EFFECT OF NITROGLYCERIN ON INTRACELLULAR CALCIUM CONCENTRATION IN HUMAN PLATELETS

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Among the urgent problems in contemporary cardiology tolerance to nitrates (TN) arouses particular interest as a factor significantly limiting the efficacy of pharmacotherapy [4, 7]. The study of the biochemical mechanisms of development of TN has encountered considerable difficulties due to the absence of adequate experimental models with which to study this phenomenon [5]. Nitroglycerin (glyceryl trinitrate – GTN), the standard preparation for arresting attacks of angina pectoris, is known to possess a marked Ca-blocking action on inducer-stimulated increase in the free intracellular Ca^{2+} concentration in the cytoplasm of human platelets [2, 3]. Improvements in fluorescence techniques, and the appearance of a new generation of Indo-1 and Fura-2 probes have opened up new opportunities in the study of exchange of Ca^{2+} ions, as universal messengers mediating the action of various compounds controlling platelet activity.

The aim of this investigation was to study the effect of nitroglycerin on the basal and ADP-induced Ca^{2+} concentrations in the cytoplasm of human platelets, measured with the aid of the Fura-2/AM fluorescent probe. At the same time, the possibility of using parameters of calcium exchange in the platelets as an experimental model with which to study tolerance to nitrates in vitro also appeared to be very interesting.

EXPERIMENTAL METHOD

The test objects were platelets from healthy male volunteers. Blood was taken with a siliconized needle with wide bore, without a tourniquet, from the ulnar vein into plastic centrifuge tubes, containing citrate anticoagulant in the ratio of 6:1 (5.92 g Na citrate·$5H_2O$, 2.73 g citric acid, 2 g glucose to 200 ml H_2O_2). Platelet-enriched plasma was obtained by centrifugation for 10 min at 200*g*. The platelets were washed free from plasma components by a modified method in [6] in medium containing 2.8 mM KCl, 2 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 138 mM NaCl, 0.2 mM EGTA, 10 mM HEPES, 5 mM glucose, 0.35 g/liter bovine serum albumin (BSA), and 0.1 U/ml apyrase, pH 6.55. Incorporation of the fluorescent probe Fura-2/AM ("Calbiochem") into platelets was carried out by the method in [8]. To washed platelets in medium containing 2.8 mM KCl, 0.5 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 138 mM NaCl, 1 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose, 0.17 g/liter BSA, and 0.1 U/ml apyrase, pH 7.4, was added a solution of the fluorescent indicator Fura-2/AM up to a final concentration of 3 M, after which the sample was incubated for 30 min at 37°C, when the cells were washed and resuspended in the same medium. 2 ml of cell suspension ($2 \cdot 10^6$ cells in 1 ml) was introduced into the cell of an MPF-3 spectrofluorometer ("Hitachi"), kept at a constant temperature of 37°C. The wavelengths of excitation were 350 and 385 nm, and of recording 500 nm. Intracellular Ca^{2+} was calculated by the equation:

$$Ca_{in}^{2+} = K_d(R - R_0)/(R_1 - R) \text{ [9]},$$

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TABLE 1. Effect of Nitroglycerin (40 μM) on Calcium Ion Concentration in Human Platelets (M ± m, n = 6)

Experimental conditions	Ca^{2+} level, nM	
	basal	ADP-induced (10^{-5} M)
Control	127±5	614±31
Nitroglycerin	73±4	360±29
p	<0,01	<0,01

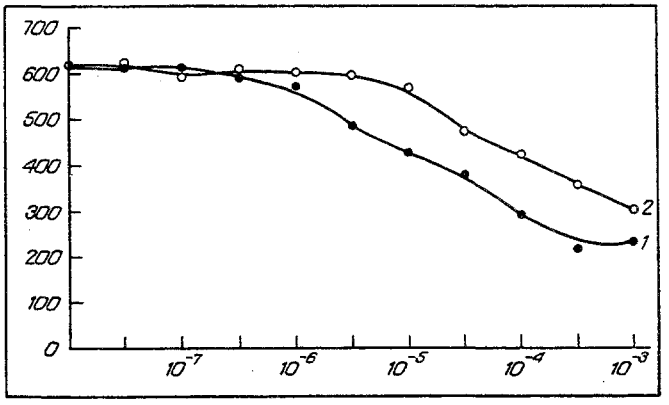

Fig. 1. Effect of nitroglycerin on ADP-stimulated increase in Ca^{2+} concentration in platelets. Abscissa, nitroglycerin concentration (in M); ordinate, Ca^{2+} concentration in platelets (in nM). 1) Without preincubation (n = 3); 2) with preliminary 30-min incubation with 60 μM nitroglycerin (n = 3).

where R denotes the ratio F_{350}/F_{385} for the test sample (F_{350} and F_{385} denote the intensity of fluorescence at excitation wavelengths of 350 and 385 nm respectively); R_0 the ratio F_{350}/F_{385} when the intracellular Ca^{2+} concentration is minimal [9]; R_1 the ratio F_{350}/F_{385} with the probe saturated with calcium, and which was determined by adding digitonin (40 μM) to the cell suspension; K_d the equilibrium constant of complex formation between Fura-2 and Ca^{2+} ions, namely 225 nm at 37°C [8].

Basal and ADP ("Serva") induced Ca^{2+} levels in the cytoplasm of the platelets were determined in the control and in the experiment with addition of 40 μM nitroglycerin. The effect of nitroglycerin on the intracellular Ca^{2+} concentration, induced by 10^{-5} M ADP, was measured in two series of experiments: without preincubation and after preliminary incubation for 30 min at 37°C with 60 μM nitroglycerin. The efficacy of action of nitroglycerin was estimated from the inhibitory effect on the increase in Ca^{2+} concentration in the cells, using the IC_{50} criterion [2]. Aliquots with ADP in a final concentration of 10^{-4} M were kept in volumes of 1.5 ml at −35°C and thawed on the day they were required for use. The duration of the experiments did not exceed 4 h from the time of taking the blood. The results were analyzed by statistical methods. The significance of differences between means was estimated by Student's paired test.

EXPERIMENTAL RESULTS

Addition of nitroglycerin in a final concentration of 40 μM to the platelet suspension led to a significant lowering of the basal level of intracellular Ca^{2+}, recorded with the aid of Fura-2/AM. In this same concentration nitroglycerin effectively blocked the increase induced by ADP in the Ca^{2+} concentration (Table 1).

Besides, as will be clear from Fig. 1, the inhibitory effect of nitroglycerin on elevation of the intracellular Ca^{2+} level, induced by ADP, within the concentration range of nitroglycerin of 10^{-7}-10^{-3} M, was distinctly dose-dependent in character. Preincubation of the platelets for 30 min with 60 μM nitroglycerin led to a significant increase in the value of IC_{50} (the nitroglycerin concentration at which half the maximal inhibition of the increase in Ca^{2+} concentration induced by

ATP is observed), from 44 to 320 μM (n = 3), evidence of the development of partial tolerance to nitroglycerin. Under these circumstances the curve remained dose-dependent in character.

The results obtained in relation to the Ca-blocking action of nitroglycerin are in agreement on the whole with data obtained by other workers [2, 3]. The absence of any significant effect of nitroglycerin on the basal intracellular Ca^{2+} level in one publication [3] is probably linked with the fact that the authors cited used the Quin-2 probe, which has a number of important disadvantages, not shared by Fura-2/AM [9].

Previous investigations showed that platelets can be used as a model with which to estimate the Ca-blocking action of nitrates and other compounds [1-3]. If this approach is used and the new possibilities afforded by fluorescence techniques are adopted, the results of the present investigation suggest that human platelets can be used as a convenient experimental model with which to study tolerance to nitrates in vitro.

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