using two different affinity-purified, polyclonal rabbit antibodies directed against either the pleckstrin-homology (PH) domain or the C-terminus of PKB, an appropriately sized 60 kDa protein was visualized in immunoblots of rat ventricular extracts from 1- to 365-day-old rats (Fig. 6A,B). Although the expression of this

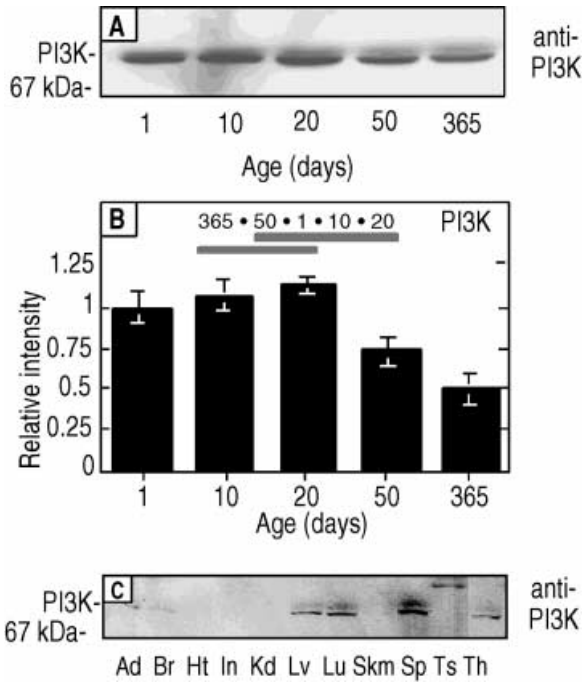

Fig. 4. Expression of PI3K during postnatal development of rat ventricle. **A**: Protein extracts (100 µg) from 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE and immunoblot analysis was performed with a mouse monoclonal antibody against the 85-kDa subunit of PI3K. **B**: The means ± SEM (n = 3 to 5) of the relative immunoreactivities of the 85-kDa subunit of PI3K as determined by densiometric analysis. Means that were not significantly different with an α value of more than 0.05 are underscored by the same grey bar. **C**: Extracts (50 µg of total protein) from 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 the PI3K monoclonal antibody.

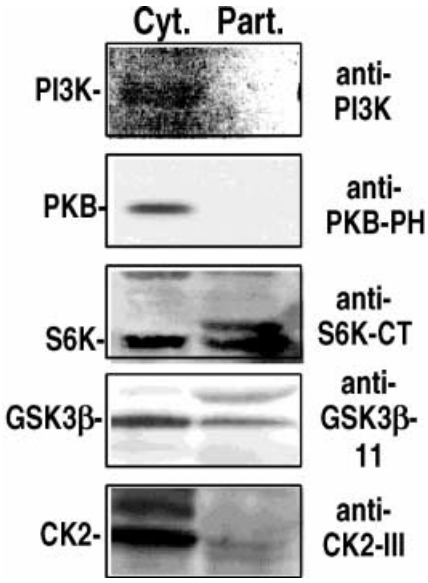

Fig. 5. Subcellular expression of insulin-regulated protein kinases in isolated ventricular myocytes. Cytosolic (Cyto.) and particulate extracts (Part.) (100 µg protein) from isolated adult (50-day-old) ventricular myocytes were subjected to 11% SDS-PAGE and Western blotted for immunoreactivity against antibodies for PI3K, PKB, S6K, GSK3β, and CK2.

protein was down-regulated by up to 60% in 1-year-old as compared to newborn rats (Fig. 6C), the basal specific enzyme activity of immunoprecipitated PKB from heart was unchanged during postnatal development (Fig. 6D). Cardiac expression of PKB was comparable to levels found in almost all of the adult rat tissues tested (Fig. 6E).

Although the intermediary steps are unclear with the exception of the dependence on the rapamycin-sensitive kinase FRAP, PKB acts upstream of p70 S6K in an insulin signaling pathway. The postnatal expression patterns of S6K in rat ventricular extracts were investigated with antibodies raised against either the N- or C-termini of this kinase (Fig. 7A,B). With both antibodies, the S6K protein level was observed to be modestly (~25%) reduced during the first year of postnatal development (Fig. 7C), and there was no significant change in the basal specific enzyme activity of immunoprecipitated S6K (Fig. 7D). Adult rat heart featured one of the highest levels of S6K that was measured, with quantities that were only exceeded by brain (Fig. 7E). In cardiomyocytes, S6K was present in both the cytosolic and particulate fractions (Fig. 5).

Developmental Regulation of GSK3 and CK2 in Rat Ventricles

GSK3 has been proposed to be directly phosphorylated and inactivated by PKB and S6K, and this could account in part for how insulin may prevent phosphorylation and inactivation of glycogen synthase [Cross et al., 1995; Burgering and Coffer, 1995; Sutherland and Cohen, 1994]. The relative expression of the β-isoform of GSK3 was low in adult rat heart as compared with brain, liver, lung, and testes (Fig. 8C). Within the myocytes, GSK3β was principally found in the cytoplasmic fraction (Fig. 5). There were no significant changes in the level of GSK3β expression during the first year of postnatal development in rat ventricles (Fig. 8A,B).

In contrast to GSK3β, the ventricular expression of CK2 was decreased up to 63% between days 10 and 365 in the rat (Fig. 9B,C). The

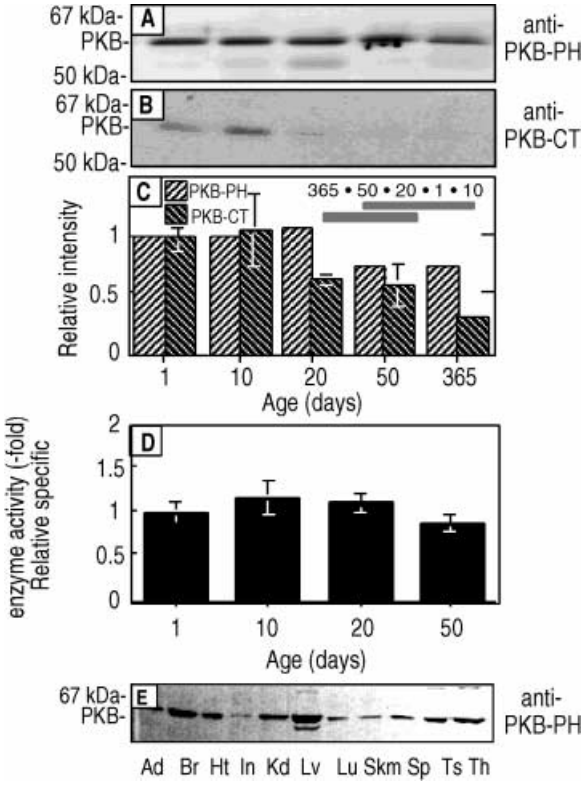

Fig. 6. Expression and activity of PKB during postnatal development of rat ventricle. Protein extracts (100 µg) of 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE and immunoblot analysis was performed with PKB-PH (**A**) and PKB-CT (**B**) polyclonal antibodies. **C:** The means ± SEM (n = 3 to 5) of the relative immunoreactivities with the PKB-PH and PKB-CT antibodies as determined by densiometric analysis. Means that were not significantly different with an α value of more than 0.05 are underscored by the same grey bar. **D:** PKB was immunoprecipitated from the ventricular extracts pepared from 1- to 50-day-old rats, and subsequently assayed for MBP phosphotransferase activity. Values are the relative enzyme activities and are the mean ± SEM of four separate experiments. **E:** Extracts (50 µg of total protein) from 11 adult rat tissues (see legend to Fig. 4) were subjected to 11% SDS-PAGE and immunoblot analysis was performed with the PKB-PH polyclonal antibody.

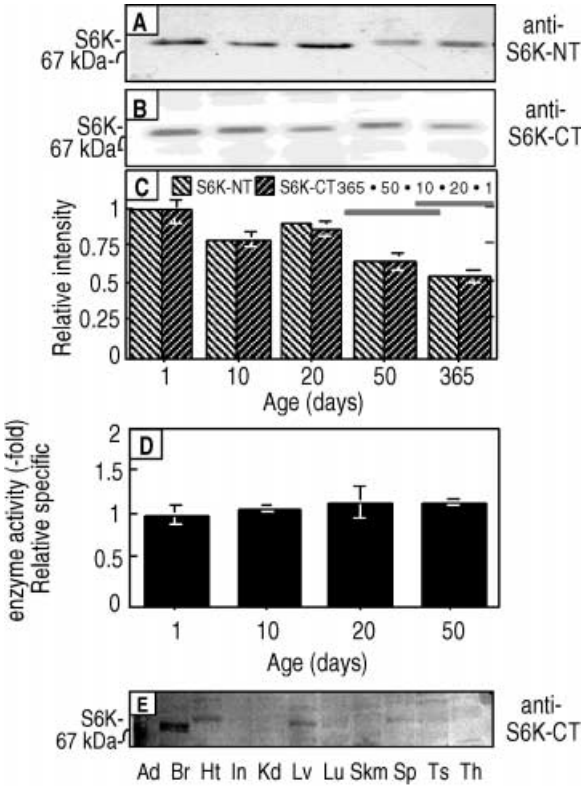

Fig. 7. Expression and activity of p70 S6K during postnatal development of rat ventricle. Protein extracts (100 µg) of 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE and immunoblot analysis was performed with S6K-NT (**A**) and S6K-CT (**B**) polyclonal antibodies. **C**: The means ± SEM (n = 3 to 5) of the relative immunoreactivities with the S6K-NT and S6K-CT antibodies as determined by densiometric analysis. Means that differed with an α value of more than 0.05 are encompassed by the same grey bar. **D:** p70 S6K was immunoprecipitated from the ventricular extracts prepared from 1- to 50-day-old rats, and subsequently assayed for S6–10 peptide (RRLSSLRA) phosphotransferase activity. Values are the relative enzyme activities and are the mean ± SEM of four separate experiments. **E:** Extracts (50 µg of total protein) from 11 adult rat tissues (see legend to Fig. 4) were subjected to 11% SDS-PAGE and immunoblot analysis was performed with the S6K-CT polyclonal antibody.

reduced casein phosphotransferase activity in older rats that was measured after Resource Q fractionation (Fig. 9A) could largely be attributed to a decrease in the amount of CK2 protein. In the neonatal and adult rat heart, the 42-kDa α subunit was the principal isoform detected in Western blots (Fig. 9B). The higher molecular mass species at 44-kDa probably corresponded to a hyperphosphorylated form of the α subunit, whereas the weaker CK2-immunoreactive band at 38-kDa was due to the presence of a smaller amount of the α' subunit [Fingar and Birnbaum, 1994]. Adult heart had some of the highest levels of CK2 expression detected in rat tissues (Fig. 9D). Although brain, thymus and testes also possessed high concentrations of CK2, the α' subunit appear to predominant in these tissues. Only intestines seemed to have comparable levels of the α subunit as compared with heart. Within myocytes, most of the CK2 was located in the cytosolic fraction (Fig. 5).

DISCUSSION

Insulin stimulates a wide array of signaling cascades, resulting in the increased synthesis

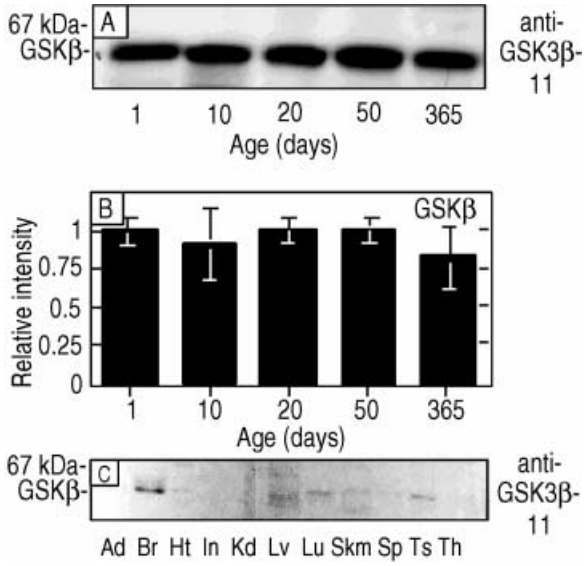

Fig. 8. Expression of GSK3 during postnatal development of rat ventricle. **A**: Protein extracts (100 µg) of 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE and immunoblot analysis was performed with GSK3β-11 polyclonal antibodies. **B**: The means ± SEM (n = 3 to 5) of the relative immunoreactivities with GSK3β-11 antibodies as determined by densiometric analysis. **C**: Extracts (50 µg of total protein) from 11 adult rat tissues (see legend to Fig. 4) were subjected to 11% SDS-PAGE and immunoblot analysis was performed with the GSK3β-11 polyclonal antibody.

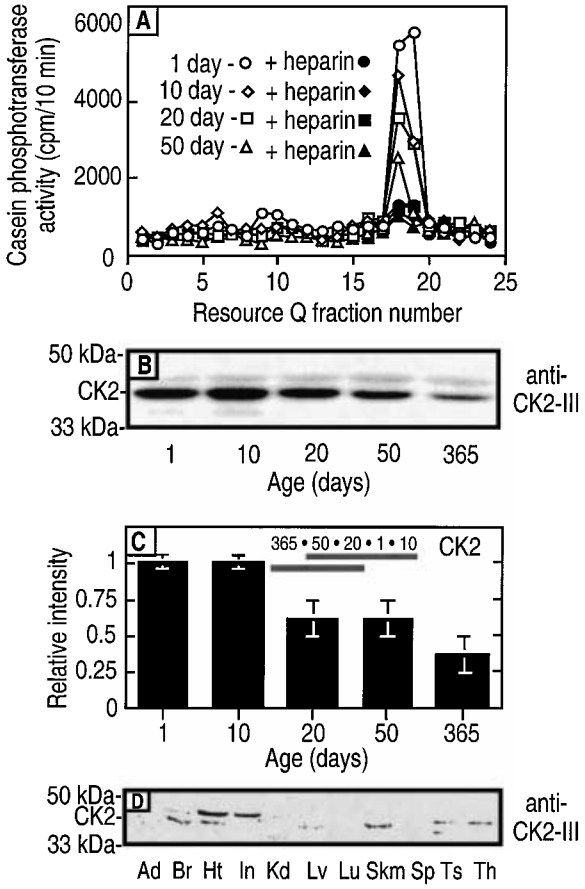

Fig. 9. Basal activity of casein kinase 2 (CK2) during postnatal development of rat ventricle. **A**: Cytosolic ventricular extracts (2 mg protein) from 1-day- (○, ●) 10-day- (◇,◆), 20-day- (□,■), and 50-day- (△,▲) old rats were applied to a Resource Q column, which was eluted with a linear 0–0.8 M NaCl gradient, and the 0.5 ml fractions were assayed for casein phosphotransferase activity in the absence (open symbols) and presence (closed symbols) of 10 µg/ml heparin. Data is representative of three separate experiments. **B**: Protein extracts (100 µg) of 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE and immunoblot analysis was performed with antibody raised against subdomain III regions of CK2. **C**: The immunoreactivity was plotted against the relative density for 1 day ventricles and values represent mean ± SEM. Means that differed with an α value of more than 0.05 are encompassed by the same grey bar. **D**: Extracts (50 µg of total protein) from 11 adult rat tissues (see legend to Fig. 4) were subjected to 11% SDS-PAGE and immunoblot analysis was performed with CK2-III antibody.

of protein, lipid and glycogen, and enhanced glucose uptake [Denton and Tavare, 1995]. Instead of employing high doses of insulin (10 U/kg or more) as performed in most previous studies, we used a lower dose of insulin (2 U/kg) that approximated more closely physiological conditions. Under these circumstances, insulin could activate PKB, S6K, and CK2 without stimulation of the Erk1 and Erk2 MAP kinases. These results are consistent with a previous report of insulin treatment in isolated cardiomyocytes that led to activation of PI3K and S6K without increasing MAP kinase activity [Lefebvre et al., 1996]. A role for MAP kinase signaling cascades in the metabolic actions of insulin has been disputed on several grounds [Denton and Tavare, 1995]. For example, stimuli such as epidermal growth factor, which can potently activate MAP kinases, or introduction of activated Raf1 failed to increase glucose transport or to stimulate glycogen synthase in fat and liver cells [Robinson et al., 1993; Fingar and Birnbaum, 1994; Gould et al., 1994].

During the development of heart, the glycogen content and dependence on glucose and lactate decrease with age and the normal adult heart predominantly utilizes fatty acids, while the metabolic pathway for glycolysis is inhibited. Since insulin has been shown to be involved in the regulation of glucose uptake, glycogen metabolism, and fatty acid synthesis, we investigated how kinases implicated in insulin signal transduction are affected during the postnatal development of the heart. There were

modest reductions in the expression levels of PI3K, PKB, and S6K after birth during the first year of development. These reductions in expression in conjunction with decreasing in circulating insulin level during development [Girard et al., 1992] may contribute the adult specific glucose metabolism in the heart. However, in the adult ventricle samples, PKB and S6K were still highly expressed (Figs. 4,6,7), which indicates that the adult rat heart is still quite responsive to insulin and is achieved in part through the PKB/S6K signaling pathway. Likewise, as GSK3β levels were maintained during postnatal development, inhibition of this kinase by PKB may also mediate some of the actions of insulin in adult heart.

Some of the most profound changes in kinase expression during heart development was observed for CK2. The known in vitro substrates for CK2 include over 100 proteins and many of them are involved in cell proliferation [Allende and Allende, 1995]. The signaling cascades for CK2 and its precise role are not clear, but it has been proposed that its expression and activity are dependent on the proliferation state of cell [Ulloa et al., 1993; Gruppuse and Boylan, 1995; Mestres et al., 1994]. While there was a reduction of CK2 protein and activity that correlated with the cessation of myocyte proliferation after day 10, the adult heart still had one of the highest levels of CK2 detected in the rat. This supports additional roles for CK2 besides control of cell proliferation.

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