EFFECT OF NITROGLYCERIN, NITROSORBIDE, AND NITROPRUSSIDE ON INTRACELLULAR Ca^{2+} ION CONCENTRATION IN HUMAN LYMPHOCYTES

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The muscle relaxing action of nitrates is mediated through cyclic guanosine-3′,5′-monophosphate (cGMP) and Ca^{2+} ions [5]. Lowering of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) under the influence of nitroglycerin (glyceryl trinitrate; GTN), nitrosorbide (isosorbide dinitrate; ISDN), and sodium nitroprusside (SNP) is observed not only in vascular smooth-muscle cells but also in platelets [2]. However, there as yet no data on the action of the above-mentioned compounds on the Ca^{2+} ion concentration in human lymphocytes. Nevertheless, we know that lymphocytes are an adequate model with which to study the effect of biologically active compounds on calcium metabolism [1], and their receptor system is largely identical with the tissue receptors of internal organs [4].

The aim of this investigation was a comparative study of the effect of GTN, ISDN, and SNP on the Ca^{2+} ion concentration in human lymphocyte cytosol, and also to investigate parameters of calcium response of cells after preincubation with GTN.

EXPERIMENTAL METHOD

To tackle the problem enunciated above, in the first stage we investigated the effect of these nitro preparations on the basal and mitogen-induced levels of intracellular Ca^{2+} in two series of experiments: 1) in a standard medium containing 1 mM $CaCl_2$; 2) in calcium-free buffer. In the second stage, in experiments simulating conditions of tolerance, the calcium-blocking action of GTN, ISDN, and SNP was evaluated after preliminary incubation of the cells with 10^{-4} M GTN under different conditions of incubation at 37°C.

The value of IC_{50} was calculated from the dose/effect curve [8]. On the graphs the value of the calcium-blocking action of nitrates was expressed in percent; for convenience, a 50% reduction in the increase in $[Ca^{2+}]_i$ induced by a mitogen, was taken as the 100% inhibitory effect of a given preparation.

Peripheral blood lymphocytes from healthy donors were isolated in a Histopak-1077 density gradient ("Sigma") by the method in [3]. The washed cells were placed in 10 ml of HEPES buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_5$, 5 mM Glu, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.35. Incorporation of the fluorescent Ca^{2+} ion indicator Fura-2/AM ("Calbiochem") into the cells was carried out by the method in [9] by incubating the cells for 20 min with a solution of the probe in a final concentration of 3 μM at 37°C. After washing and resuspension in HEPES buffer, 2 ml of the cell suspension was introduced into the measuring cell of a "Hitachi" spectrofluorometer, and thermostated at 37°C; the kinetics of fluorescence was recorded for 10 min. The wavelengths of excitation were 340 and 385 nm and of recording 500 nm. The intracellular Ca^{2+} ion concentration was calculated as described in [9]. The mitogen concanavalin A (con A, "Sigma") was used in a concentration of 25 μg/ml.

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TABLE 1. Effect of GTN, ISDN, and SNP on Basal and Mitogen-Induced (con A) Level of $[Ca^{2+}]_i$ in Lymphocytes (M ± m)

Preparation 10^{-4} M	$[Ca^{2+}]_i$ level, nm		
	Basal	Mitogen-induced (con A, 25 μg/ml)	
		in calcium-free medium	in presence of 1 mM $CaCl_2$
Control (n=12)	123±14	211±27	384±31
GTN (n=6)	122±11	202±16	221±19*
ISDN (n=4)	124±16	209±11	246±22*
SP (n=4)	119±22	205±24	208±16*

Legend: *$p < 0.05$: difference from control significant.

TABLE 2. Effect of Preincubation Time of Lymphocytes with GTN (10^{-4} M) on Index of Calcium-Blocking Action of Nitro Preparations (IC_{50})

Preparation	Preincubation time, min	IC_{50}, M
GTN	0	$9{,}8 \cdot 10^{-6}$
	30	$3{,}7 \cdot 10^{-5}$
	60	$4{,}9 \cdot 10^{-4}$
	120	$5{,}5 \cdot 10^{-4}$
ISDN	0	$1{,}1 \cdot 10^{-5}$
	30	$4{,}1 \cdot 10^{-5}$
	60	$6{,}2 \cdot 10^{-4}$
	120	$7{,}1 \cdot 10^{-4}$
SNP	0	$3{,}4 \cdot 10^{-7}$
	30	$3{,}5 \cdot 10^{-7}$
	60	$3{,}0 \cdot 10^{-7}$
	120	$3{,}2 \cdot 10^{-7}$

The results were subjected to statistical analysis and presented in the form of the mean value ± standard error of the mean (M ± m). The significance of differences was estimated by Student's paired t test.

EXPERIMENTAL RESULTS

As Table 1 shows, the basal Ca^{2+} ion concentration in the lymphocytes averaged 123 ± 14 nM and was unchanged by the action of the nitrocompounds.

On addition of the mitogen to the lymphocyte suspension an increase in the intensity of fluorescence proportional to the increase in $[Ca^{2+}]_i$ concentration was observed after 30 sec, and reached a maximum after incubation for 4 min. GTN, ISDN, and SNP, if added to the cell suspension 1 min before the mitogen, within the concentration range from 10^{-8} to 10^{-4} M, led to dose-dependent inhibition of the increase in the $[Ca^{2+}]_i$ level in the lymphocytes induced by con A (Fig. 1). On comparison of GTN, ISDN, and SNP by the degree of their effect on calcium metabolism in the lymphocytes, it was found that SNP had the highest activity under these experimental conditions ($IC_{50} = 4.1 \cdot 10^{-7}$ M), followed by GTN and ISDN (for which IC_{50} was $9.8 \cdot 10^{-6}$ M and $1.5 \cdot 10^{-5}$ M respectively).

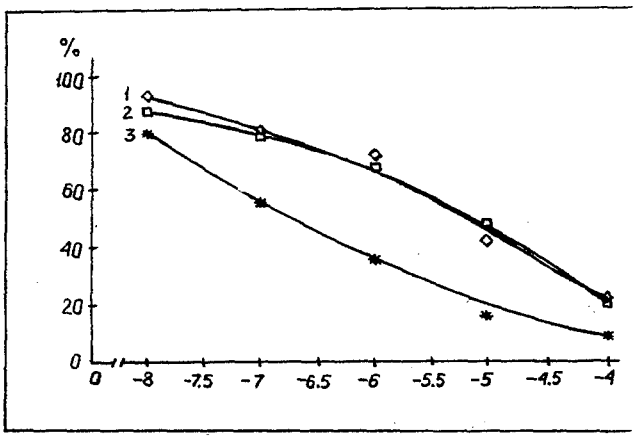

Fig. 1. Inhibitory action of nitro preparations on mitogen-induced increase in $[Ca^{2+}]_i$ concentration in human lymphocytes. Abscissa, logarithm of concentration of nitro compounds, M; ordinate, change in calcium concentration, percent. Legend: 1) GTN; 2) ISDN; 3) SNP.

The higher SNP activity was evidently due to the ability of this nitro compound to release NO, a powerful stimulator of guanylate cyclase, directly, whereas the organic nitrates GTN and ISDN have to undergo biotransformation and require coenzymes of metabolic activation in order to generate NO [6].

It was shown previously in experiments with radioactive $^{45}Ca^{2+}$ that the leading component of the calcium-blocking action of GTN in platelets is blockade of the Ca^{2+} inflow into the cell through chemosensitive calcium channels [2]. To test this hypothesis on a model of lymphocytes we carried out a series of experiments in calcium-free medium (Table 1). As the results show, the inhibitory effect of GTN, ISDN, and SNP on mitogen-induced growth of $[Ca^{2+}]_i$ is clearly manifested in standard medium containing 1 mM $CaCl_2$ but virtually absent in calcium-free buffer. Consequently, lowering of the level of Ca^{2+} ions under the influence of the nitro compound can be explained by a decrease in Ca^{2+} inflow into the lymphcoytes from the extracellular space, as a result of blocking of receptor-dependent calcium channels of the plasma membrane (PM), mediated through cGMP, and to a lesser degree by inhibition of mobilization of Ca^{2+} ions from the intracellular compartment [5]. An additional component of the calcium-blocking action of nitrates, mediated through the secondary messenger cGMP, is activation of the "pumping" of Ca^{2+} out of the cell through stimulation of Ca^{2+}, (Mg^{2+})-ATPase of PM by cGMP-dependent protein kinase [7].

In the next series of experiments we studied the effect of the duration of preliminary incubation of the cells in medium with a high GTN concentration (10^{-4} M) on the efficacy of the calcium-blocking action of the compounds. As the results in Table 2 show, appreciable weakening of the inhibitory effect of GTN and ISDN as the result of the development of tolerance is observed after a period of preincubation as short as 30 min. Increasing the incubation time to 120 min led to total loss of the calcium-blocking action of the compounds (IC_{50} for GTN and ISDN increased by 1-2 logarithmic orders of magnitude). The absence of any inhibitory effect of ISDN against the background of preincubation of the cells for 120 min with GTN is evidence of the existence of crossed tolerance between these preparations. Conversely, the efficacy of SNP was not reduced after incubation of the lymphocytes with GTN for 120 min, in agreement with the observations of other workers in experiments on isolated segments of the rat aorta [6].

The results of the present investigation suggest that parameters of the Ca^{2+}-response of human lymphocytes can be used as an indicator of the sensitivity of the cells to nitro compounds in vitro. This model can be used to study the molecular mechanisms of action of nitrates and the development of tolerance to them.

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