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BIOPHYSICS OF COMPLEX SYSTEMS

The Contribution of Protein Kinase C and Rho-Kinase to the Regulation of Receptor-Dependent Contraction of Arteries Decreases with Age Independently of Sympathetic Innervation

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Abstract—The age-related dynamics of the activity of signaling pathways coupled with α_1-adrenoreceptors and their dependence on sympathetic innervation of arterial smooth muscle has been studied. For this purpose, the effects of protein kinase C inhibitor (GF 109203X, 10^{-6} M and Rho-kinase inhibitor (Y27632, 10^{-5} M) on isometric contraction of the rat saphenous artery in response to the α_1-adrenoreceptor agonist methoxamine were determined. The rats in the age of two weeks (with partial sympathetic innervation) had the lower vascular sensitivity to methoxamine than adult rats, but the effects of both inhibitors were more prominent. The denervation induced by excision of sympathetic ganglia in adult rats increased the arterial sensitivity to methoxamine but the sensitivity to inhibitors was unchanged. Thus, the postnatal development of the arterial smooth muscle is accompanied by diminution of the role of protein kinase C and Rho-kinase in the regulation of contraction, but these changes do not correlate with the changes in arterial sensitivity to α_1-adrenergic stimulation and development of sympathetic innervation.

Key words: smooth muscle, methoxamine, GF 109203X, Y27632, postnatal development, denervation
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Contractive characteristics of vascular smooth muscles vary in the course of postnatal development [1], but the mechanisms of these changes have been studied insufficiently. It is known that smooth muscle differentiation is regulated to a great extent due to the trophic impact of sympathetic nerves on the expression of specific proteins and, consequently, the regulatory mechanisms of contraction [2, 3]. Sympathetic innervation of blood vessels in many mammals is formed in the early postnatal period [4], which concurs with smooth muscle maturation. The similar dynamics of the two processes suggests that the age-related changes of vascular contractility may be associated with the development of sympathetic innervation of vessels.

The main mediator of postganglionic sympathetic fibers is noradrenalin; its vasomotor effect on vessels is associated mainly with activation of α_1-adrenoreceptors (α_1-AR) [5]. Long-term trophic effects of noradrenalin are also mediated by α_1-AR [6]. However, mRNA expression, the quantity and affinity of α_1-adrenoreceptors in smooth muscle cells of vessels do not depend on the state of sympathetic innervation [7–9], which means that the trophic effect of sympathetic nerves is realized at post-receptor stages of the regulation of contraction.

The main signal pathways coupled with α_1-adrenoreceptors include activation of three-dimensional GTP-binding proteins: $G_{q/11}$ and $G_{12/13}$. The signaling via $G_{q/11}$ leads to activation of phospholipase C and formation of inositol triphosphate and diacyl glycerol, release of Ca^{2+} from intracellular depots, and activation of protein kinase C (PKC) [5]. Via the $G_{12/13}$ proteins, the signal from α_1-adrenoreceptors is coupled with activation of small G protein RhoA and its target, Rho-kinase (RhoK) [10]. At present, quite a lot of data show that protein kinase C and Rho-kinases are involved in regulation of sensitivity of the contractile apparatus to Ca^{2+} ions and, probably, homeostasis of Ca^{2+} in smooth muscle cells [11-1–4-].

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Body weight, inner diameter, maximal contractile force in response to methoxamine and sensitivity of the saphenous artery to methoxamine in rats from different experimental groups

Group of rats	Body weight, g	Diameter, μm	Maximal contractile force, mnewton	$-\log EC_{50}$ for methoxamine
Two-week (n = 14)	34 ± 2*	262 ± 10*	9.3 ± 0.7*	5.27 ± 0.06*
Adult (n = 10)	338 ± 12	532 ± 13	29.3 ± 2.5	5.66 ± 0.08
Pseudooperated (n = 12)	398 ± 15	518 ± 31	25.9 ± 3.1	5.81 ± 0.04
Denervation (n = 12)	403 ± 13	530 ± 31	28.9 ± 2.6	6.11 ± 0.05#

Note: Diameter values are given at the optimal spread (calculated on the assumption that the studied vascular segment has a rounded section). Since two preparations were obtained from each rat, the size of sampling for body weight is twice lower than for other parameters; * $p < 0.05$ as compared with adults; ** $p < 0.05$ as compared with pseudooperated rats.

Variations in expression or activity in the course of smooth muscle differentiation were shown for some components of the signal pathways coupled with α_1-adrenoreceptors [15-1–9-]. It was also shown that the activity of these components could be different in vessels with different density of innervation [20] or change after chronic denervation [21, 22]. However, the association between the maturation of sympathetic innervation and the change of activity of the signal cascades coupled with α_1-adrenoreceptors in the developing vascular smooth muscle is still unclear.

The goal of this work was to study the contribution of protein kinase C and Rho-kinase to the regulation of contraction of the saphenous artery of rat induced by the agonist of α_1-adrenoreceptors depending on the state of sympathetic innervation. The work was performed using two experimental models. First, the contribution of these protein kinases was studied in the vessels of rats of different age, with different degree of development of sympathetic nervous system. Second, an attempt was made to invert the age-related changes of contraction regulation by way of chronic denervation of this artery in adult rats. We have established that at maturation of vascular smooth muscle its sensitivity to the agonist of α_1-adrenoreceptors (methoxamine) increases. At the same time, the activities of protein kinase C and Rho-kinase decrease, but these changes are independent of the development of sympathetic innervation.

MATERIALS AND METHODS

Four groups of Wistar rats were used: in the age of 12–15 days ("two–week"), 2–3 months ("adult"), adult rats with denervated vessels and the control (pseudooperated). The body weight of animals is given in the Table.

The saphenous artery (a. saphena), an artery of muscular type, is located in a shin and carries blood to a foot. In adult rats, this artery is densely innervated by sympathetic fibers [4, 23]. For denervation of the saphenous artery, sympathetic ganglia (L1–L6) were removed in the rats anaesthetized with Nembutal (40 mg/kg, intraperitoneally) three weeks before the experiment. Such operation was shown to result in practically complete and irreversible denervation of the saphenous artery (the data not presented). On the day of the experiment, the rats were decapitated by guillotine and the saphenous artery was isolated.

Staining of adrenergic nervous fibers. A segment of the artery (10–12 mm in length) was put into 0.1 M phosphate buffered saline solution (pH 7.2) with the addition of glyoxylic acid (2%), sucrose (10%), and pontamine sky blue (0.03%). After 30–min incubation, the preparation was spread on a slide plate, dried (30 min in warm air stream, 5 min at 100°C), embedded in mineral oil, and covered with a cover glass. A LUMAM P3 microscope (LOMO, USSR; objective ×20) was used. The light excitation wavelength was 380–440 nm; the studied luminescence excitation wavelength was 480–700 nm.

Experiments on isolated vessels. Circular artery segments (2 mm long) were fixed in a two-channel myograph for registration of contraction in the isometric mode (Danish Myo Technology A/S, Denmark). Two adjacent segments of the same artery were used for adult rats and segments of the arteries from the two rear extremities were used for two–week rats. The spread of the preparation optimal for displaying the contractile activity was determined after the myograph heating to 37°C [25]. The used solution contained (mM): NaCl, 120; $NaHCO_3$, 26; KCl, 4.5; $CaCl_2$, 1.6; $MgSO_4$, 1.0; NaH_2PO_4, 1.2; D-glucose, 5.5; EDTA, 0.025; HEPES, 5.0. It was continuously aerated by carbogene (5% CO_2 + 95% O_2) to maintain pH 7.4. The preparations were activated by twofold addition of noradrenalin (10^{-5} M; duration of each contraction, 5 min; interval, 10 min).

The contraction of the preparations in response to methoxamine and its variations under the action of inhibitors of Rho-kinase (Y27632, 10^{-5} M) and protein kinase C (GF109203X, 10^{-6} M) was studied.

Methoxamine, in contrast to noradrenalin, is not subject to recapture in the nerve terminals. Hence, it is possible to adequately compare the reactions of vessels with different innervation density. The reactions to methoxamine were studied cumulatively; the action of

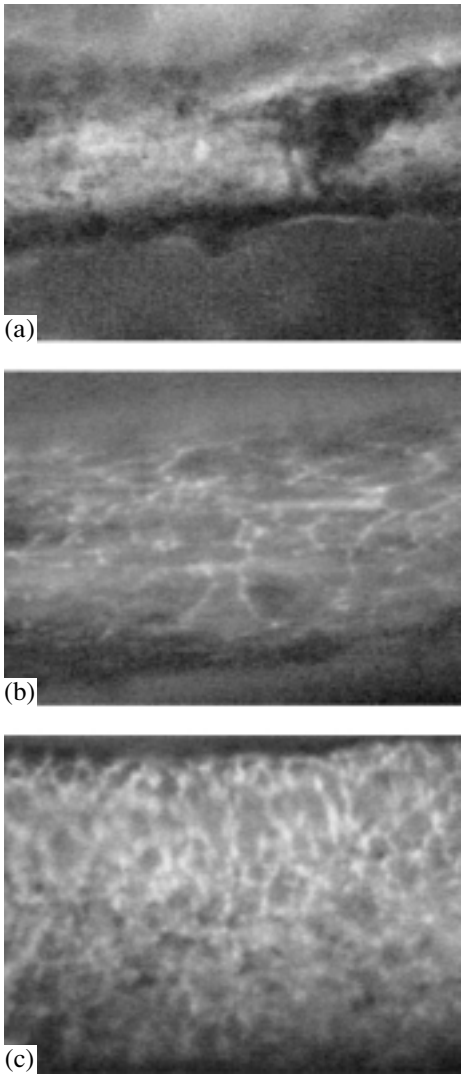

Fig. 1. Formation of the plexus of adrenergic nervous fibers in the saphenous artery of rat. The typical images of total plane artery preparations are presented, which illustrate the picture of vascular innervation in rats in the age of 5 days (a), 12 days (b), and 28 days (c) (the study was performed for three–five animals of each group). Nervous fibers (Figs. b and c) are light-colored. The image size is 170 × 120 μm.

each concentration lasted 3–5 min till stabilization of the contractile force. With methoxamine or other agonists of α_1-adrenoreceptors, the experiments can be performed in the preparations with intact endothelium, because α_1-adrenoreceptors are located only in smooth muscle cells and are absent in endothelium [5].

After registration of the first "concentration–effect" dependence, Y27632 was added to the preparation in one of the myograph channels and GF109203X was added to the other preparation. After a 15–min incubation, the reactions to methoxamine were tested repeatedly. All pharmacological preparations were produced by Sigma (USA).

Data registration and processing. The contractile force was registered by PC using an ADC (digitization frequency 0.5 Hz) and Myodag 2.02 software (Danish Myo Technology A/S, Denmark).

The contractile force values at various methoxamine concentrations were approximated by the equation: $E_A = E_{max}A^p(A^p + EC_{50}^p)$, where E was the value of reaction at the antagonist concentration equal to A; E_{max} was the maximal reaction; P was the Hill coefficient; EC_{50} was the agonist concentration inducing 50% of the maximal response. The sensitivity of vessels to methoxaime was estimated by $-\log EC_{50}$. Calculations were performed in the GraphPad Prism software (version 4.00).

At assessment of the effect of inhibitors on each preparation, all E_A values, both initial and in the presence of inhibitors, were normalized by the value of maximal reaction registered in the absence of inhibitors.

The results were statistically processed using the Mann–Whitney and Willcockson criteria. The data are presented as the mean ± standard deviation.

RESULTS

The plexus of postganglionic sympathetic fibers in the rat saphenous artery wall is formed in the first month of postnatal development (Fig. 1). During the first week, vascular innervation is absent (Fig. 1a) or represented by single nervous fibers only. In the age of two weeks, the nervous plexus already emerges but its density is much less than in adult animals (Fig. 1b). Innervation density reaches the level of an adult animal only in four weeks after birth (Fig. 1c) and then is unchanged (the data not shown). The similar dynamics of development of the rat saphenous artery innervation has been described previously [4].

The inner diameter and maximal contractile force of preparations in two-week rats is less than in rats of other three groups (Table). The sensitivity to methoxamine in the age of two weeks is lower as well. On the contrary, after denervation, the sensitivity of the artery to methoxamine is higher as compared with the vessels of pseudooperated rats (Table). The diameter and maximal contractile force do not vary after denervation.

Rho-kinase inhibition under the action of Y 27632 results in significant suppression of the artery contraction in rats from all groups, but the degree of inhibition is different. In the two-week rats, the contractile response to methoxamine in the concentration of 10^{-4} M decreases much more (to 10% of the initial level) than in adult rats (only to 60%) (Fig. 2a). At the same time, the effects of Y27632 on denervated and control preparations do not differ (Fig. 2c). Originally, the "concentration–effect" dependence for denervated preparations is located more to the left than for the control, which corresponds to the higher sensitivity to methoxamine. Under the

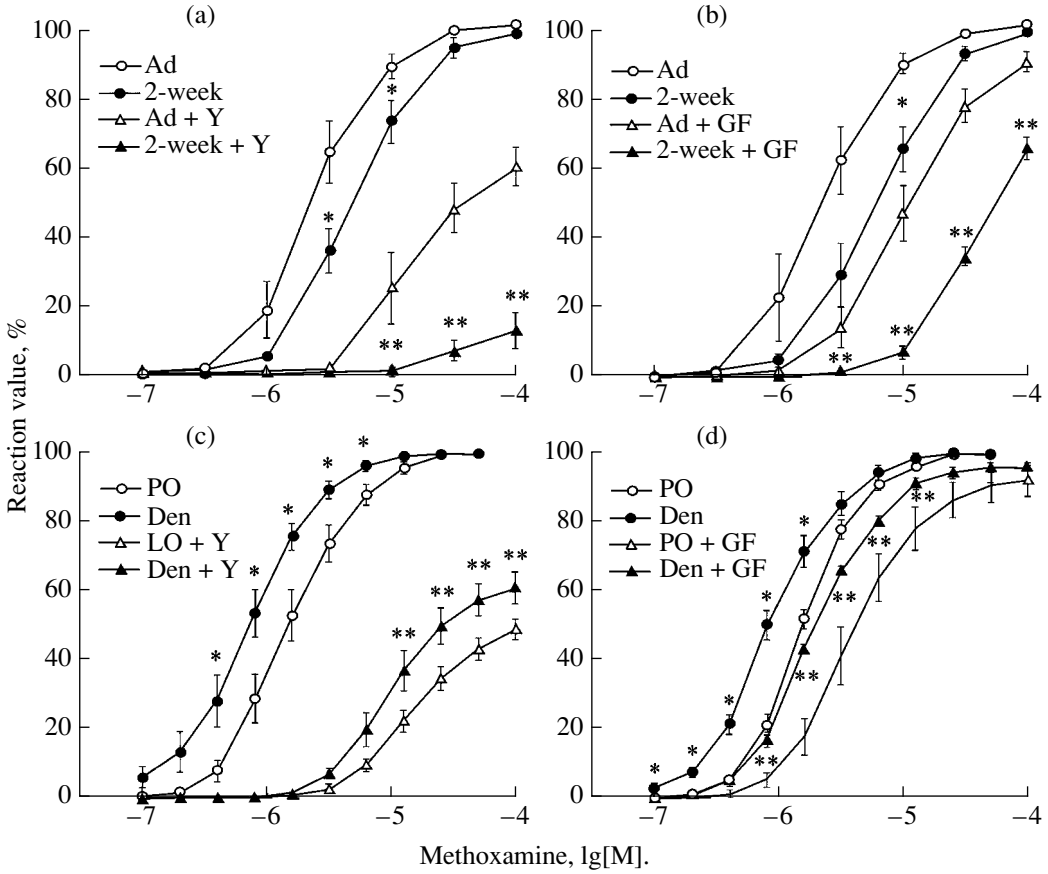

Fig. 2. The effect of inhibitors of Rho-kinase (Y27632, 10^{-5} M) (a, c) and protein kinase C (GF109203X, 10^{-6} M) (b, d) on contraction of the saphenous artery preparations in response to methoxamine in two-week rats (2-week, $n = 7$) as compared with adults (Ad., $n = 5$) (a, b) or at denervation (Den., $n = 6$) as compared with the pseudooperated control (PO, $n = 6$) (c, d). The contractile force values are given as a percentage of the maximal reaction to methoxamine in the absence of inhibitors. * $p < 0.05$ as compared with adult or pseudooperated rats in the absence of inhibitors; # $p < 0.05$ as compared with adult or pseudooperated rats in the presence of respective inhibitor.

effect of Y27632, both dependences shift to the region of higher agonist concentrations but their mutual position is unchanged.

Inhibition of protein kinase C by GF 109203X does not influence the contraction so significantly as in the case of Y27632. In adult rats, both in the control and after denervation, GF 109203X almost does not change the maximal response to methoxamine (Fig. 2b, d). However, the comparison of GF109203X effects on the vessels of rats from different groups reveals the same regularities as at the effect of Y27632. The contractile force of blood vessels in two-week rats decreases to a greater extent than in adult rats (Fig. 2b) and just as in the control after denervation (Fig. 2d).

DISCUSSION

The results of the present work demonstrate that the sympathetic innervation of the saphenous artery in two-week rats is not completely formed. The artery preparations of such animals develop the lower contractile force and are less sensitive to the agonist of α_1-adrenoreceptors but characterized by the higher activity of signal pathways involving Rho-kinase and protein kinase C, as compared with the artery preparations of adult rats. Sympathetic denervation of the saphenous artery of adult rats results in the increase of smooth muscle sensitivity to the agonist of α_1-adrenoreceptors; however, it is not associated with the enhancement of the regulatory role of Rho-kinase and protein kinase C.

We believe that the decrease of vascular sensitivity to methoxamine in two-week rats is not associated with the change of α_1-adrenoreceptors themselves. Indeed, the high level of expression of α_1-adrenoreceptors in blood vessels of rats is revealed already in the first days after birth [7]. Consequently, immaturity of the mechanisms of smooth muscle contraction at activation of α_1-adrenoreceptors may show itself at the stage of enhancement of intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) and at interaction of Ca^{2+} with the contractile apparatus.

The basic mechanism of $[Ca^{2+}]_i$ increase in blood vessels of muscular type is the entrance of Ca^{2+} into cells through potential-dependent potassium channels of L-type. It has been shown that these channels mark the differential state of smooth muscle cells [26]. In this context, it is not surprising that the activity of L-type channels in smooth muscle cells of blood vessels in the newborn rats is very low [27]. Besides, immature smooth muscle is characterized by the low expression and activity of kinase of the light myosin chains (LMC) [16, 18, 19], which is the main activator of Ca^{2+}-dependent contraction of smooth muscle cells [11, 12].

The above data suggest that the low sensitivity of blood vessels of two-week rats may be associated with the low activity of the mechanisms of Ca^{2+}-dependent regulation of contraction. Our results indicate that the insufficiency of Ca^{2+}-dependent regulation in the developing smooth muscle may be partially compensated by activation of the mechanisms providing the increase of the contractile apparatus sensitivity to Ca^{2+}. The higher sensitivity of contraction to Ca^{2+} is actually typical of the developing smooth muscle [17, 18], and the arteries of muscular type are characterized just by the increase of Ca^{2+}-dependent sensitivity but not basal Ca^{2+}-sensitivity [18].

The main mechanisms of Ca^{2+} sensitization of smooth muscle contraction are direct inhibition of LMC phosphatase activity under the action of Rho-kinase and activation of the endogenous inhibitor of phosphatase of light myosin chains (CPI-1–7-) under the effect of protein kinase C [11, 12]. In our experiments, inhibition of Rho-kinase and protein kinase C resulted in a more significant suppression of contractile force in two-week rats than in adult ones. The similar data on the effect of Rho-kinase [16, 17] or protein kinase C [15] inhibitors on immature smooth muscle were obtained by other authors.

The striking manifestation of the effects of activation of protein kinase C and Rho-kinase in immature smooth muscle tissue may be associated with the change of expression of components of these signal cascades and/or their final target: the phosphatase of light myosin chains [17–19]. So, the increase of activity of protein kinase C itself [15] and expression of CPI-1–7- [28] have been described for the cascade activated by protein kinase C.

It is interesting that the rats of all groups showed a greater decrease of vascular contraction at inhibition of Rho-kinase as compared with the inhibition of protein kinase C. Probably, it is due to the low content of CPI-1–7-, the expression of which in the arteries of muscular type is many times less than in elastic arteries [20]. Besides, the activation of protein kinase C may result in Ca^{2+} sensitization of contraction also without the involvement of CPI-1–7-, namely by activation of mitogen-activated protein kinases (MAPK), the main targets of which are the kinase of light myosin chains and caldesmon [11, 12]. Such action of protein kinase C in immature smooth muscle seems possible, because the expression of mitogen-activated protein kinases in the latter is much higher [29] but requires experimental verification.

We believe that the enhanced activity of signal systems including Rho-kinase and protein kinase C is an important mechanism of regulation of the contraction of smooth muscle until it reaches the differentiated state. Taking into account the data on inconsistency of the mechanisms providing the increase of $[Ca^{2+}]_i$ in smooth muscle cells [27] and the low activity of the kinase of light myosin chains [16, 18, 19], we believe that the procontractive effect of these signal systems is directed mainly to the increase of the contractile apparatus sensitivity to Ca^{2+}.

Postnatal maturation of the saphoneous artery smooth muscle is accompanied by decrease of the contributions of Rho-kinase and protein kinase C to the regulation of contraction, which is concurrent with the period of development of sympathetic innervation (see Fig. 1 and the work [4]). While the age-related reduction of the activities of these kinases is regulated by sympathetic nerves, denervation of the artery must result in reversible changes, i.e. increase of the activity of these signal cascades. Therefore, we have studied their role in contractive responses of the artery before and after denervation.

Denervation was accompanied by the increase of sensitivity of the saphenous artery to methoxamine. Hypersensitivity of vascular smooth muscle at denervation has been described previously [2, 23, 30]. It is displayed under the effect of quite different vasoconstrictors and is not connected with the change of the receptor apparatus of smooth muscle cells [8, 9]. Persistent depolarization of the outer membrane observed at denervation [30] facilitates the activation of L-type potassium channels and, probably, the increase of the contractile apparatus sensitivity to Ca^{2+} [31] or increase of the activity of protein kinase C [21].

However, our results suggest that the contribution of protein kinase C and Rho-kinase to the regulation of contraction of vascular smooth muscle of is unchanged after denervation. It is an evidence that the age-related decrease of the activity of these pathways is independent of the trophic effect of sympathetic innervation and conforms to the fact that the higher Rho-kinase activity is typical of the immature smooth muscle and the organs, for which sympathetic regulation is not basic [16, 17]. Apparently, the increase of contraction sensitivity to Ca^{2+} after denervation [31] is associated with the changed activity of other signal pathways.

As a whole, the results of this work lead to a conclusion that the postnatal smooth muscle maturation is accompanied by decrease of the regulatory role of the two major signal cascades coupled with the activation of α_1-adrenoreceptors, and these changes are independent of the trophic effect of sympathetic nerves. How-

ever, for understanding of the mechanisms of these changes it is necessary to experimentally determine the Ca^{2+} sensitivity of the vascular contractile apparatus and its variations at inhibition of the main signal cascades and then to compare the results of functional testing with the results of measurement of the expression and activity levels of the main participants of these cascades. Taking into account the importance of the considered problem both for fundamental science and practice (for correction of possible disturbances of vascular activity in early neonatal age), the relevance of further studies is obvious.

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SPELL: 1. denervation, 2. denervated, 3. anaesthetized, 4. digitization