EFFECT OF ETHAPHON ON THE CALCIUM ION CONCENTRATION IN SMOOTH-MUSCLE CELLS OF THE AORTA

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Ethaphon (2-diethylaminoethoxy-3-phenylpropiophenone hydrochloride) has been shown to possess antianginal, local-anesthetic, antiarrthymic, and spasmolytic activity [1, 3, 4]. It has accordingly been suggested that ethaphon may disturb intracellular and transmembrane transport of Ca^{2+} ions in the cell, which leads to relaxation of the smooth muscle and to a corresponding reduction of vascular tone, both coronary and peripheral [2].

The aim of this investigation was to study the effect of ethaphon on the Ca^{2+} ion concentration in smooth-muscle cells.

EXPERIMENTAL METHOD

Isolated smooth-muscle cells were obtained from the aorta. Isolation of smooth-muscle cells from the thoracic part of the rabbit's aorta was carried out by the method in [9] with modifications. Under control of a binocular microscope the adventitia was carefully separated from the surrounding tissues. The endothelium was removed by means of a gauze pad. The muscular layer was divided longitudinally, fixed with slight stretching to a plastic plate, and immersed in calcium-free HEPES-buffer (NaCl 140 mM, KCl 4 mM, $MgCl_2$ 2 mM, K_2HPO_4 1 mM, glucose 10 mM, HEPES 10 mM, pH 7.4). After incubation for 1 h at 37°C the tissue was transferred into calcium-free HEPES-buffer containing collagenase (1 mg/ml), trypsin inhibitor (0.5 mg/ml), and bovine serum albumin (2 mg/ml). As a result of enzyme treatment for 1 h the muscle tissue became friable, it was divided into small pieces with a razor blade, and treated with versene. The resulting homogenate was resuspended through a plastic pipette for 15 min and filtered through nylon gauze. The dispersed cells were sedimented by centrifugation at 30*g* for 5 min. The residue was washed twice and finally resuspended in 10 ml of HEPES-buffer (cell concentration $1 \cdot 10^6$/ml). The cells were counted in a Goryaev's chamber. Viability was assessed by the trypan blue test [8].

The intracellular concentration of free Ca^{2+} ions in the cytoplasm of the myocytes (Ca_{in}^{2+}) was determined. Loading of the cells with the fluorescent calcium ion indicator fura-2/AM ("Calbiochem") was carried out as in [7]. A solution of fura-2/AM was added to the isolated myocytes to a final concentration of 3 μM and the cells were incubated for 30 min at 37°C, after which they were washed and resuspended in medium containing 140 mM NaCl, 1 mM Na_2HPO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM glucose, and 10 mM HEPES-NaOH, pH 7.35. Next, 2 ml of the suspension (10^6 cells in 1 ml) was transferred into the cell of an MPF-3 spectrofluorometer (Hitachi) and thermostatted at 37°C. The wavelengths of excitation were 350 and 385 nm and of recording 500 nm. Ca_{in}^{2+} was calculated by the formula [6]:

$$Ca_{in}^{2+} = K_d(R - R_0)/(R_1 - R),$$

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TABLE 1. Effect of Ethaphon on Basal Ca^{2+} Level (in nM) in Smooth-Muscle Cells of Rabbit Aorta (M ± m)

Experimental conditions	Ethaphon concentration in incubation medium, M						
	$1 \cdot 10^{-7}$	$2 \cdot 10^{-6}$	$5 \cdot 10^{-6}$	$1 \cdot 10^{-5}$	$2 \cdot 10^{-5}$	$5 \cdot 10^{-5}$	$1 \cdot 10^{-4}$
Ethaphon	123±5	117±4	106±6	93±3	89±2	147±7	151±6
p				<0,05	<0,01		<0,05
Control (ethaphon absent)	120±6						

Legend. Results of 4-6 independent experiments; p) statistical significance of difference from control. Here and in Table 2, effect of ethaphon was recorded 5 min after beginning of incubation of preparation with cells.

where R denotes the ratio F_{350}/F_{385} for the test specimen (F_{350} and F_{385} denote the intensity of fluorescence at wavelengths of excitation of 350 and 385 nm, respectively); R_0, the ratio F_{350}/F_{385} under conditions when Ca_{in}^{2+} is minimal [7]; R_1, the ratio F_{350}/F_{385} under conditions of calcium saturation of the probe, which was determined by adding digitonin (40 μM) to the cell suspension; K_d, the equilibrium constant of formation of a complex between fura-2 and calcium ions, with a value of 225 nM at 37°C [6]. The results were analyzed by statistical methods on the Amstrad PC 1640 personal computer, using the "Microstat" package of applied statistical programs.

EXPERIMENTAL RESULTS

The Ca^{2+} concentration in resting aortic smooth-muscle cells was 120 ± 21 nM (n = 8). Addition of ethaphon to the cell suspension up to a final concentration of $1 \cdot 10^{-7}$-$3 \cdot 10^{-6}$ M did not change the basal Ca_{in}^{2+} level, at least during incubation for 20 min with the preparation. In concentrations of $5 \cdot 10^{-6}$-$2 \cdot 10^{-5}$ M ethaphon caused a dose-dependent decrease in Ca_{in}^{2+}, recorded as a decrease in the intensity of fluorescence of fura-2 during excitation of the sample in the short-wave region (350 nm) and an increase in the intensity of fluorescence F_{385} (Table 1). The decrease in Ca_{in}^{2+} began after 1-2 min and reached a maximum 4-6 min after addition of the compound. In high concentrations ($0.5 \cdot 10^{-4}$-10^{-4} M) ethaphon, on the contrary, raised the intracellular Ca^{2+} level. This effect was observed immediately after the addition of ethaphon to the incubation medium, due evidently to disturbance of the structure of the plasma membranes of the aortic smooth-muscle cells by it.

It was decided to study the effect of ethaphon on the elevated Ca_{in}^{2+} level, for we know that this compound has a direct myotropic action, and exhibits spasmolytic activity [5].

The Ca_{in}^{2+} level was raised by two methods: 1) by activation of the cells with a 10^{-5} M solution of acetylcholine; 2) by placing the smooth-muscle cells in medium containing 118 nM KCl, by replacing the NaCl with an equimolar concentration of KCl.

Incubation of the cells with acetylcholine for 2 min almost doubled the Ca_{in}^{2+} level (185 ± 7 nM). The effect of a high potassium ion concentration (118 mM) on Ca_{in}^{2+} also was exhibited quickly (in the course of 1 min the intensity of fluorescence F_{350} reached a maximum and remained almost unchanged during 10 min of observation).

Against the background of an elevated Ca_{in}^{2+}, the action of ethaphon began to be manifested with a concentration of $5 \cdot 10^{-7}$ M. As the data in Table 2 show, ethaphon lowered Ca_{in}^{2+}, and this action was most marked against cells kept in hyperpotassium medium. In that case, the maximal inhibitory activity of ethaphon was seen in a concentration of $5 \cdot 10^{-6}$ M. A further increase in the dose of ethaphon caused no significant change in Ca_{in}^{2+}.

The increase in the Ca_{in}^{2+} concentration observed when the cells are placed in medium with a high K^+ concentration is known to be depolarization of the cytoplasmic membrane and to the entry of Ca^{2+} ions inside the cell from outside, through voltage-dependent calcium channels. The increase of Ca_{in}^{2+} in smooth-muscle cells under the influence of acetylcholine is due to interaction between the neurotransmitter and membrane receptors, followed by opening of chemically sensitive Ca^{2+}-channels and mobilization of calcium ions from their intracellular depots (the sarcoplasmic reticulum).

TABLE 2. Effect of Ethaphon on Increase in Ca_{in}^{2+} (in nM) Due to Action of Acetylcholine and of 118 mM K^+

Experimental conditions	Ethaphon concentration in incubation medium, M						
	0	$1 \cdot 10^{-7}$	$5 \cdot 10^{-7}$	$1 \cdot 10^{-6}$	$2 \cdot 10^{-6}$	$5 \cdot 10^{-6}$	$1 \cdot 10^{-5}$
Acetylcholine 10^{-5}	185±7	191±9	174±12(94)	166±8(90)	159±8*(86)	152±7*(82)	158±10*(85)
KCl 118 mM	197±11	193±9	182±10(92)	171±12*(87)	153±10*(78)	134±8*(68)	133±11*(67)

Legend. Mean values and confidence intervals at the p = 0.05 level shown. Fall in Ca^{2+} level (in % of initial level) given in parentheses; significant differences marked by an asterisk.

The action of ethaphon is thus most marked against transmembrane transport of Ca^{2+} ions from the extracellular space. Meanwhile, the effect of the drug on the concentration and transport of calcium ions from the intracellular pool is exhibited less strongly.

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