Molecular Mechanisms of Vasoconstrictor Action of Imidazo[1,2-α]benzimidazole Derivative RU-1117 Possessing Local Anesthetic Properties

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We studied the mechanisms of action of imidazobenzimidazole derivative RU-1117 on calcium homeostasis in myocytes isolated from rat thoracic aorta. In therapeutic concentrations, RU-1117 increased the content of free calcium ions due to their mobilization from intracellular depot via the IP_3-dependent mechanisms. Antagonists of angiotensin II AT_1-receptors irbesartan and, to greater extent, eprosartan abolished the calcium-mobilizing action of RU-1117. Selective antagonist of endothelin ET_A-receptors sitaxsentan and α1-adrenoceptor agonist prazosin produced no effect on calcium mobilization caused by novel local anesthetic RU-1117.

Key Words: *myocytes; calcium; ET_A-endothelial receptors; α1-adrenoceptors; AT_1-angiotensin receptors*

Many routine and novel local anesthetics (LA) does not exert anesthetizing effect of sufficient strength and duration and can induce side effects. The search for agents possessing unique pharmacokinetic and pharmacodynamic properties is a way for creation of efficient and safe LA. It was recently found that RU-1117, a representative of imidazo[1,2-α]benzimidazole family synthesized in Research Institute of Physical and Organic Chemistry, Rostov State University possesses not only anesthetizing potency, but also vasoconstrictor activity after local application [2]. The increase in the vascular tone caused by application of local anesthetic with an adrenoceptor agonist significantly prolongs the anesthetizing effect and improves safety by reducing the risk of systemic toxic effects on electrically excitable cells of the myocardium and nervous system [10]. Thus, vasoconstrictor activity of RU-1117 is its important pharmacodynamic advantage obviating the need of adding vasoconstrictor agents, *e.g.* epinephrine, to LA solutions.

The molecular targets and the mechanisms underlying the vasoconstrictor effect of RU-1117 are little known. Our aim was to study the effects of RU-1117 on intracellular Ca^{2+} and to identify possible targets of its action in smooth muscle cells of rat thoracic aorta.

MATERIALS AND METHODS

Suspension of smooth muscle cells from rat thoracic aorta was isolated as described elsewhere [8] in our modification. The adventitia and the adjacent tissues were separated under a binocular microscope. The endothelium was removed with a gauze tampon. The muscle layer was cut longitudinally, slightly stretched, fixed on a plastic plate, and placed in a Ca-free HEPES buffer containing (in mM): 4 KCl, 2 $MgCl_2$, 1 K_2HPO_4, 140 NaCl, 10 HEPES, 10 glucose, pH 7.4. After 1-h incubation at 37°C

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the specimen was transferred into Ca-free HEPES buffer containing 1 mg/ml collagenase, 2 mg/ml BSA, and 0.5 mg/ml trypsin inhibitor. After 1 h the muscle tissue became loose, it was cut into small pieces with a blade and teased. The homogenate was resuspended with a polyethylene pipette for 15 min and filtered through a nylon mesh. The dispersed cells were precipitated by centrifugation at 30*g* for 5 min. The precipitate was washed two times and finally resuspended in 10 ml HEPES buffer to a concentration 10^6/ml. The myocytes were counted in a Goryaev chamber; their viability was assessed by trypan blue staining. The myocytes were loaded with fluorescent probe FURA-2/AM and the cytoplasmic concentration of calcium ions was calculated [1].

The data were processed using Pharmacological Basic Statistics software. The confidence intervals for experimental values and significance of differences were assessed by Student's *t* test at $p<0.05$.

RESULTS

In resting smooth muscle cells isolated form rat thoracic aorta, the cytoplasmic concentration of free calcium ions $[Ca^{2+}]_{cyt}$ was 116±8 nM (n=4). Addition of RU-1117 to the cell suspension led to a dose-dependent elevation of $[Ca^{2+}]_{cyt}$ (Fig. 1).

The study of the dynamics of RU-1117-induced elevation of $[Ca^{2+}]_{cyt}$ showed that calcium level rose as early as during the first minute of incubation and peaked during incubation minutes 2-3 (Fig. 2). During the following 15 minutes, $[Ca^{2+}]_{cyt}$ gradually decreased, but remained above the initial value by 60%.

RU-1117-induced elevation of $[Ca^{2+}]_{cyt}$ did not result from calcium entry via sarcolemmal Ca^{2+}-channels (Fig. 3). Indeed, when the myocytes were transferred into Ca-free medium (containing 2 mM EDTA), the calcium response to RU-1117 applied within the examined concentration range only slightly decreased by 8% (p=0.12). Moreover, selective blockers of L-type Ca^{2+}-channels cadmium ions (0.2 mM $CdCl_2$) or nickel ions (1 mM $NiCl_2$) also had no effect on $[Ca^{2+}]_{cyt}$ elevation induced by RU-1117 (Fig. 2).

Low cytoplasmic calcium concentration in myocytes is maintained by the following cell systems: calcium ATPases of the sarcoplasmic reticulum (SPR), Ca^{2+}-ATPase of the cytoplasmic membrane, and Na^+/Ca^{2+} antiport system. For evaluation of the role of SPR in the regulation of $[Ca^{2+}]_{cyt}$ under the action of RU-1117 we used ryanodine (30 μM), an activator of IP_3-dependent mobilization of Ca^{2+} ions from intracellular depot. Ryanodine dramatically changed the kinetics of Ca^{2+}-response: the increase in $[Ca^{2+}]_{cyt}$ after application of RU-1117 in the maximum concentration did not exceed 4% (p=0.44). At the same time, inhibitor of Na^+/Ca^{2+} transport amiloride (40 μM) did not modify pharmacodynamics of RU-1117 effect. Blockers of the phosphoinositide metabolism LiCl (10 mM) abolished the effect of RU-1117 on $[Ca^{2+}]_{cyt}$, which also attested to IP_3-dependent mechanism of Ca^{2+} rise.

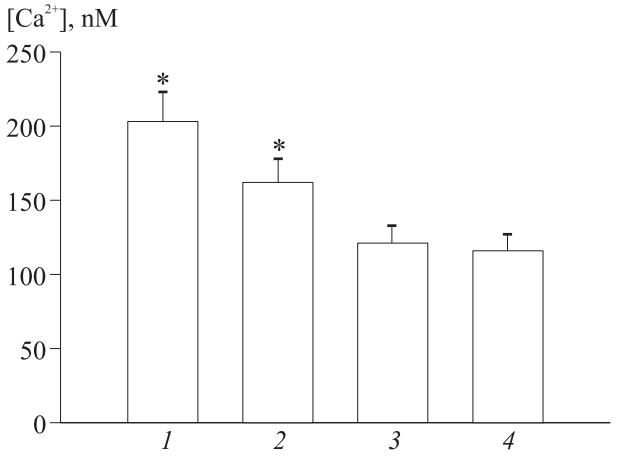

Fig. 1. Dose-dependent effect of RU-1117 on $[Ca^{2+}]_{cyt}$ in myocytes. *1*) 80 μM; *2*) 40 μM; *3*) 20 μM; *4*) 0 μM. Calcium response was recorded 2 min after addition of RU-1117 to the culture medium. *$p<0.05$ compared to the control.

There are several pathways and mechanisms regulating calcium homeostasis in myocyte cytoplasm: potential-dependent passive inward flow of Ca^{2+} ions into the cell, ATP-dependent transport of Ca^{2+} ions from the cell into the extracellular space (sarcolemmal Ca^{2+}-pump), Na^+-dependent transport of Ca^{2+} ions from the cell into extracellular space (Na^+/Ca^{2+} exchange), ATP-dependent compartmentalization of Ca^{2+} ions in SPR (thapsigargin-sensitive Ca^{2+}-pump of SPR), accumulation of Ca^{2+} ions in

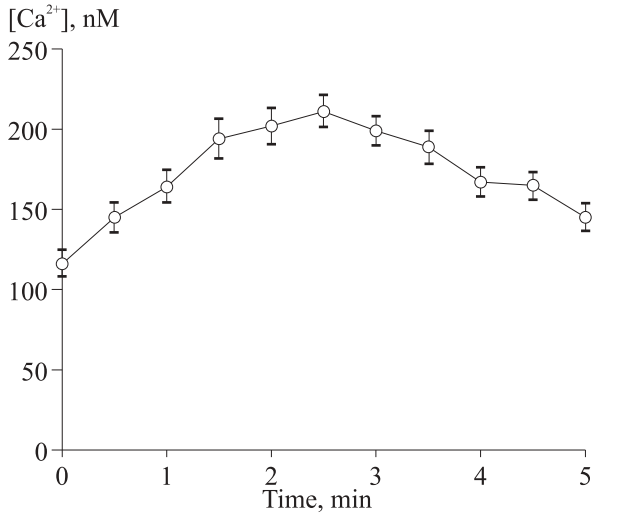

Fig. 2. Time course of the effect of RU-1117 (80 μM) on $[Ca^{2+}]_{cyt}$ in myocytes.

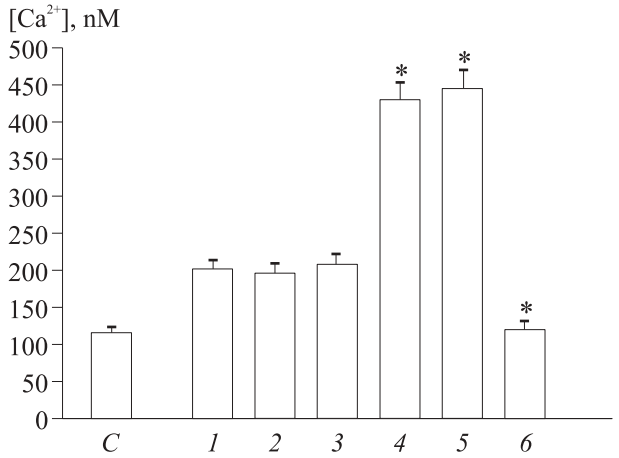

Fig. 3. Calcium response of myocytes to addition of 80 μM RU-1117 under different experimental conditions. C: control. *1*) calcium-free medium; *2*) 1 mM $NiCl_2$; *3*) 0.2 mM $CaCl_2$; *4*) 30 μM ryanodine; *5*) 30 μM ryanodine+RU-1117; *6*) 10 mM LiCl. **p*<0.05 compared to the control.

mitochondria driven by ATP or electrochemical proton potential ($\Delta\mu H^+$). $[Ca^{2+}]_{cyt}$ is directly proportional to the contractile response of myocytes and determines the tone of arterial and venous vessels [5]. Reversible drop in $[Ca^{2+}]_{cyt}$ underlies smooth muscle relaxation.

The vasoconstrictor effect of natural (endothelins, angiotensin II, norepinephrine) or pharmacological (adrenoceptor agonists phenylephrine, metaraminol, methoxamine) agents can be mediated by the following pathways: receptor-mediated or direct activation of Ca^{2+}-channels in the plasmalemma, elevation of $[Ca^{2+}]_{cyt}$ due to calcium mobilization from the intracellular depots (SPR, mitochondria), and inhibition of potassium channels (4-aminopyridines).

For evaluation of the mechanisms of RU-1117 action on calcium homeostasis in myocytes, we carried out pharmacological analysis with selective antagonists of ET_1-subtype endothelin receptors (sitaxsentan), α_1-adrenoceptors (prazosin), and AT_1-receptors for angiotensin II (AII-receptors; noncompetitive antagonist irbesartan and competitive antagonist eprosartan).

Pharmacological efficiency was assessed by IC_{50}, *i.e.* concentration reducing the Ca-response by 50%.

Antagonists of AII-receptors inhibited elevation of $[Ca^{2+}]_{cyt}$ in a dose-dependent manner starting from the concentration of 2.5 nM. IC_{50} values for eprosartan and irbesartan were 14±3 and 58±11 nM, respectively. Adrenoceptor antagonist prazosin exhibited low Ca^{2+}-blocking activity in this experimental model (IC_{50}>10 μM). Antagonist of ET_1-subtype endothelin receptors sitaxsentan in the examined concentration range (1-100 μM) produced no significant changes in the basal and induced $[Ca^{2+}]_{cyt}$.

According to published data, irbesartan and eprosartan bind to AT_1-receptors with high affinity. In rat mesenteric artery, the values of IC_{50} (the ability of antagonists to replace ^{125}I from its complex with AT_1-receptors) were 1.3 and 1.5 nM for irbesartan and eprosartan, respectively [6]. No partial antagonistic activity was found. Eprosartan is a derivative of imidazole-5-acryl acid; it differs from other antagonists of AII-receptors by competitive mechanism of AT_1-receptor blockade [7].

Apart from postsynaptic membrane of myocytes, AT_1-receptors are present in the presynaptic membrane of sympathetic adrenergic nerve terminals, where they participate in activation of norepinephrine release [3,4]. The pre- and postsynaptic AT1-receptors are characterized by different sensitivity to their specific agonists and antagonists [9,11]. Thus, the observed vasoconstrictor effect of RU-1117 can result from its direct action on myocytes and indirect action on the vascular tone via sympathetic neural regulation. The latter mechanism is more probable, because the concentration of AII in the blood is an order of magnitude higher than in the synaptic cleft.

Thus, evaluation of the molecular pharmacology of the novel local anesthetic RU-1117 showed that this drug in therapeutic doses increases intracellular concentration of free calcium ions due to their mobilization from intracellular depots via IP_3-dependent mechanism. The vasoconstrictor effect of RU-1117 can be mediated via presynaptic AT_1-receptors.

REFERENCES

1. V. V. Ponomarev, A. P. Galenko-Yaroshevskii, A. S. Dukhanin, and V. A. Anisimova, *Byull. Eksp. Biol. Med*., **135**, No. 6, 570-572 (2003).
2. V. V. Ponomarev, A. P. Galenko-Yaroshevskii, A. S. Dukhanin, and V. A. Anisimova, *Ibid*., **136**, No. 1, 53-55.
3. A. Dendorfer, P. Dominiak, K. Òåìðål, and W. Raasch, *Hypertens.*, **23**, No. 10, 1861-1867 (2005).
4. M. E. Fabiani, M. Sourial, W. G. Thomas, *et al*., *J. Endocrinol*., **171**, No. 1, 97-108 (2001).
5. M. M. Givertz, W. S. Colucci, T. H. LeJemtel, *et al*., *Circulation*, **101**, No. 25, 2922-2927 (2000).
6. S. Guimaraes, C. Carneiro, F. Brandao, *et al*., *Naunyn Schmiedebergs Arch. Pharmacol*., **370**, No. 4, 262-269 (2004).
7. L. Hunyady, T. Balla, and K. Catt, *Trends Phamacol. Sci*., **17**, No. 4, 135-140 (1996).
8. K. Kariya, Y. Kawahara, H. Fukuzaki, *et al*., *Biochem. Biophys. Res. Com*., **161**, No. 3, 1020-1027 (1989).
9. A. Nap, J. C. Balt, M. P. Mathy, *et al*., *J. Cardiovasc. Pharmacol*., **43**, No. 1, 14-20 (2004).
10. H. P. Rang, M. M. Dale, and J. M. Ritter, *Pharmacology*, L. (1999).
11. R. G. Tilton, C. L. Munsch, S. J. Sherwood, *et al*., *Pulm. Pharmacol. Ther*., **13**, No. 2, 87-97 (2000).