BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1998 Mar; 19 (2): 148 – 150

Effects of verapamil on down-regulation of norepinephrine-induced β adrenoceptors in cultured rat cardiomyocytes[1]

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KEY WORDS beta adrenergic receptors; verapamil; calcium; norepinephrine; myocardium; cultured cells; dihydroalprenolol; Fura-2

AIM: To determine whether verapamil (Ver) inhibits norepinephrine (NE)-induced β adrenoceptors down-regulation in cultured rat cardiomyocytes. **METHODS:** [^{3}H]-Dihydroalprenolol (DHA) radiobinding assay was used to measure β adrenoceptor density, fluorescent indicator Fura 2-AM was used to estimate levels of free cytosolic calcium ($[Ca^{2+}]_i$). **RESULTS:** Ver reduced $[Ca^{2+}]_i$ and increased β adrenoceptor density, NE increased $[Ca^{2+}]_i$ and reduced β adrenoceptor density of cultured cardiomyocytes, these effects were time and concentration dependent. Ver inhibited the above effects of NE. **CONCLUSIONS:** Ver increased β adrenoceptor density and inhibited NE-induced β adrenoceptor down-regulation of cardiomyocytes.

Long-term exposure of myocardial tissue to β adrenoceptor agonists reduces β adrenoceptor number and β adrenoceptor-mediated adenylate cyclase activity[1,2]. An inverse relation is also presented between the number of adrenoceptor and the level of NE in blood plasma of heart failure patients[3,4]. But the mechanisms underlying adrenoceptor down-regulation induced by agonists are incompletely understood.

Calcium-channel blockers, such as verapamil (Ver), are useful in treatment of several cardiovascular disorders. Hedberg[5] *et al* reported a 46 % – 65 % increase in the number of β adrenoceptors in human atria treated with calcium antagonist. Yonemochi[6] *et al* observed that β adrenoceptors of cultured rat ventricular myocytes increased by 45 % after 24 h of incubation with Ver 1 $\mu mol \cdot L^{-1}$. But the mechanisms of this effect were still unclear. Some researchers[6,7] supposed that verapamil's ability to increase β adrenoceptor density was partly attributed to a decrease in $[Ca^{2+}]_i$. However, all of them did not directly measure exact level of intracellular calcium ion. The purpose of present study was to determine whether calcium antagonist Ver could increase β adrenoceptor density of normal cardiomyocytes and inhibit the NE-induced down-regulation of β adrenoceptors as well as their possible mechanism.

MATERIALS AND METHODS

Cell culture Cardiomyocytes were prepared from neonatal rat ventricles. Hearts were removed from 2 – 3 d old neonatal SD rats of either sex under ether anesthesia. Ventricles were minced into 1-mm^3 pieces in phosphate-buffered solution (PBS) The pieces were washed 3 times with PBS, incubated with 0.06 % collagenase (Type IV) for 5 min in 37 ℃ water bath with shaking. The supernatant containing erythrocytes and cell debris was discarded, the remaining pieces were treated with above method for 3 – 5 times. The free-floating cardiomyocytes were stored in Dulbecco's modified Eagle's medium (DMEM), centrifuged at 500 × *g* for 5 min to collect the isolated cardiomyocytes. The collected cardiomyocytes were seeded in glass culture bottles (Shanghai, China), and incubated for 90 min at 37 ℃ in 5 % CO_2. The fibroblasts attached to the bottom of the culture bottles after 1 – 2 h, and the floating cardiomyocytes were collected by decantation. The cell numbers were adjusted to 2×10^9 cells/L in DMEM supplemented with 5 % fetal bovine serum, benzylpenicillin 1×10^5 $U \cdot L^{-1}$ and streptomycin 100 $mg \cdot L^{-1}$. Cell suspension 10 mL was inoculated into a 25-mL glass culture bottle. After 48 h culture, more than 90 % of the cells adhered to the bottom. Thereafter, the cultures were refed daily.

Measurement of β adrenoceptors The cardiomyocytes were scraped off with a rubber policeman to make a cell suspension, incubated with 100 μL [^{3}H]dihydroalprenolol ([^{3}H]DHA, 5 – 100 $\mu mol \cdot L^{-1}$) for 12 h at 4 ℃. A threefold volume of ice-cold Tris $MgCl_2$ buffer was then added to stop binding, the samples were collected on the F49 filter papers (Xinhua, Shanghai) through a filter. The filter papers were

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Received 1996-08-19 Accepted 1997-10-14

then dried for 20 min at 80 ℃. The radioactivity was counted with Packard 4000 scintillator. Nonspecific binding was defined by using propranolol 100 $\mu mol \cdot L^{-1}$.

Measurement of intracellular calcium Free cytosolic calcium was estimated with the fluorescent dye Fura 2-AM. Cardiomyocytes 2 mL were loaded with Fura 2-AM 5 $\mu mol \cdot L^{-1}$ for 35 – 40 min at 37 ℃. BSA-Hanks' solution 2 mL was then added. The samples were centrifuged ($500 \times g$) to collect the cardiomyocytes. The cardiomyocytes were incubated in 0.2 % BSA-Hanks' solution and then counted. Fura-2-Ca^{2+} fluorescence (F) was measured using an F-3000 spectrophotometer (Hitachi, Japan) with λ_{ex} 340 nm and λ_{em} 540 nm. Addition of 0.1 % triton-100, Fura-2-Ca^{2+} maximal fluorescence (F_{max}) was measured. Then, by addition of $MnCl_2$ 2 $\mu mol \cdot L^{-1}$ to extinguish fluorescence, F_{min} was measured. Free calcium concentration was calculated by the equation $F = K_d(F - F_{min})/(F_{max} - F)$.

Protocol The experiments were done after cardiomyocytes cultured for 4 d. The whole experiments were divided into 3 groups. First group, the different concentrations of NE or Ver were added into cultured bottles to incubate for 24 h to determine the concentration-response curves of NE and Ver on β adrenoceptors and $[Ca^{2+}]_i$. Second group, Ver 1 $\mu mol \cdot L^{-1}$ or NE 10 $\mu mol \cdot L^{-1}$ were added into cultured bottles to incubate for different time to determine the time-response curves. Third group, cardiomyocytes were cultured with NE 10 $\mu mol \cdot L^{-1}$ for different time to induce β adrenoceptor down-regulation, Ver 1 $\mu mol \cdot L^{-1}$ was added first, 30 min before adding NE to determine whether Ver can inhibit NE-induced β adrenoceptor down-regulation.

Animals and reagents Neonatal SD rats were purchased from the Experimental Animal Center of Railway Ministry. Ver, NE, Fura 2-AM, collagenase type IV, and propranolol were purchased from Sigma Chemical Co. [^{3}H]DHA (specific activity 1.55 $PBq \cdot mol^{-1}$) was obtained from Beijing Nuclear Research Institute of China.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and analyzed with the paired t test.

RESULTS

A concentration-dependent increase of β adrenoceptors and a concentration-dependent decrease of $[Ca^{2+}]_i$ were observed after cardiomyocytes were incubated with Ver for 24 h (Tab 1).

A time-dependent increase of β adrenoceptors and a time-dependent decrease of $[Ca^{2+}]_i$ were seen after cardiomyocytes were incubated with Ver 1 $\mu mol \cdot L^{-1}$. There was a time-relation between $[Ca^{2+}]_i$ decrease and β adrenoceptor increase, with $[Ca^{2+}]_i$ decreasing first and β adrenoceptors increasing later (Tab 2).

Tab 1. Intracellar Ca^{2+} and β adrenoceptors after incubation with Ver for 24 h. $n = 6$ rats, $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Verapamil/ $nmol \cdot L^{-1}$	$[Ca^{2+}]_i$/ $nmol \cdot L^{-1}$	β Adrenoceptors, pmol/g protein
0	117 ± 10	139 ± 15
0.05	115 ± 10	142 ± 15^c
0.1	110 ± 8^c	159 ± 16^c
0.5	104 ± 15^c	170 ± 16^c
1	99 ± 7^c	196 ± 17^c

Tab 2. Intracellular Ca^{2+} and β adrenoceptors after incubation with Ver 1 $\mu mol \cdot L^{-1}$. $n = 6$ rats, $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Incubation time	$[Ca^{2+}]_i$/ $nmol \cdot L^{-1}$	β Adrenoceptors, pmol/g protein
5 min (control)	120 ± 9	
10 min	109 ± 9^c	
30 min	99 ± 8^c	
60 min	99 ± 8^c	
2 h (control)		136 ± 14
4 h		147 ± 15^c
12 h		166 ± 15^c
24 h		186 ± 17^c
48 h		193 ± 17^c

A time-dependent increase of $[Ca^{2+}]_i$ and a time-dependent decrease of β adrenoceptors were noted after cardiomyocytes were incubated with NE 10 $\mu mol \cdot L^{-1}$. There was also a time-ralation between $[Ca^{2+}]_i$ increase and β adrenoceptor decrease, with $[Ca^{2+}]_i$ increasing first and β adrenoceptor reducing later. If Ver was added beforehand, the effects of reducing β adrenoceptor density induced by NE was inhibited, but the number of β adrenoceptor was still lower. Ver almost completely prevented the increase of $[Ca^{2+}]_i$ induced by NE (Tab 3).

DISCUSSION

Our results showed that Ver induced increase of β adrenoceptor density and decrease of intracellular calcium ion both in time- and concentration-dependent manner. There was a time-relation presented between the change of intracellular calcium ion and that of β adrenoceptors, with intracellular free calcium reducing first and β adrenoceptors increasing later. These results suggested that the effect of increase β

Tab 3. Effects of Ver 1 μmol·L^{-1} on NE 10 μ mol·L^{-1} induced intracellular Ca^{2+} increase and β adrenoceptor decrease. $n = 6$ rats, $\bar{x} \pm s$.
$^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* NE + Ver.

Time	$[Ca^{2+}]_i$/nmol·L^{-1}		β Adrenoceptors, pmol/g protein	
	NE	NE + Ver	NE	NE + Ver
2 min	144 ± 14^b	121 ± 12		
5 min	182 ± 15^b	139 ± 12		
10 min	256 ± 19^c	158 ± 15		
30 min	281 ± 30^c	169 ± 20		
1 h			132 ± 15^a	135 ± 15
2 h			110 ± 14^a	116 ± 15
6 h			50 ± 20^c	76 ± 19
12 h			25 ± 10^c	63 ± 19

adrenoceptors of calcium antagonists might be attributed to the decrease of intracellular calcium ion.

The mechanisms of agonists-induced down-regulation of β adrenoceptors remain unclear. Some research[3,8] suggested that loss of receptors might be results of receptor internalization and degration or the decrease of β adrenoceptor gene expression, but our present research found that NE-induced decrease of β adrenoceptors followed the significantly increase of intracellular $[Ca^{2+}]_i$. Moreover, it seems that there was a negative correlation presented between increase of intracellular $[Ca^{2+}]_i$ and decrease of β adrenoceptors. Calcium antagonist Ver could inhibited the increase of intracellular $[Ca^{2+}]_i$ of cardiomyocytes induced by NE, meanwhile, NE-induced reduction of β adrenoceptor density also partly reversed. These phenomena suggested that agonists-induced β adrenoceptor down-regulation might be partly caused by the increase of intracellular $[Ca^{2+}]_i$. Calcium antagonists Ver might have the effect of inhibiting NE-induced β adrenoceptor reduction and intracellular calcium ion increase.

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1998 – 150 (13)

维拉帕米对去甲肾上腺素诱导的培养大鼠心肌细胞β受体下调的影响

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关键词 β肾上腺素能受体；维拉帕米；钙；去甲肾上腺素；心肌；培养的细胞；双氢阿普洛尔；Fura-2

目的：研究维拉帕米(Ver)是否能抑制去甲肾上腺素(NE)诱导的培养大鼠心肌细胞β受体下调及其可能机制. **方法**：β受体密度用[^{3}H]-DNA放射配基标记法，细胞内游离钙用钙离子荧光探针 Fura 2-AM 法测定. **结果**：Ver 能明显降低培养大鼠心肌细胞内游离钙水平，增加β受体密度；NE 增加细胞内游离钙水平，降低β受体密度；Ver 能明显抑制 NE 引起的细胞内游离钙增加和β受体密度的降低. **结论**：Ver 能增加正常心肌细胞β受体密度，抑制 NE 产生的心肌β受体下调.