A Study of the Mechanism of Action of Befol on Ca^{2+} Metabolism in Cardiomyocytes Using a FURA-2 Fluorescent Probe

P. V. Sergeev, P. A. Galenko-Yaroshevskii, A. I. Khankoeva, and A. S. Dukhanin

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 121, № 3, pp. 288-291, March, 1996
Original article submitted March 28, 1995

The dynamics of the Ca-response of cardiomyocytes is studied and the efficiency of befol, verapamil, and amiodarone is compared using various experimental models of stimulation of $[Ca^{2+}]_i$. Befol (1-5 μM) is shown to inhibit the caffeine- and strophanthin G-induced rise of $[Ca^{2+}]_i$. Unlike verapamil and amiodarone, befol exhibits no Ca-blocking activity in modeled K-depolarization. It is concluded that the cardiotropic effect of befol is mediated through its primary action on Na^+/Ca^{2+} exchange in cardiomyocytes, while the cardioplegic effect of verapamil and amiodarone is due to their ability to block the slow Ca^{2+} inward current.

Key Words: *calcium; befol; amiloride; strophanthin; caffeine; cardiomyocytes*

Calcium ions participate in the coupling between electrical excitation and muscle contraction in cardiomyocytes. In resting cardiomyocytes (CMC) the diastolic concentration of free Ca^{2+} ions in the cytoplasm ($[Ca^{2+}]_i$) does not exceed 150 nM, whereas the Ca^{2+} content in the extracellular space is several orders of magnitude higher (1-2 mM). Stimulation of the cells by various agents (electrical pulse, activation of α1-, β_1-, and β_2-adrenoreceptors) leads a rise of $[Ca^{2+}]_i$ which is directly proportional to the contractile response of CMC and determines the strength of cardiac contractions [10]. The reversible drop of $[Ca^{2+}]_i$ underlies the relaxation process.

A positive inotropic effect of pharmacological preparations may be realized through: 1) receptor-mediated (isoproterenol, histamine) or direct (forskolin) activation of adenylate cyclase; 2) elevation of the intracellular concentration of Na^+ and Ca^{2+} (cardiac glycosides); and 3) inhibition of K^+ channels (4-aminopyridines). However, the molecular mechanisms of pharmacological regulation of excitation and contractile activity of the heart have been little studied.

Apart from the traditional electrophysiological methods of recording Ca fluxes in the myocardium, a new approach to the measurement of $[Ca^{2+}]_i$ has recently gained broad acceptance. This method is based on the use of the quin-2, indo-1, and FURA 2-AM fluorescent probes for Ca^{2+} ions. This highly sensitive fluorescent technique has helped elucidate the main regularities of Ca homeostasis in isolated CMC [8].

The aim of the present study was to investigate the effect of befol, a new Russian-manufactured antidepressant which has recently been found to possess also antiarrhythmic activity [2], on the basal and stimulated $[Ca^{2+}]_i$ levels in a CMC suspension and to compare this effect with that of verapamil and amiodarone.

MATERIALS AND METHODS

CMC were isolated from the left ventricle of rat hearts as described previously [9]. The cells were resuspended in a Krebs-Ringer bicarbonate buffer solution ($10\text{-}15\times10^5$ cells/ml) and incubated with FURA 2-AM in

Department of Molecular Pharmacology and Radiobiology, Russian State Medical University, Moscow; Department of Pharmacology, Kuban Medical Academy, Krasnodar

0007-4888/96/0003-0265$15.00

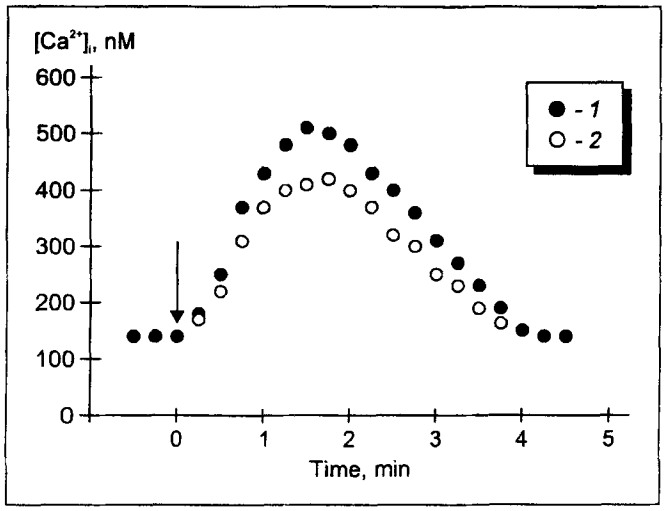

Fig. 1. Effect of 1 μM verapamil (*2*) on the KCl (40 μM)-induced rise of $[Ca^{2+}]_i$ (*1*) in cardiomyocytes. Verapamil was added 1-1.5 min prior to KCl. Here and in Figs. 2 and 3: the arrow indicates when the inductor was added.

a final concentration of 5 μM at 20°C for 1 hour. The cells were then washed twice with fresh Krebs-Ringer buffer and resuspended in HEPES buffer containing (in mM) 145 NaCl, 5 KCl, 1 Na_2HPO_4, 1 $CaCl_2$, 0.5 $MgSO_4$, 5 glucose, and 10 Na-HEPES, pH 7.4, at 37°C.

Aliquots of the suspension (1 ml, 0.5×10^6 cells/ml) were placed in a thermocontrolled cell (37°C) of an MPF-3 Hitachi spectrofluorimeter, and fluorescence (500 nm) was recorded at excitation wavelength 340 nm, corresponding to the maximal absorption of the FURA-Ca^{2+} complex [7]. The concentration of free Ca^{2+} was calculated by the formula:

$$[Ca^{2+}]_i=K_d\times(F-F_{min})/(F_{max}-F),$$

where F is the fluorescence of the sample, F_{max} is the fluorescence of FURA 2-AM at the saturating Ca^{2+} concentration in the presence of digitonin (40 μM), F_{min} is the fluorescence of the probe in the presence of 1 mM $MnCl_2$, which binds with FURA 2-AM, displacing Ca^{2+}, and quenches the fluorescence of the probe. K_d is an equilibrium dissociation constant of the FURA-Ca^{2+} complex determined previously in control experiments (130 nM).

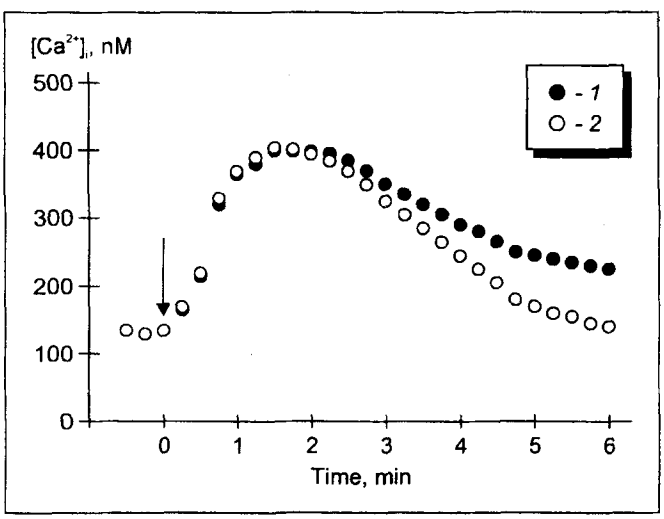

Fig. 2. Dynamics of $[Ca^{2+}]_i$ in cardiomyocytes in response to the addition of 10 mM caffeine (*1*) alone and in combination with 40 μM amiloride (*2*).

For elucidation of their effect on the basal $[Ca^{2+}]_i$ level the preparations were incubated with cells for 15 min.

The efficacy of the test substances was assessed using the IC_{50} parameter, i.e., the concentration of pharmacological preparation which reduces the Ca response of the cells by 50%.

The data were processed statistically using the Student *t* test.

RESULTS

The basal (diastolic) level of $[Ca^{2+}]_i$ in CMC in our experiments was 135±12 nM (n=4). Within the concentration range of 10^{-7}-5×10^{-6} M none of the preparations affects $[Ca^{2+}]_i$. Starting from a concentration of 5 μM, befol increased $[Ca^{2+}]_i$ in a dose-dependent manner. The maximal dose of befol (10^{-4} M) increased this parameter to 204±13 nM (n=4). Amiodarone and verapamil (50 and 70 μM, respectively) produced a stable rise of $[Ca^{2+}]_i$, and increasing the doses in the incubation medium did not affect this parameter. These shifts of $[Ca^{2+}]_i$ are probably due to the earlier described nonspecific toxic effect of these substances [6].

The dose dependence of the effect of befol on the basal Ca^{2+} level implies the presence of a specific component in the mechanism of its action.

In the next experimental series we studied the effect of the preparations (10^{-7}-5×10^{-6} M) on induced $[Ca^{2+}]_i$ in CMC. A rise of $[Ca^{2+}]_i$ was induced by either increasing the extracellular concentration of K^+ ions or adding caffeine or ouabain (strophanthin G) to the cell suspension.

A rise of the concentration of K^+ ions from 20 to 80 mM leads to partial depolarization of the CMC membrane accompanied by opening of the sarcolemmal voltage-dependent Ca channels and Ca^{2+} inflow [8]. The dynamics of $[Ca^{2+}]_i$ during K depolarization is presented in Fig. 1. The content of Ca^{2+} starts to rise 5-10 sec after the addition of KCl (final concentration 40 mM), rapidly (in 1-2 min) attains 510±20 nM, and then drops back down to the initial level during the next 2-2.5 min.

Verapamil, starting from a concentration of 0.5 μM, inhibited the rise of $[Ca^{2+}]_i$ in a dose-dependent manner, IC_{50} being 2.8±0.3 μM. The effect of verapamil is determined by its ability to block L-type Ca channels which possess a binding site for 1,4-dihydropyridines [1].

The Ca-blocking activity of amiodarone in this experimental model was less expressed in comparison with verapamil (IC_{50}=4.7±0.6 μM). Befol had no effect on $[Ca^{2+}]_i$ within the studied concentration range.

Apart from Ca^{2+} entry from the extracellular space, Ca^{2+} ions may be delivered to the myoplasm from cisternae of the sarcoplasmic reticulum (SPR). Under physiological conditions this mobilization of Ca^{2+} from the SPR may be triggered by inositol triphosphate and by a rise in the concentration of Ca^{2+} itself, the so-called calcium-induced calcium release (CICR) [11]. In an experimental setting Ca^{2+} mobilization from the SPR may be induced by caffeine [5] and ryanodine [4]. These substances also deplete Ca^{2+} stores by inhibiting the accumulation of Ca^{2+} in the SPR.

Addition of caffeine (10 mM) to the cell suspension induces a rapid rise of $[Ca^{2+}]_i$ which is 20% lower than the maximal Ca^{2+} level produced by K depolarization (Fig. 2). The kinetics of $[Ca^{2+}]_i$ also has some peculiarities: for an approximately equal rate of $[Ca^{2+}]_i$ rise, the subsequent drop under the influence of caffeine occurred more slowly. This is due to the fact that caffeine interferes with the uptake and accumulation of Ca^{2+} in the SPR, and Ca^{2+} removal from the cytosol is effected mainly through Na^+/Ca^{2+} exchange [3]. Combined application of caffeine and amiloride, an inhibitor of Na^+/Ca^{2+} exchange (40 μM), prolongs the drop of $[Ca^{2+}]_i$ even further (Fig. 2).

Neither verapamil nor amiodarone exhibits Ca-blocking activity in the caffeine model. Befol in concentrations of 1-5 μM reduced the rate of Ca^{2+} removal, thus exhibiting an amiloride-like activity. The dynamics of $[Ca^{2+}]_i$ was similar in control (caffeine+befol) and amiloride-treated CMC.

Na^+/Ca^{2+} antiport is electrogenic: 3 Na^+ ions are exchanged per Ca^{2+} ion, thereby lowering the sarcolemma membrane potential [1]. Normally, depolarization of the membrane is prevented by the work of Na^+/K^+-ATPase, but under conditions of reduced energy production (ischemia, acidosis) the Na^+/Ca^{2+} exchange becomes arrhythmogenic [12]. It may be assumed that the described antiarrhythmic properties of befol stem from its effect on Na^+/Ca^{2+} exchange.

To verify this assumption, we used a strophanthin model. Strophanthin (5 μM) blocks Na^+/K^+-ATPase, increases the intracellular concentration of Na^+ ions, and activates the Na^+/Ca^{2+} transporter in CMC [12]. The direction of ion transport is reversed: 3 Na^+ ions are transported out and 1 Ca^{2+} ion enters the cell, resulting in a rise of $[Ca^{2+}]_i$.

The dynamics of the Ca response of CMC to the addition of strophanthin is presented in Fig. 3. $[Ca^{2+}]_i$ slowly rises 3-4 min after the addition of strophanthin and then returns to the initial level after a 7-8-min incubation. Befol reliably inhibited the rise of $[Ca^{2+}]_i$, IC_{50} being 12±3 μM. No effect of amiodarone and verapamil was observed in this model.

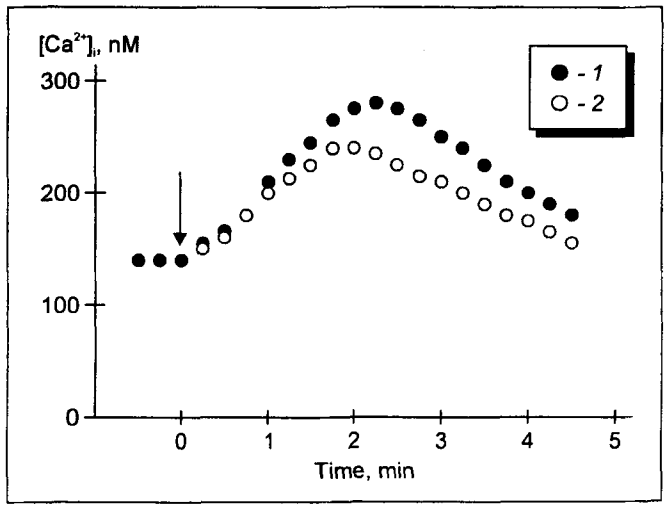

Fig. 3. Effect of 10 μM befol (*2*) on the strophanthin (5 μM)-induced rise of $[Ca^{2+}]_i$ (*1*). Befol was added 1-1.5 min before strophanthin.

Hence, the data obtained using various experimental models suggest that the cardiotropic action of befol is related to its predominant effect on Na^+/Ca^{2+} exchange in CMC, while the cardioplegic effect of verapamil and amiodarone is determined by their ability to blocks the slow Ca^{2+} inflow.

REFERENCES

1. M. D. Kurskii, S. A. Kosterin, and Z. D. Vorobets, *Regulation of the Intracellular Calcium Concentration in Muscles* [in Russian], Kiev (1987).
2. V. V. Skibitskii and S. G. Kanorskii, *Eksp. Klin. Farmakol.*, **56**, № 5, 23-27 (1993).
3. R. A. Bassani, J. W. Bassani, and D. M. Bers, *J. Physiol. (Lond.)*, **476**, № 2, 295-308 (1994).
4. M. Chiesi, A. Wrzosek, and S. Grueninger, *Mol. Cell. Biochem.*, **130**, № 2, 159-171 (1994).
5. J. P. Gomez, D. Potreau, and G. Raymond, *Cell Calcium*, **15**, № 4, 265-275 (1994).
6. L. S. Gotzsche and E. M. Pedersen, *J. Cardiovasc. Pharmacol.*, **23**, № 1, 13-23 (1994).
7. G. Grynkievich, M. Poenie, and R. Y. Tsien, *J. Biol. Chem.*, **206**, 3440-3450 (1985).
8. T. Powell, P. E. Tatham, and V. W. Twist, *Biochem. Biophys. Res. Commun.*, **122**, № 3, 1012-1019 (1984).
9. L. Ransnas, Hjalmarson, E. Sabler, and B. Jacobson, *Pharmacol. Toxicol.*, **61**, 107-110 (1987).
10. H. Scholz, *Basic Res. Cardiol.*, **84**, Suppl. 1, 3-7 (1989).
11. T. Tameyasu, H. Kasugai, M. Tanaka, and H. Harada, *J. Gen. Physiol.*, **103**, № 4, 625-645 (1994).
12. R. D. Vaughhan-Jones, *Br. Med. Bull.*, **42**, № 4, 413-420 (1986).