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Dual Effect of Heparin on Cultured Adult Rat Cardiomyocytes

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Abstract Heparin has been widely reported to inhibit the growth of several cell types including neonatal rat cardiac myocyte (NRCM) but its effect on adult rat ventricular myocyte (ARVM) is unknown. To determine whether heparin is able to modulate ARVM protein synthesis capacity and if so which pathway is involved in this response, ARVM were cultured in presence or absence of 5% human serum and exposed to heparin (2–2,000 μg/ml) or its analogue xylan (0.5 and 50 μg/ml), and either the Ca^{2+} chelator BAPTA/AM (10 μg/ml), or the calcineurin inhibitor FK506 (10 μg/ml), and heparinase I (0.1–10 U/ml) for 2 days. The protein synthesis (PS) was measured after 24 h incorporation of [14C]-Phenylalanine in ARVM. Independently of the serum presence, heparin and xylan altered PS in a bimodal dose-dependent manner. At high doses, heparin and xylan (2,000 and 50 μg/ml, respectively) either had no effect (without serum) or inhibited PS (with serum). In absence of serum, low doses of heparin or xylan (20 and 0.5 μg/ml, respectively) amplified the PS process in ARVM (2-fold, $P < 0.05$). FK506 inhibited the trophic response to 20 μg/ml heparin alone (−39%, $P < 0.05$). In presence of serum, the heparin induced-trophic effect, that was not significantly altered by FK506, was inhibited by BAPTA/AM (−32%, $P < 0.05$). Finally, heparinase I that increased PS in NRCM had no effect on ARVM growth. This study strongly suggests that heparin dose-dependently modulated PS in ARVM, this result being not observed in neonatal cells. Different mechanisms involving intracellular Ca^{2+} play a role in the PS response of ARVM to low concentrations of heparin, the intracellular pathways depending on the presence of serum. J. Cell. Biochem. 92: 1212–1220, 2004.

Key words: heparin; calcium; heart; protein synthesis

In vivo, the development of cardiac hypertrophy is triggered by numerous factors including mechanical forces and cardiac or humoral growth factors [Swynghedauw, 1999]. Most of the studies have been focused on the identification of growth-stimulating factors but little is known about the factors that may negatively regulate the process of cardiomyocyte growth [Corda et al., 1997, 2000].

Interestingly, exogeneous heparin and endogeneous heparan sulfate inhibit cell growth and proliferation in a variety of cell types, including cultured vascular smooth muscle [Peterson et al., 1999; Kalmes et al., 2000; Underwood and Mitchell, 2000] and endothelial cells [Pintus et al., 1998, 1999]. Based on a study with neonatal rat cardiac myocyte (NRCM), Akimoto et al. [1996] proposed that endogenous heparin-like molecules, possibly heparan sulfate, exert a negative regulatory role in the development of cardiomyocyte hypertrophy. However, it is difficult to extend these observations to adult cells since the responses of cardiomyocytes to growth factors and hormones strongly differ depending on the developmental stage of cardiac myocytes [Parker et al., 1990; Schneider et al., 1990; Samuel et al., 1995]. As an example, α1-adrenergic stimulation promotes hypertrophy of NRCM [Simpson, 1983] but not in adult rat ventricular myocytes (ARVM) [Dubus et al., 1990]. Therefore, the question arises whether heparin modulates ARVM protein synthesis capacity, as observed in NRCM.

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Heparin has been shown to modulate different signaling pathways. For instance, it can inhibit inositol 1,4,5-trisphosphate ($InsP_3$)-induced Ca^{2+} release [Ghosh et al., 1988; Kobayashi et al., 1988, 1989] in different cells including cardiomyocytes [Perez et al., 1997]. Heparin can also selectively block the PKC pathway of mitogenic signaling as well as the phosphorylation and activation of mitogen-activated protein kinase (MAPK) [Castellot et al., 1989; Pukac et al., 1992]. Both pathways are known to be implicated in the control of cardiac growth. Indeed the signaling pathways that couple the demand for increased contractile power to increased myocyte growth involved Ca^{2+}-dependent pathways. It has been argued that elevated levels of cytosolic Ca^{2+} in cells activate Ca^{2+}-dependent enzymes, such as the Ca^{2+}-dependent phosphatase calcineurin [Molkentin et al., 1998]. On the other hand, several signaling pathways leading to protein phosphorylation (mainly catalyzed by Ser-/Thr- or Tyr-specific protein kinases) participate in the development of cardiomyocyte hypertrophy [Sugden, 1999]. A further question arises by which mechanism heparin may control growth response of ARVM.

To analyze the effect of heparin on cardiac cell growth capability, we used a well-established model of cultured myocytes isolated from either adult or neonatal rat hearts. In order to better characterize the pathways involved in the cardiomyocyte response to heparin, we studied Ca^{2+}-dependent pathways by means of the intracellular Ca^{2+} chelator BAPTA/AM and the calcineurin inhibitors FK506 and Cyclosporin A (CsA).

We demonstrate that heparin dose-dependently modulates protein synthesis in ARVM. The response varied according to the developmental stage of the cells. Different mechanisms involving intracellular Ca^{2+} play a role in the trophic response of ARVM to low concentrations of heparin but the mechanisms involved depend on the presence of serum.

MATERIALS AND METHODS

Isolation and Culture of rat Cardiomyocytes

ARVM. ARVM were obtained from 2-month-old male Wistar rats weighing 200–250 g (Iffa Credo, Lyon, France) as previously described [Dubus et al., 1990, 1993]. Cells (80,000 cells/ml) were seeded in culture plates (Falcon BD, VWR, Strasbourg, France), precoated with 1.5 μg/cm^2 laminin (Sigma-Aldrich, L'Ile d'Abeau Chesnes, France), and they were cultured for 2 days in a serum free medium (BM86 Wissler, Cryo Bio System, L'Aigle, France), daily replaced. Two hours after cell plating, the percentage of rod-shaped myocytes reached $91 \pm 5\%$ of total cells. Both cell density (cells/mm^2) and percentage of rod-shaped cardiomyocytes were daily analyzed. Whatever the experimental conditions, the cardiomyocytes did not exhibit spontaneous contraction.

The cells were cultured in the presence, or not, of various concentrations (2–2,000 μg/ml) of heparin (Pharmacie centrale des hôpitaux de Paris, France), (0.5 and 50 μg/ml) of xylan (Sigma Chem. Co., St Louis, MO), 10 μg/ml of BAPTA/AM (Calbiochem, VWR, Strasbourg, France), 10 μg/ml of FK506 (Fujisawa Healthcare, Deerfield, IL) or 500 ng/ml of CsA (Sandoz Laboratories, Schonenwerd, Switzerland) supplemented, or not, with 5% human serum AB (Valbiotech, Paris, France). In addition, the effects of heparinase I (from *Flavobacterium heparinum*, EC 4.2.2.7), or heparinase III (from *F. heparinum*, EC 4.2.2.8, Sigma) were tested.

NRCM. Primary cultures of NRCM were conducted according to Knowlton et al. [1991]. Ventricular cells from 2-day-old Wistar rat hearts were dispersed with collagenase A (0.45 mg/ml) (Boehinger, Mannheim, Germany) and pancreatin (0.05 mg/ml) (GIBCO/BRL, Invitrogen Life Technologies, Cergy Pontoise, France) and then NRCM were purified on a discontinuous Percoll gradient. Cells were seeded in plates at the density of 2.10^5 cells/ml. After 48 h culture in presence of fetal calf serum and then 24 h in a serum free medium, NRCM were treated with heparin or heparinase as described above.

Protein Synthesis in Cardiomyocytes

Protein synthesis in cultured cardiac myocytes, was evaluated by the incorporation of 1 μCi [^{14}C]-Phenylalanine (specific activity, 472 mCi/mmol, Amersham Biosciences, Little Chalfont, UK) for 24 h, as previously described [Dubus et al., 1990; Corda et al., 1997]. Protein samples were obtained by precipitation with 10% TCA, and then solubilisation in 1 N NaOH. Protein content was measured with Bradford, and the radioactivity contained in an aliquot was counted. The rate of protein synthesis was expressed as the amount of [^{14}C]-Phenylalanine

that has been incorporated in 10^3 cells (cpm/10^3 cells). For each point, experiments were performed independently in triplicate.

Isolation of Cardiac Cytosolic and Cytoskeleton Proteins

Cytosolic and cytoskeletal proteins were prepared as previously described [Kuppuswamy et al., 1997], with minor modifications. Briefly, to obtained cytosolic fraction, ARVM cultures were incubated 1 h at 4°C with 100 μl of ice-cold Tris-Triton extraction buffer containing 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.2 mM Na_3VO_4, 50 mM NaF, 40 mM β-glycerophosphate, 100 mM NaCl, 5 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin. The Triton X-100-soluble fraction was collected and centrifuged at 15,000*g* for 5 min at 4°C, the insoluble fractions were treated with 100 μl of hot Tris-SDS extraction buffer (10 mM Tris-HCl, pH 7.4, 1% SDS, 1 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 mg/ml PMSF). After scraping, the extracts were centrifuged at 15,000*g* for 5 min at 15°C and supernatants were collected.

The protein concentrations were measured using the BCA protein assay kit, and the samples were stored at −20°C until use.

Western Blot Analysis

Triton X-100 soluble and insoluble fractions (20 μg) were resolved by electrophoresis on 10% SDS-acrylamide gel and transferred to nitrocellulose membrane. After blocking, the membranes were successively incubated (1 h at 21°C) with monoclonal anti-Phosphotyrosine antibody (PY20, Transduction Laboratories, Santa Cruz Biotechnology, Santa Cruz, CA) and with anti-mouse-IgG conjugated to horseradish peroxidase (1:5,000). After washing, immunoreactive bands were visualized by enhanced chemiluminescence ECL$^+$ and quantified by densitometry using a computer-based imaging system (Fuji).

Statistical Analysis

Results are expressed as mean ± standard error of the mean. ANOVA tests were used in statistical evaluation of the data. A Scheffe-test, except when indicated, was used, and a value of $P < 0.05$ was considered as significant.

RESULTS

Effects of Treatments on ARVM Density, Shape, and Protein Content

As shown in Table I, the addition 2,000 μg/ml heparin either alone or in presence of 5% human serum to ARVM increased significantly the percentage of rod-shaped cells at day 2. Similar results were obtained in presence of 10 μg/ml BAPTA/AM (+50% as compared to control condition) whereas other treatments had no effect when compared with control.

Only high dose of heparin (2,000 μg/ml) prevented cell death as indicated by the highest cell density ($P < 0.05$, vs. control, *t*-test) whereas other treatments were ineffective (Table I).

In addition, it should be notice that whatever the experimental conditions, the amount of total protein in ARVM did not vary significantly except in the presence of BAPTA/AM (Table I).

TABLE I. Effects of Exogenous Heparin (20 and 2,000 μg/ml), Xylan (0.5 and 50 μg/ml), BAPTA/AM (10 μg/ml), and FK506 (10 μg/ml) With or Without 5% Human Serum (HS) on the Percentage of Rod-Shaped ARVM, Total ARVM Density, and Protein Content After 2 Days of Culture

		Heparin (μg/ml)		Xylan (μg/ml)		BAPTA/AM (μg/ml)	FK506 (μg/ml)
	Control	20	2,000	0.5	50	10	10
Rod-shaped cell (%)							
Medium	54 ± 5	51 ± 7	94 ± 2*	54 ± 8	49 ± 7	92 ± 1*	55 ± 7
5% HS	51 ± 5	45 ± 5	84 ± 4*	48 ± 9	39 ± 10	53 ± 6	36 ± 1
Cell density (cell/mm^2)							
Medium	50 ± 4	47 ± 5	64 ± 7	41 ± 5	49 ± 8	45 ± 6	44 ± 4
5% HS	44 ± 4	43 ± 6	61 ± 7**	39 ± 9	56 ± 9	41 ± 6	48 ± 4
Total protein (μg/10^3 cells)							
Medium	6.4 ± 0.6	6.4 ± 1.0	5.7 ± 0.6	6.0 ± 1.0	6.2 ± 0.5	3.6 ± 0.7*	6.3 ± 0.4
5% HS	7.2 ± 0.5	7.2 ± 0.7	6.1 ± 1.2	5.5 ± 0.5	5.8 ± 0.9	4.8 ± 0.2**	7.1 ± 1.0

Values are mean ± SEM with N = 4 to 10 independent experiments, each done in triplicate.
*$P < 0.05$, versus control medium.
**$P < 0.05$, versus 5% HS.

Effects of Heparin and Xylan on ARVM Protein Synthesis

The dose-responses curves of heparin on ARVM indicated that low doses of heparin (20 μg/ml) induced a significant increase in protein synthesis (+80% vs. control, $N = 7$, $P < 0.05$) whereas the highest concentrations (2,000 μg/ml) had no effect (Fig. 1A). The heparin structural analogue, xylan at low (0.5 μg/ml) and high (50 μg /ml) concentrations induced similar effects as observed with heparin (Fig. 1B).

Then, we determine whether the growth response of cardiomyocytes to 20 μg/ml heparin was mediated by the calcineurin pathway. Pretreatment of ARVM with either FK506 or CsA (data not shown) had no effect on the basal rate of protein synthesis but completely abolished the increase in protein synthesis induced by 20 μg/ml heparin alone (−39%, heparin + FK506 vs. heparin, $N = 5$, $P < 0.05$) (Fig. 1C).

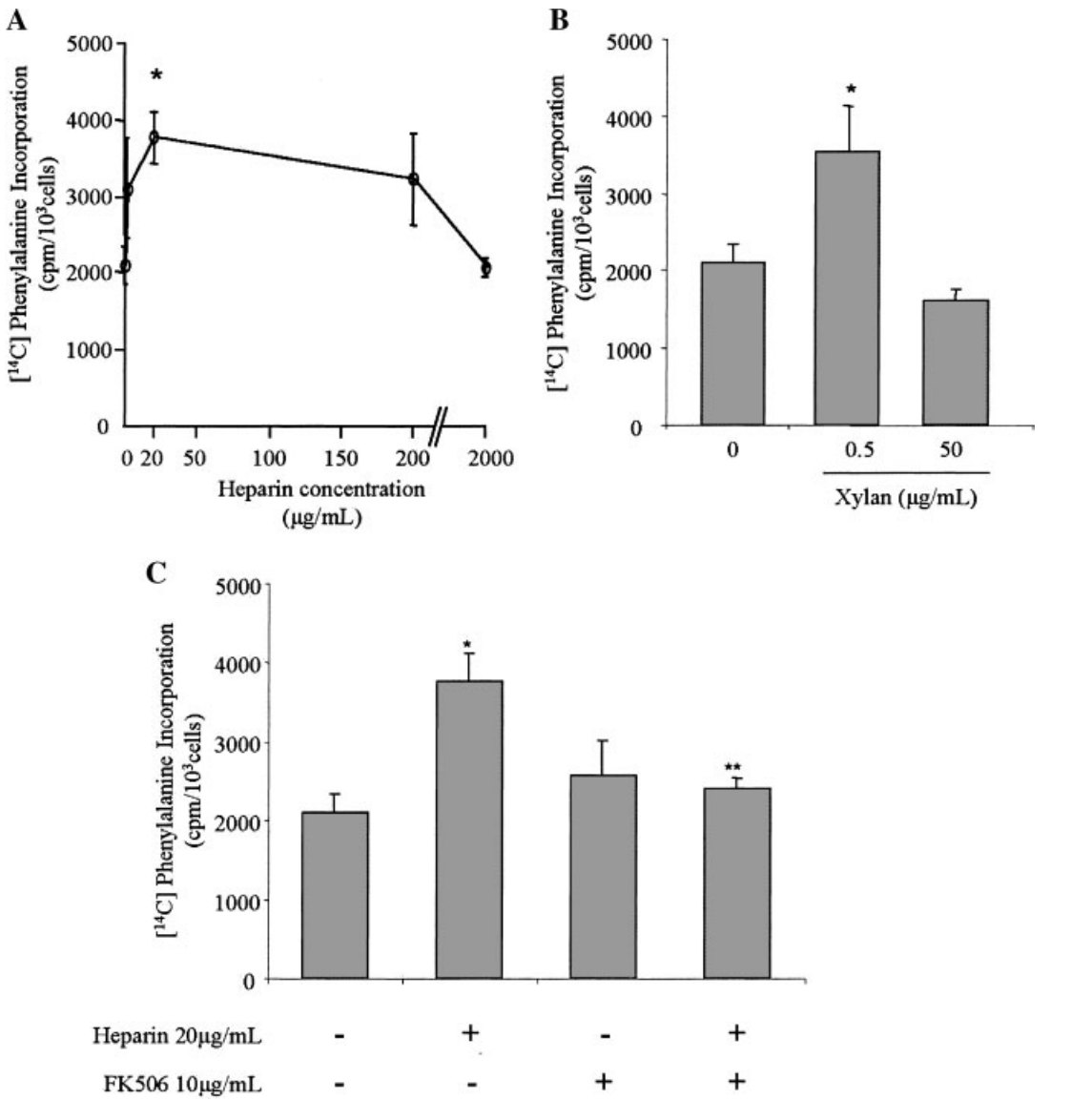

Fig. 1. Dual effects of either heparin or xylan on the adult rat ventricular myocytes (ARVM) protein synthesis. **A**: Effects of increasing concentrations of heparin (2–2,000 μg/ml) for 48 h on ARVM protein synthesis. **B**: Effects of low and high concentrations of xylan (0.5 and 50 μg/ml, respectively for 48 h) on ARVM protein synthesis. **C**: Involvement of calcineurin-dependent pathway through inhibitory effect of FK506 (10 μg/ml), an inhibitor of calcineurin, on the trophic effect of low concentration of heparin. The protein synthesis was evaluated by [^{14}C]-Phenylalanine relative incorporation for 24 h. Values are mean ± SEM of five to seven independent experiments, each done in triplicate. *$P < 0.05$ versus control, **$P < 0.05$ versus low concentration of heparin.

These results indicated that the trophic effect of ARVM to low doses of heparin is calcineurin dependent.

Additional Effects of Heparin and Xylan on Human Serum-Induced Protein Synthesis of ARVM

Human serum (5% in culture medium) induced an increase in protein synthesis (3,569 ± 239 cpm/10^3 cells vs. 2,101 ± 239 cpm/10^3 cells, $P < 0.05$). In the presence of 5% human serum, the dose-response curve of heparin showed that a 20 μg/ml concentration markedly enhanced the protein synthesis of ARVM (+60% vs. human serum, N = 7, $P < 0.05$) (Fig. 2A) whereas the highest doses (2,000 μg/ml) decreased human serum-induced protein synthesis (−50% vs. serum alone, N = 5, $P < 0.05$) (Fig. 2A). Likewise xylan at low (0.5 mg/ml) and high (50 μg/ml) concentrations respectively

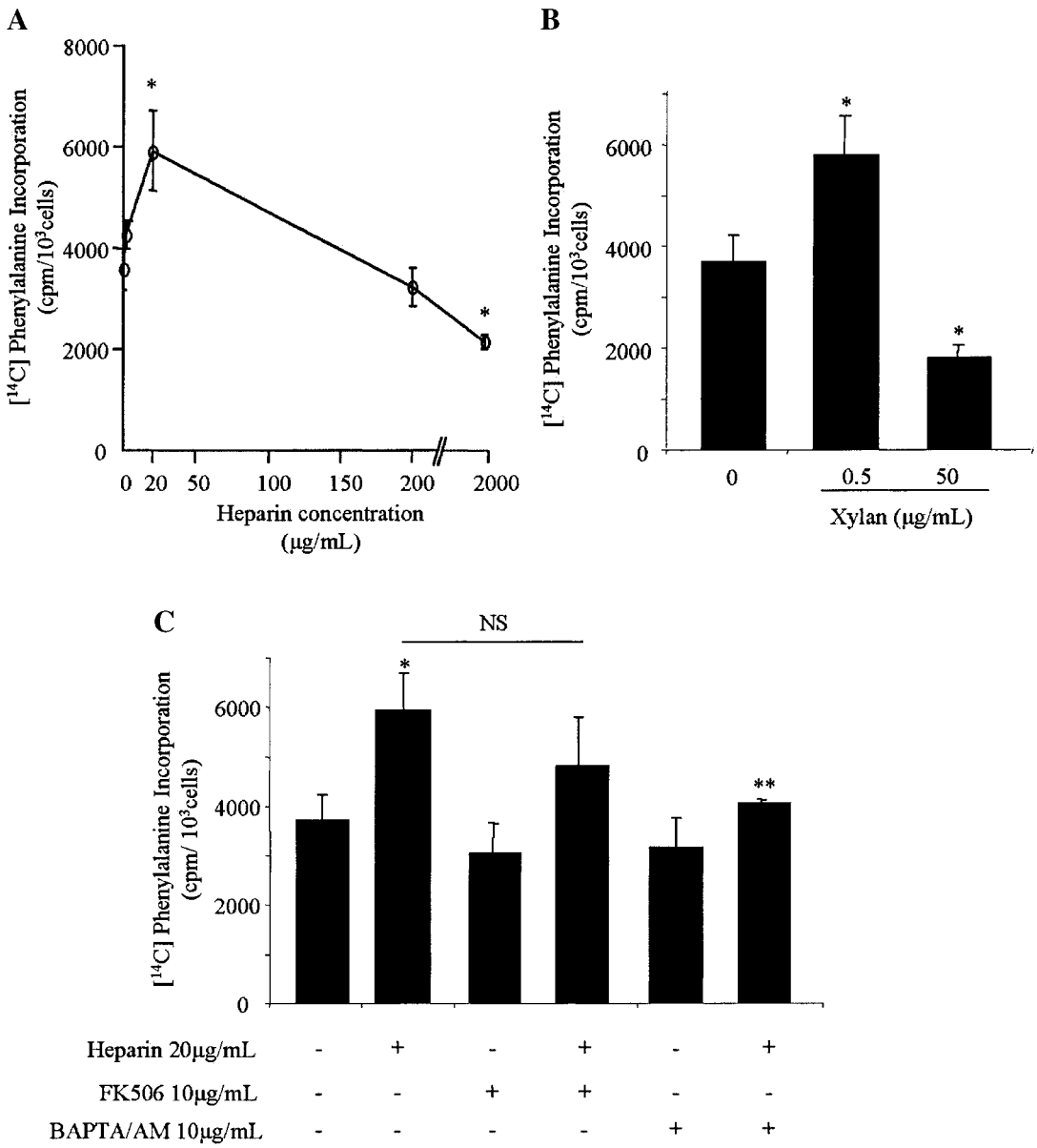

Fig. 2. Effects of heparin and xylan on ARVM protein synthesis in the presence of human serum. **A**: Effect of increasing doses of heparin (2–2,000 μg/ml) on human serum-stimulated ARVM protein synthesis. **B**: Effect of low and high doses of xylan (0.5 and 50 μg/ml) on human serum-stimulated ARVM protein synthesis. **C**: Respective effects of FK506 and BAPTA/AM calcineurin on protein synthesis of ARVM stimulated with both human serum and low doses of heparin. Values are mean ± SEM of five to seven independent experiments, each done in triplicate. *$P < 0.05$ versus control, **$P < 0.05$ versus low concentration of heparin.

enhanced (+55% vs. human serum, N = 7, $P < 0.05$) and inhibited (−50% vs. serum alone, N = 5, $P < 0.05$) the protein synthesis of ARVM (Fig. 2B).

In presence of human serum, ARVM showed no significant difference in protein synthesis after 48 h of exposure to FK506 (Fig. 2C) or CsA alone (data not shown). Furthermore, FK 506 tend to decrease heparin-induced protein synthesis, albeit values did not reach statistical significance. In contrast, 10 μg/ml BAPTA/AM significantly decreased the trophic effect induced by the association of human serum and heparin (20 μg/ml) (−32%, N = 5, $P < 0.05$) (Fig. 2C).

These data indicate that the synergistic effect of heparin on human serum-induced protein synthesis did not involve the calcineurin pathway, but other Ca^{2+}-mediated mechanism.

Tyrosine Phosphorylation of Cytoskeleton and Cytosolic-Associated Proteins After Heparin Stimulation of ARVM

Both Triton X-100 soluble (cytosolic) and Triton X-100 insoluble (membrane and cytoskeleton) fractions contained several tyrosine-phosphorylated proteins, however, no significant changes between these lanes were noted in response to 20 μg/ml heparin 10 min and 24 h after the onset of stimulation, respectively (Fig. 3).

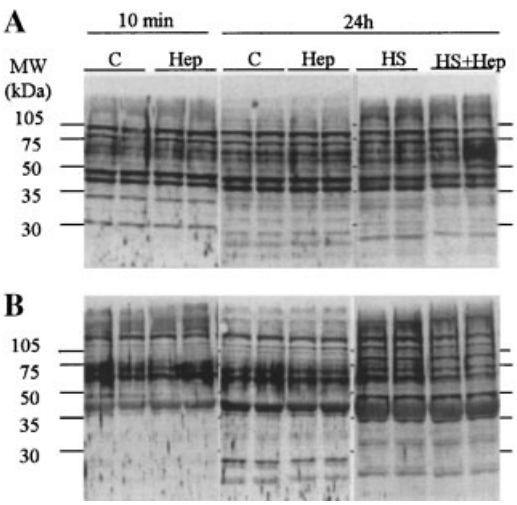

Fig. 3. Heparin did not alter the tyrosine-phosphorylated protein profiles in ARVM. Profiles of tyrosine-phosphorylated proteins present in the Triton X-100-soluble (**A**) and insoluble (**B**) subfractions after 10 min and 24 h of heparin (Hep) (20 μg/ml) stimulation with or without 5% human serum (HS) on ARVM as analyzed by western blot with anti-phosphotyrosine antibody (PY20). Note that heparin did not modify the phosphotyrosine profile whatever the time and the experimental conditions.

Effect of Heparin on NRCM Protein Synthesis

To further investigate the trophic effect of heparin according to the developmental stage, we stimulated NRCM with low and high concentrations of heparin in the presence and absence of human serum (Fig. 4). Human serum strongly increased the [^{14}C]-Phenylalanine relative incorporation in immature myocytes (5-fold, human serum vs. control, N = 3, $P <$ 0.01) (Fig. 4). Heparin alone had no effect on [^{14}C]-Phenylalanine relative incorporation on NRCM (Fig. 4). However, the growth effect of human serum was strongly inhibited by heparin (20 and 2,000 μg/ml) in a dose dependent-manner (−16 or −48%, respectively, N = 3, $P < 0.01$) (Fig. 4).

Effects of Heparinase I and III on Basal Protein Synthesis of ARVM and NRCM

To assess whether intrinsic heparin-like molecules may participate in the control of the cardiomyocytes growth process [Akimoto et al., 1996], we examined the effects of heparinase I (Fig. 5) and heparinase III (data not shown) on both neonatal and adult cardiac myocytes growth. In our culture conditions, heparinase I (10 U/ml) upregulated protein synthesis in NRCM (+20%, N = 5, $P < 0.05$) (Fig. 5A). In ARVM, various concentrations of heparinase I

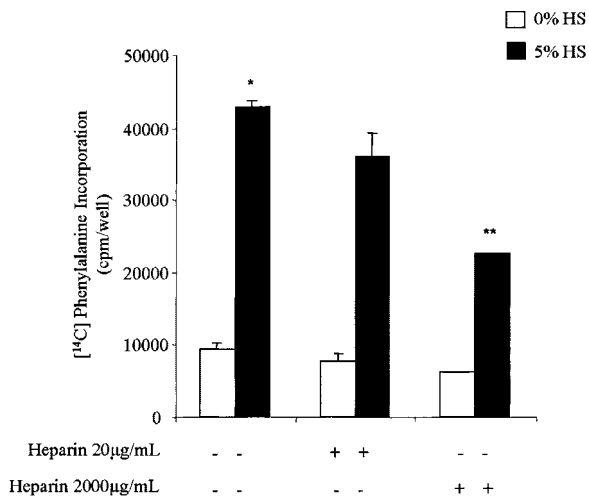

Fig. 4. Effects of low and high concentrations of heparin on neonatal rat cardiac myocyte (NRCM) growth stimulated by 5% human serum. Cells were incubated in the presence (dark column) or absence (white column) of 5% human serum, with heparin (20 and 2,000 μg/ml) for 48 h. Mean ± SEM of three independent experiments, each done in triplicate, $*P < 0.01$ versus control medium, $**P < 0.01$ versus human serum.

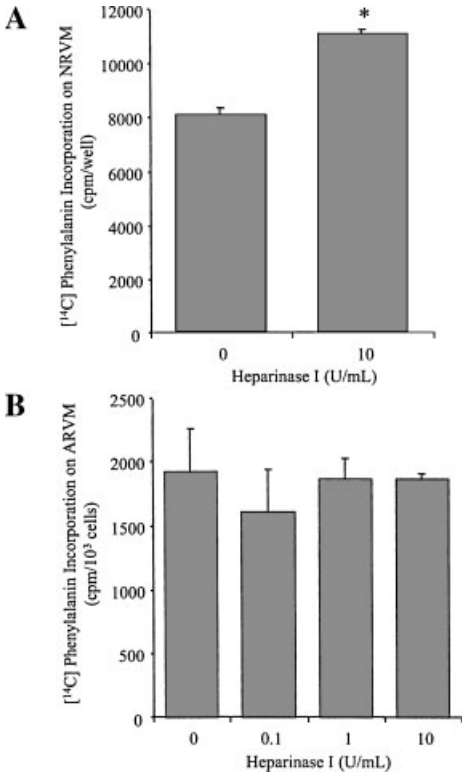

Fig. 5. Effects of heparinase I on protein synthesis of both NRCM and ARVM. **A**: Effects of high concentration of heparinase I (10 U/ml) on protein synthesis of neonatal rat cardiomyocytes. Data were obtained with five independent experiments, each done in triplicate. Results represent mean ± SEM. $*P < 0.05$ versus control medium. **B**: Effects of several concentrations heparinase I (0.1, 1, and 10 U/ml) on the growth process of ARVM. Results represent mean ± SEM with five independent experiments.

(0.1–10 U/ml) or heparinase III (10^{-2}–1 U/ml) (data not shown), in the culture medium for 48 h, had no effect on protein synthesis (Fig. 5B), showing that, in contrast with findings on NRCM, endogenous heparin-like molecules do not seem to participate directly in the regulation of ARVM growth.

DISCUSSION

In the present study, we report that heparin has a dual effect on adult rat cardiomyocyte growth: at low concentrations (20 μg/ml), heparin increases protein synthesis and potentiates the growth effect of human serum in isolated adult rat cardiomyocytes, at high concentrations, it induces reverse effects. We provide also evidence that the heparin growing effect (i) is specific of the adult developmental stage and (ii) involved the calcineurin and/or other Ca^{2+} pathways.

One of the major findings of the present study is the increase in protein synthesis observed in adult cardiomyocytes in response to low doses of heparin and xylan either alone or in combination with human serum. The process involves different Ca^{2+}-mediated signaling pathways. As adult cardiomyocytes are quiescent in vitro, the Ca^{2+}-mediated trophic response observed herein is more likely due to a prolonged increase in intracellular Ca^{2+} rather than the transient high amplitude Ca^{2+} alteration that occurs upon each cycle of contraction-relaxation. In turn, the increase of intracellular Ca^{2+} concentration triggers adult rat cardiomyocyte hypertrophy as evidenced in numerous studies [Molkentin et al., 1998]. Indeed, Molkentin et al. [1998] have demonstrated that calcineurin Ca^{2+}/calmodulin-dependent phosphatase is a major pathway that links Ca^{2+}signaling in the cytosol with changes in gene expression. In line with this finding, our data indicated that the calcineurin pathway plays a pivotal role in the growth effect of low doses of heparin in adult rat cardiomyocytes.

Interestingly, high doses of heparin (1,500 μg/ml) has been used as a potent competitive inhibitor of $InsP_3$-binding to $InsP_3$ receptors ($InsP_3$-R) to block $InsP_3$-gated Ca^{2+}-channels and to reduce the activity of the type 2 $InsP_3$-R in rat cardiomyocytes [Perez et al., 1997]. On the other hand, heparin at low doses induces Ca^{2+} release from the terminal cisterns of skeletal muscle sarcoplasmic reticulum [Ritov et al., 1985], likely via the opening of the Ca^{2+} release channel (ryanodine receptor RYR) [Bezprozvanny et al., 1993]. Taken together the data indicate that heparin, alone and at low doses, activates calcineurin pathway through changes in intracellular Ca^{2+} in adult cardiomyocytes.

Previous in vivo study suggested that Cyclosporine/FK506 effectiveness differed depending on the models of cardiac hypertrophy [Bueno et al., 2002]. In support of this view, we show that FK506 was unable to significantly block heparin-increased protein synthesis when serum was present. However, intracellular Ca^{2+} was still involved in the process since the intracellular Ca^{2+} chelator BAPTA/AM blocked the heparin-induced increase in protein synthesis in these experimental conditions (Fig. 2C). The respective effects of

FK506 and BAPTA/AM when serum is associated with heparin supports the hypothesis that serum reroutes heparin signaling toward a different Ca^{2+} dependent signaling pathway.

In smooth muscle cells, heparin has been shown to selectively block the PKC pathway of mitogenic signaling as well as the phosphorylation and activation of MAPK [Castellot et al., 1989; Hedin et al., 1998; Pintus et al., 1999]. In fact, these effects are mediated through the inhibition of the phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II via the activation of protein phosphatase PP2A, independently of the effect of heparin on Ca^{2+} transients [Mishra-Gorur et al., 2002]. The profiles of both cytosolic and cytoskeleton fractions in response to low doses of heparin, either alone or in the presence of serum showed no changes in the P-tyr profiles due to heparin, suggesting that heparin preferentially targets signaling pathways through calcineurin and other Ca^{2+}- dependent mechanisms rather than acting through the activation of tyrosine-protein kinase to increase protein synthesis in adult rat cardiomyocytes.

Finally, the inhibitory effect of high doses of heparin on serum-induced protein synthesis observed in both adult and neonatal cardiomyocytes is consistent with previous study showing that heparin blocks Angiotensin II-induced increased protein synthesis in neonatal rat cardiomyocytes [Akimoto et al., 1996]. This inhibitory response is likely mediated through a reduced activity of the $InsP_3$-R [Perez et al., 1997]. In addition, in neonatal cardiomyocytes but not in adult cells, exogeneous heparin acts in combination with the endogeneous heparin-like molecules as indicated by the effect of heparinase I in the former cell type (Fig. 5). Thus, the involvement of endogenous heparin-like substances found in neonatal cardiomyocyte growth [Akimoto et al., 1996] is not relevant at the adult stage.

In conclusion, this study demonstrates that the heparin dose-dependently interacts with serum factors to modulate growth in rat cardiomyocytes, the responses differ according to the developmental stage of the cells.

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