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Effect of Nitroglycerine on Content of Second Messengers in Myocytes of Rat Thoracic Aorta

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We evaluated the concentration dependence and time dependence of the effect of nitroglycerine on intracellular content of cAMP, cGMP, and free Ca^{2+}. It was shown that after norepinephrine stimulation, nitroglycerine exhibited calcium-blocking activity in lower concentrations (starting from 10^{-7} M). Under conditions of experimental nitrate tolerance the dose-dependent effect of nitroglycerine on intracellular cGMP and Ca^{2+} was less pronounced. Calcium-blocking activity of nitroglycerine decreased most significantly upon stimulation of myocytes with norepinephrine.

Key Words: *calcium ions; cAMP; cGMP; nitrates; myocytes*

Recent studies showed that the major biochemical mechanisms of vasodilation induced by organic nitrates include the formation of nitric oxide (NO), stimulation of guanylate cyclase, accumulation of cGMP, and decrease in Ca^{2+} concentration in smooth muscle cells [8,9,10]. The role of other second messengers in pharmacological activity of nitrates is poorly understood.

The effectiveness of nitrates in patients with coronary heart disease decreases due to the development of drug tolerance and other complications of pharmacotherapy. These changes are most pronounced during long-term therapy with nitrates. The molecular mechanisms of this effect remain unknown [4,5,6]. Little is known about the relationship between changes in the concentration of second messengers and development of nitrate tolerance. The study of fundamental mechanisms underlying the action of nitrates and development of new biochemical methods for objective monitoring of drug efficacy will allow us to diagnose the decrease in nitrate efficacy and perform pharmacological correction of drug dosage.

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Here we compared the effects of nitroglycerine (NTG) on the concentration of Ca^{2+} and cyclic nucleotides in smooth muscle cells under normal conditions and during experimental nitrate tolerance.

MATERIALS AND METHODS

The suspension of smooth muscle cells from rabbit thoracic aorta was isolated as described elsewhere [7]. The adventitia and surrounding tissues were carefully isolated under a binocular microscope. The endothelium was removed with a gauze tampon. The muscle layer was cut longitudinally, stretched slightly, fixed on a plastic plate, and put in calcium-free HEPES buffer containing 4 mM KCl, 2 mM $MgCl_2$, 1 mM K_2HPO_4, 140 mM NaCl, 10 mM HEPES, and 10 mM glucose (pH 7.4). The tissue was incubated at 37°C for 1 h and placed in calcium-free HEPES buffer containing 1 mg/ml collagenase, 2 mg/ml bovine serum albumin, and 0.5 mg/ml trypsin inhibitor. Muscle tissue loosened after 1-h enzyme treatment was cut with a razor into small fragments and then crisped. The homogenate was resuspended using a polyethylene pipette (15 min) and filtered through a Nylon grid. Dispersed cells

were precipitated by centrifugation at 30*g* for 5 min. The pellet was washed and resuspended in 10 ml HEPES buffer (cell concentration 10^6/ml). Myocytes were counted in a Goryaev chamber. Cell viability was estimated in trypan blue test.

Loading of cardiomyocytes with FURA-2 fluorescent probe and measurement of Ca^{2+} concentration in the cell cytoplasm ($[Ca^{2+}]_i$) were performed as described previously [1]. The intracellular concentration of cyclic nucleotides cAMP and cGMP was measured by the radioligand method using Amersham kits. The cells were washed and placed in balanced buffer solution with test substances. The final volume of samples was 1 ml cell suspension. They were incubated at 37°C for 10 min. Radioactivity of samples was measured on an Intertechnique liquid scintillation radiometer.

The results were analyzed using Pharmacological Basic Statistics software. The confidence intervals and significance of differences were evaluated by Student's *t* test (significance level 0.05).

RESULTS

We studied the effect of NTG on the concentration of major intracellular transmitters in the suspension of rat aortic myocytes. The vascular smooth muscle cell serves as a target for chemically different nitrogen-containing drugs and other cardiotropic compounds. We evaluated the effect of nitrates on the intracellular concentration of cyclic nucleotides (cAMP and cGMP) and free Ca^{2+}. Inositol triphosphate concentration was not measured due to a small volume of cell material.

The effect of nitrates on intracellular Ca^{2+} concentration was studied after stimulation of cells with 10^{-5} M norepinephrine (NE). Otherwise, the cells were maintained in a high-K^+ buffer with 118 mM KCl. This buffer was obtained by substitution of NaCl for KCl in an equimolar concentration.

The intracellular concentration of free Ca^{2+} in resting smooth muscle cells from rat aorta was 116±12 nM. Addition of NTG in increasing concentrations (final concentration 10^{-4} M) to the cell suspension had no effect on basal $[Ca^{2+}]_i$ (at least over 15 min of incubation).

Basal levels of cAMP and cGMP in myocytes were 0.5±0.1 and 0.65±0.20 pmol/10^6 cells, respectively.

Addition of NTG in increasing concentrations to the cell suspension was followed by a dose-dependent increase in cGMP concentration in the cytosol of smooth muscle cells from rat aorta (Table 1). However, cAMP concentration remained practically unchanged after treatment with NTG in these concentrations. The exception was treatment with NTG in a concentration of 10^{-4} M. It should be emphasized that this concentration of NTG is 2-3 orders of magnitude higher than its therapeutic dose *in vivo*.

TABLE 1. Effect of NTG on the Basal and NE-Induced (10^{-5} M) or KCl-Induced (118 mM) Intracellular Concentration of Ca^{2+}, cAMP, and cGMP in Rat Aortic Myocytes (*M*±*m*)

Concentration of substance, group	NTG, log M					Significance of between-concentration differences
	-8	-7	-6	-5	-4	
$[Ca^{2+}]_i$, nM						
group 1 (without stimulation)	118±11	121±9	111±18	117±13	128±32	
group 2 (NE)	212±26^{*x}	196±17*	152±23*	134±29*	135±21	<0.05
group 3 (KCl)	236±34^{+xx}	301±19^{+}	212±22^{+}	198±16	160±28	<0.01
$[cAMP]_i$, pmol/10^6 cells						
group 1 (without stimulation)	1.2±0.1^{x}	2.9±0.2	3.4±0.1	4.9±0.4	6.7±0.4	<0.05
group 2 (NE)	1.5±0.2^{x}	2.7±0.1	2.9±0.2	5.2±0.2	6.9±0.3	<0.05
group 3 (KCl)	2.1±0.05^{+x}	2.0±0.3^{+}	3.1±0.1	6.1±0.4^{+}	5.9±0.3^{+}	<0.05
$[cGMP]_i$, pmol/10^6 cells						
group 1 (without stimulation)	0.7±0.2^{x}	0.8±0.1	0.75±0.25	1.1±0.4	2.6±0.35	<0.05
group 2 (NE)	1.2±0.25	1.4±0.4	1.1±0.25	1.4±0.3	1.8±0.3	
group 3 (KCl)	1.1±0.3	1.2±0.2	1.3±0.5	1.3±0.2	2.4±0.6	

Note. Incubation for 3 min. $p<0.05$: *compared to group 1; +compared to group 2. $^{x}p<0.05$ and $^{xx}p<0.01$ compared to a NTG concentration of log 10^{-8}.

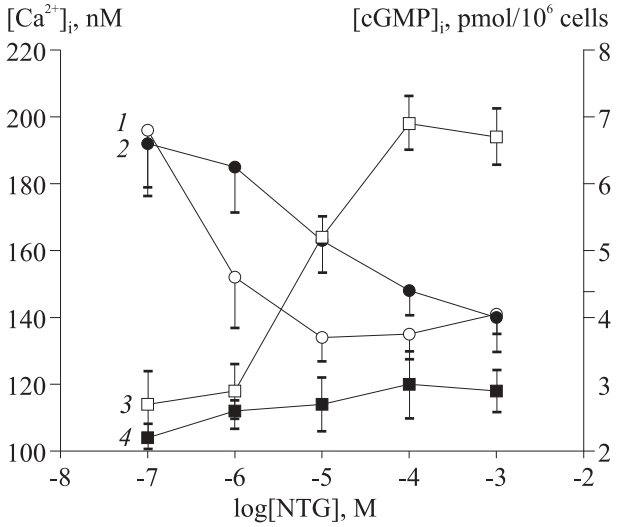

Fig. 1. Effect of nitroglycerine (NTG) on the increase in intracellular concentrations of Ca^{2+} and cGMP induced by norepinephrine (10^{-5} M) before and after 30-min preincubation of myocytes with NTG (10^{-4} M). Basal concentration of Ca^{2+} (*1*); Ca^{2+} concentration after preincubation in the buffer with high content of NTG (*2*); basal concentration of cGMP (*3*); cGMP concentration after preincubation in the buffer with high content of NTG (*4*).

We studied the increase in cGMP concentration in smooth muscle cells under the influence of NTG. cGMP concentration increased over the first minute of incubation and reached maximum after 2-3 min (Table 2). Intracellular cGMP concentration progressively decreased over the next 15 min, but 2-fold exceeded the basal level. The dynamics of variations in cGMP concentration differed during stimulation of cells with NE (10^{-5} M). cGMP concentration increased slowly, reached maximum by the 10th minute, and remained unchanged over 15 min of incubation (Table 2). The increase in cGMP concentration under these conditions is a secondary event, which reflects activation of feedback cellular effector mechanisms in response to agonist treatment. Our results are consistent with published data on cultured rat lungs fibroblasts and bovine coronary myocytes [2,3].

For evaluation of the molecular mechanisms underlying the action of nitrates, we studied the effect of NTG on variations in cGMP and Ca^{2+} concentrations induced by KCl and NE.

In series I, smooth muscle cells were placed in a medium with 118 mM KCl. NaCl was substituted for KCl in an equimolar concentration. In series II, the cells were activated with NE in a concentration of 10^{-5} M. Incubation of cells in the medium with 118 mM KCl was followed by a rapid increase in fluorescence of Fura-2/AM (over 1 min). These changes reflect the increase in $[Ca^{2+}]_i$ in myocytes. This parameter remained unchanged over 10 min. Ca^{2+} concentration increased to 236±34 nM. NE had an immediate effect and 2-fold increased $[Ca^{2+}]_i$ compared to the basal level.

Under conditions of high intracellular Ca^{2+} concentration, the minimum effective dose of NTG was 10^{-7} M. NTG prevented the increase in $[Ca^{2+}]_i$ induced by NE treatment or maintenance of cells in a high-K^+ medium (Table 1). The dose-dependence of calcium-blocking activity was most pronounced for NTG in concentrations of 10^{-7}-10^{-4} M. Further increase in the concentration of NTG above 10^{-4} M was not accompanied by significant changes in $[Ca^{2+}]_i$.

Biochemical mechanism underlying the action of nitrates on smooth muscle cells includes biotransformation of nitrates with the formation of NO or S-nitrosothiols and activation of cytosolic (soluble) guanylate cyclase with NO or S-nitrosothiols. These changes result in an increase in the intracellular concentration of another second messenger (cGMP) and decrease in $[Ca^{2+}]_i$ in the cytoplasm of smooth muscle cells, which leads to vasodilation.

The increase in $[Ca^{2+}]_i$ under the influence of NE and KCl was followed by a rise in cGMP concentration (Table 2). Addition of NTG in increasing concentrations to cell suspension was accompanied by further increase in cGMP level. Under these conditions cGMP concentration exceeded not only the basal (by 15 times), but also the NE-induced level (by more than 3 times).

After administration of NTG a strong negative correlation was revealed between the increase in $[cGMP]_i$ and decrease in $[Ca^{2+}]_i$ (r=-0.79, without preincubation of myocytes with NTG).

TABLE 2. Intracellular cGMP concentration (pmol/10^6 cells) under the Influence of NTG and NE

Experimental conditions	Control	Time of incubation, min					
		1	2	3	5	10	15
NTG (100 μM)	0.5±0.1	2.8±0.2	4.9±0.3	6.7±0.4	4.1±0.4	2.4±0.2	2.3±0.4
NE (10 μM)	0.5±0.1	1.5±0.3	2.4±0.2	2.8±0.3	2.6±0.4	4.4±0.4	4.3±0.4
NE (10 μM)+NTG (100 μM)	0.5±0.1	3.0±0.1	5.1±0.4	6.9±0.3	4.5±0.4	4.3±0.4	4.5±0.5

Preincubation of myocytes in a medium with NTG in high concentration (10^{-4} M, 30 min) resulted in the development of drug tolerance. It was manifested in a decrease in calcium-blocking activity of NTG and prevention of cGMP accumulation (Fig. 1). After 30-min preincubation in HEPES buffer, IC_{50} for $[Ca^{2+}]_i$ increased by 80 times compared to the control.

The decrease in cGMP accumulation under the influence of NTG (preincubation of cells in the buffer with high concentration of NTG) was observed in resting and NE-stimulated cells (10^{-5} M, NE). This effect was most pronounced during stimulation of cells with the agonist (Fig. 1).

Our results suggest that cGMP concentration and calcium response of smooth muscle cells can serve as the biochemical criteria of the efficacy of nitrate therapy and development of drug tolerance *in vivo*.

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