# Chronotropic Effect of D-Ala$^2$,Leu$^5$,Arg$^6$-Enkephalin (Dalargin) is Associated with Activation of Peripheral κ-Opioid Receptors

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Intravenous infusion of D-Ala$^2$,Leu$^5$,Arg$^6$-enkephalin (dalargin) caused bradycardia in narcotized rats. This effect was not observed during opioid receptor blockade with naloxone, naloxone methiodide, and norbinaltorphimine. Dalargin and (-)-U-50,488 added to Krebs—Henseleit perfusion solution for isolated rat heart decreased heart rate. Ganglionic blocker hexamethonium potentiated the negative chronotropic effect of dalargin. The negative chronotropic effect of dalargin is probably associated with activation of cardiac κ-opioid receptors. It should be noted that dalargin caused tachycardia in some animals. This reaction was not observed after treatment with hexamethonium. The positive chronotropic effect of dalargin is probably related to modulation of the parasympathetic autonomic nervous system. Agonists and antagonists of δ-opioid receptors caused persistent bradycardia. We hypothesized that selective δ-opioid antagonists exhibit properties of partial δ-receptor agonists.

**Key Words:** *opioid receptors; heart; dalargin*

Opioid peptide D-Ala$^2$,Leu$^5$,Arg$^6$-enkephalin (dalargin) was approved for clinical use in the therapy of stomach ulcer. Published data show that this peptide has a positive therapeutic effect on the course of coronary heart disease [6,7]. It remains unclear which type of receptors mediates the effect of dalargin. *In vitro* studies of biological activity yielded contradictory data on the interaction of D-Ala$^2$, Leu$^5$,Arg$^6$-enkephalin with opioid receptors (OR) [2,13]. Some authors reported that this opioid peptide has similar affinity for μ-OR and δ-OR [2]. Other authors showed that dalargin exhibits high affinity for μ-OR and low affinity for δ-OR and cannot bind to κ-OR [13]. After systemic administration this peptide does not cross the blood-brain barrier (BBB) and interacts only with peripheral OR [1,2].

Here we estimated the type of receptors mediating the cardiovascular effect of dalargin.

## MATERIALS AND METHODS

We performed 2 series of experiments. *In vivo* series I involved Wistar rats weighing 200-250 g and narcotized intraperitoneally with α-chloralose in a dose of 20 mg/kg. *In vitro* series II was conducted on isolated perfused hearts from these animals.

ECG was recorded in lead I over 15-25 min after injection of OR ligands using an UBF4-03 biopotential amplifier and original software. We measured heart rate (HR) and length of *PQ*, *QRS*, and *QT* intervals. The *QT* interval was corrected by HR

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($QT_c$). Dalargin (Tyr-D-Ala-Gly-Phe-Leu-Arg, 0.1 mg/kg), selective μ-agonists DAMGO (H-Tyr-D-$Ala^2$-Gly-N-Me-Phe-$Gly^5$-ol [10], 0.08 mg/kg) and DALDA ($NH_2$-Tyr-D-Arg-Phe-Lys-$NH_2$ [10], 0.1 and 0.5 mg/kg), selective δ-agonist DPDPE (H-Tyr-D-Pen-Gly-Phe-D-Pen-OH [10], 0.09 mg/kg), and selective k-agonist (-)-U-50,488 hydrochloride (*trans*-(-)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl) cyclohexyl] benzenazeneacetamide [10], 1 mg/kg) were infused intravenously. Peptide agonists of OR were used in doses equimolar to the dose of dalargin. (-)-U-50,488 was applied in a dose producing an antiarrhythmic effect (1 mg/kg) [8]. Experiments were performed with the following OR antagonists: μ-OR preferential antagonists naloxone and naloxone methiodide not crossing BBB [12]; selective μ-antagonist $NH_2$-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-L-Pen-Thr-$NH_2$ (CTAP) [10]; selective δ-antagonists N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174,864) [10], TIPP[ψ] (H-Tyr-Ticψ [$CH_2$NH]Phe-Phe-OH) [10], and naltrindole hydrochloride [10]; and selective κ-antagonist norbinaltorphimine. All antagonists (except norbinaltorphimine) were injected intravenously 25 min before coronary artery occlusion. Norbinaltorphimine was administered 90 min before dalargin injection [9]. The test preparations were used in the following doses: naloxone, 0.05 mg/kg; naloxone methiodide, 0.5 and 2.0 mg/kg; CTAP, 0.5 mg/kg; ICI 174,864, 0.2 and 0.5 mg/kg; TIPP[ψ], 0.5 mg/kg; naltrindole, 1 mg/kg; and norbinaltorphimine, 2.5 and 9.0 mg/kg. Ganglionic blocker hexamethonium in a dose of 10 mg/kg was injected intravenously 15 min before dalargin administration. Guanethidine monosulfate in a daily dose of 50 mg/kg was injected intraperitoneally for 3 days to deplete the reserve of endogenous catecholamines. The last injection of guanethidine was performed 24 h before dalargin administration. The test preparations were dissolved in 0.9% NaCl. The doses of preparations were selected taking into account published data [9,12] and results of our experiments [3,8,11].

*In vitro* study was performed on isolated hearts from male Wistar rats weighing 250-300 g. After thoracotomy the hearts were rapidly removed and placed in a bath with cold Krebs—Henseleit solution (4°C). The harts were maintained in this solution until cessation of spontaneous contractions. After cardiac arrest the hearts were placed in a thermostabilized moistened chamber. Isotonic solution was delivered through a cannula inserted into the ascending aortic arch. Open-circuit retrograde perfusion of the heart with Krebs—Henseleit solution was performed by the method of Langendorff. Contractility of the perfused heart was measured at a constant pressure of 52 mm Hg. Krebs-Henseleit solution was saturated with carbogen (37°C, pH 7.4) and contained 120 mM NaCl, 4.8 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 20.0 mM $NaHCO_3$, and 10.0 mM D-glucose. HR of the isolated heart was recorded after a 20-min adaptation period. Cardiac OR were stimulated by 10-min perfusion of the heart with Krebs—Henseleit solution containing dalargin and (-)-U-50,488 in concentrations of 0.1 mg/kg (130 nmol/liter) and 100 nmol/liter, respectively.

Dalargin was synthesized at the Laboratory of Peptide Synthesis (Russian Cardiology Research-and-Production Complex). Peptide OR ligands CTAP, DAMGO, DALDA, DPDPE, TIPP[ψ], and ICI 174,864 were synthesized at the Multiple Peptide Systems Company. Naltrindole, (-)-U-50,488, and naloxone were obtained from the Tocris Cookson Ltd. Company. Norbinaltorphimine was obtained from the Research Triangle Institute. We also used naloxone methiodide, hexamethonium, and guanethidine (Sigma). Krebs—Henseleit solution was prepared using ICN Biomedicals reagents.

The results were analyzed by pairwise Student's *t* test.

## RESULTS

*In vivo* study showed that transitory bradycardia develops 5 min after injection of 0.1 mg/kg D-$Ala^2$, $Leu^5$,$Arg^6$-enkephalin, but disappears 10 min after treatment (Fig. 1, *a*). The length of *PQ*, *QRS*, and *QT* intervals remained unchanged after dalargin injection. HR decreased by the 15th minute after administration of DPDPE. A selective κ-agonist (-)-U-50,488 caused bradycardia, which was observed 5 min after injection and persisted over 15 min. DALDA in a dose of 0.1 mg/kg had no effect on HR. This preparation in a dose of 0.5 mg/kg caused bradycardia (Fig. 1, *a*). HR decreased 5 min after injection of DAMGO in a dose of 0.08 mg/kg. The length of *PQ*, *QRS*, and *QT* intervals remained unchanged after treatment with opioids.

OR agonists (except for 0.5 mg/kg DALDA and (-)-U-50,488) caused transitory bradycardia. The development of persistent bradycardia after administration of DALDA (0.5 mg/kg) and (-)-U-50,488 (1 mg/kg) was probably related to treatment with high doses of these preparations. The test opioids serve as standard selective agonists of OR and are used in experimental researches (except for D-$Ala^2$,$Leu^5$,$Arg^6$-enkephalin [2,13]).

OR antagonists were used to estimate the type of receptors mediating the cardiovascular effect of dalargin.

Naloxone caused transitory bradycardia, which disappeared 15 min after injection (Fig. 1, *b*). Naloxone methiodide had no effect of HR. Therefore, naloxone-induced bradycardia was related to blockade of central OR. Norbinaltorphimine also induced pronounced bradycardia on minute 5 postinjection, which disappeared by the 10th minute. Transitory bradycardia was revealed 10 min after CTAP administration. Dalargin was administered 15 min after injection of antagonists. HR was recorded by the 20th minute after treatment with antagonists. In this period CTAP, naloxone, and naloxone methiodide had no effect on cardiac activity. Therefore, antagonist-induced transitory bradycardia observed in experiments with CTAP, naloxone, naloxone methiodide, and norbinaltorphimine did not modulate the effect of dalargin.

Intravenous injection of selective peptide (ICI 174,864 and TIPP[ψ]) and nonpeptide δ-antagonists (naltrindole) induced persistent bradycardia (Fig. 1, *b*), which peaked 15 min postinjection and persisted for 25 min. These data show that the effect of selective δ-antagonists was similar to that of DPDPE. They caused persistent bradycardia. Therefore, none of these pharmacological agents can be used in combination with dalargin. It is impossible to distinguish bradycardia induced by dalargin and δ-antagonists. BBB is not permeable for peptide agonists and antagonists of OR. However, naltrindole can cross BBB [5]. It can be hypothesized that bradycardia observed after treatment with δ-antagonists results from the interaction of ICI 174,864, TIPP[ψ], and naltrindole with peripheral receptors. There are no data that these preparations can bind to non-δ-OR receptors. Probably, δ-antagonists *in vivo* exhibit properties of partial δ-agonists.

Naloxone in a dose of 0.05 mg/kg prevented the development of bradycardia induced by D-Ala$^2$, Leu$^5$,Arg$^6$-enkephalin (Fig. 2). The chronotropic effect of D-Ala$^2$,Leu$^5$,Arg$^6$-enkephalin is associated with activation of OR. Naloxone analogue naloxone methiodide was used to determine whether these receptors are located in the brain or peripheral tissues. Naloxone methiodide does not cross BBB. Affinity of naloxone methiodide for OR is 10-20 times lower than that of naloxone [13]. Naloxone methiodide was administered in a dose of 0.5 mg/kg. This dose of naloxone methiodide is 10-fold higher than the dose of naloxone. However, this dose of naloxone methiodide was insufficient to abolish the chronotropic effect of dalargin. Dalargin-induced bradycardia was not observed only after treatment with naloxone methiodide in a dose of 2 mg/kg. Our findings provide indirect evidence that the chronotropic effect of D-Ala$^2$, Leu$^5$,Arg$^6$-enkephalin is not associated with activation of μ-OR. This effect is probably related to the interaction of dalargin with peripheral δ-OR or κ-OR.

Selective antagonists of μ-OR and κ-OR were used to test this hypothesis. The chronotropic effect of D-Ala$^2$,Leu$^5$,Arg$^6$-enkephalin persisted after CTAP injection (Fig. 2). Therefore, the decrease in HR induced by dalargin under conditions of μ-OR blockade is associated with activation of δ-OR or κ-OR. Blockade of κ-OR abolished dalargin-induced bradycardia. Hence, the negative chronotropic effect of dalargin results from activation of peripheral κ-OR. These results raised doubts, since previous

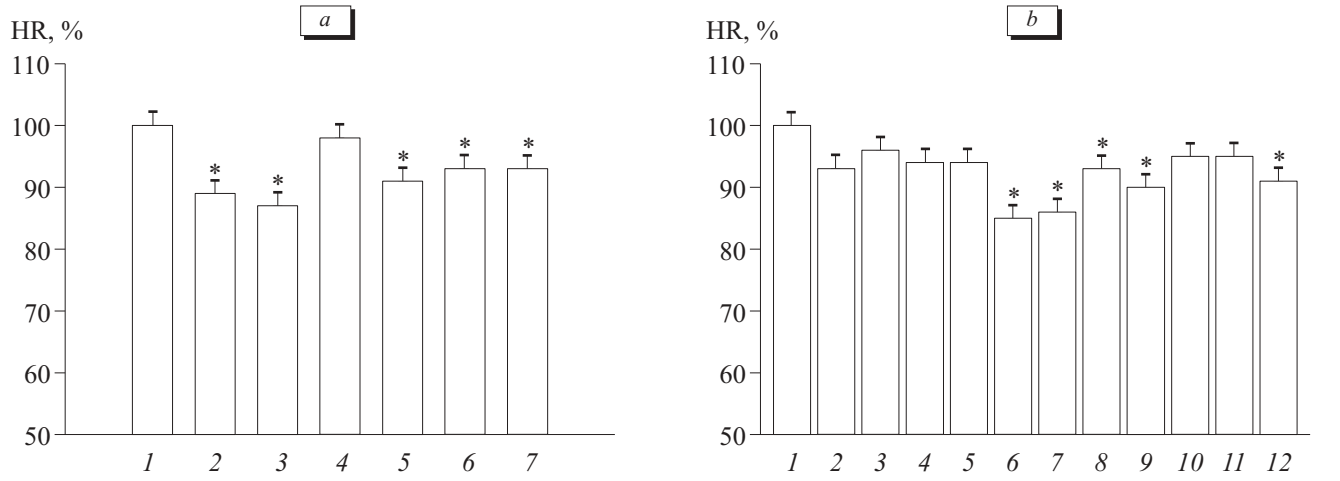


**Fig. 1.** *In vivo* effects of opioid agonists (*a*), opioid antagonists, and hexamethonium (*b*) on the heart rate (HR) in rats. *a*) Basal level, before administration of preparations (*1*); dalargin, 0.1 mg/kg (*2*); DAMGO, 0.08 mg/kg (*3*); DALDA, 0.1 mg/kg (*4*); DALDA, 0.5 mg/kg (*5*); DPDPE, 0.09 mg/kg (*6*); and (-)-U-50,488, 1 mg/kg (*7*). *b*) Basal level, before administration of preparations (*1*); naloxone, 0.05 mg/kg (*2*); naloxone methiodide, 0.5 mg/kg (*3*); naloxone methiodide, 2 mg/kg (*4*); CTAP, 0.5 mg/kg (*5*); ICI 174,864, 0.2 mg/kg (*6*); ICI 174,864, 0.5 mg/kg (*7*); TIPP[ψ], 0.5 mg/kg (*8*); naltrindole hydrochloride, 1 mg/kg (*9*); norbinaltorphimine, 2.5 mg/kg (*10*); norbinaltorphimine, 9 mg/kg (*11*); and hexamethonium, 10 mg/kg (*12*). Here and in Fig. 2: *$p<0.01$ compared to the basal level.

studies showed that D-Ala$^2$,Leu$^5$,Arg$^6$-enkephalin binds only to μ-OR and δ-OR [2,13]. We hypothesized that the selected dose of norbinaltorphimine (9 mg/kg) is too high, and the preparation in this dose blocks all OR. The dose of the test preparation was reduced to 2.5 mg/kg. However, norbinaltorphimine in a dose of 2.5 mg/kg had no effect on (-)-U-50,488-induced bradycardia. These results show that the dose of norbinaltorphimine was adequate for the purpose of our study. The negative chronotropic effect of dalargin is associated with activation of peripheral κ-receptors.

There are 2 possible reasons for the discrepancy between our results and published data [13]. First, dalargin is *in vivo* metabolized with the formation of a compound exhibiting affinity for κ-OR. And second, dalargin probably interacts with κ-OR *in vivo* and *in vitro*. It cannot be excluded that D-Ala$^2$,Leu$^5$,Arg$^6$-enkephalin binds to μ-OR (as shown in *in vitro* experiments) [2,13]. Published data show that cardiomyocyte sarcolemma carries δ-OR and κ-OR, but not μ-OR. Moreover, sympathetic and parasympathetic nerve terminals innervating the heart have δ-OR and κ-OR, but not μ-OR [3,4]. In the heart μ-OR are present only on endotheliocytes of coronary arteries [3,4], therefore the negative chronotropic effect of dalargin is associated only with activation of κ-OR (irrespective on the ability of D-Ala$^2$,Leu$^5$,Arg$^6$-enkephalin to interact with μ-receptors). Bradycardia was observed after administration of DALDA only in a dose of 0.5 mg/kg. This dose of DALDA 5-fold surpasses the dose of dalargin, although their molecular weights are similar. It can be hypothesized that DALDA in a dose of 0.5 mg/kg activates not only μ-OR, but also other types of OR. Thus, the chronotropic effect of dalargin is associated with activation of peripheral κ-receptors.

Administration of a ganglionic blocker hexamethonium results in reversible chemical denervation of organs and tissues. Pretreatment with this drug prevented the development of tachycardia induced by enkephalins [3,4]. Hexamethonium caused short-term bradycardia, which disappeared 15 min after injection (Fig. 1, *b*). Under conditions of peripheral autonomic ganglion blockade with hexamethonium, D-Ala$^2$,Leu$^5$,Arg$^6$-enkephalin caused persistent and pronounced bradycardia (Fig. 2). Therefore, this agent potentiated the negative chronotropic effect of dalargin.

Guanethidine was administered to deplete the reserve of endogenous catecholamines. This treatment caused persistent bradycardia, which was observed 1 day after the last injection of test preparation (Fig. 1, *b*). Against the background of depleted catecholamine reserves, bradycardia was observed 5 min after dalargin injection and disappeared by the 10th minute after opioid treatment (Fig. 2, *a*). In contrast to hexamethonium, guanethidine did not modulate the negative chronotropic effect of dalargin. Therefore, this effect is not mediated by the sympathoadrenal system. It can be hypothesized that bradycardia is associated with a direct effect of dalargin on the sinoatrial node. This hypothesis is consistent with our findings. We showed that HR decreases by 34% after 10-min perfusion of the heart with a solution containing 0.1 mg/kg dalargin (Fig. 3). The negative chronotropic effect of D-Ala$^2$,Leu$^5$,Arg$^6$-enkephalin probably involves cardiac κ-OR, since perfusion of the isolated heart with a solution containing (-)-U-50,488 produces the same changes (Fig. 3). Therefore, the negative chronotropic effect of D-Ala$^2$,Leu$^5$,Arg$^6$-enkephalin is related to its direct influence on the heart.

Previous studies showed that intravenous injection of dalargin in a dose of 0.1 mg/kg increased HR in narcotized rats and volunteers [6]. Our findings seem to contradict these data. We studied the individual response of each animal to D-Ala$^2$,Leu$^5$,Arg$^6$-enkephalin injection. Dalargin caused persistent bradycardia in 10 of 16 animals. Persistent tachycardia was observed in 6 animals. Different reactions of the cardiovascular system to the test preparation are probably associated with differences in basal HR. HR in animals exhibiting the positive and negative chronotropic response was 312±4 ($n$=6) and 368±6 bpm ($n$=10, $p$<0.001), respectively. Our previous experiments were performed

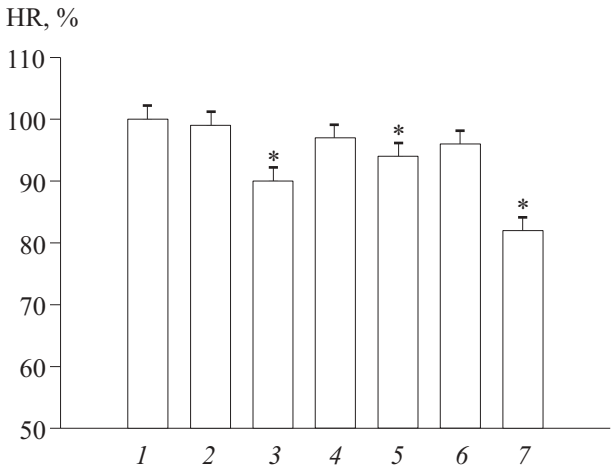


**Fig. 2.** *In vivo* effect of dalargin on HR after administration of opioid antagonists and hexamethonium. Basal level, before administration of preparations (*1*); 0.05 mg/kg naloxone and 0.1 mg/kg dalargin (*2*); 0.5 mg/kg naloxone methiodide and 0.1 mg/kg dalargin (*3*); 2 mg/kg naloxone methiodide and 0.1 mg/kg dalargin (*4*); 0.5 mg/kg CTAP and 0.1 mg/kg dalargin (*5*); 9 mg/kg norbinaltorphimine and 0.1 mg/kg dalargin (*6*); and 10 mg/kg hexamethonium and 0.1 mg/kg dalargin (*7*);

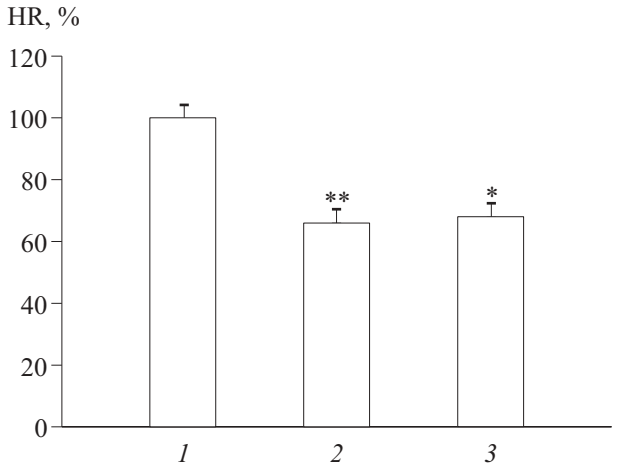


**Fig. 3.** *In vitro* effects of dalargin and (±)-U-50,488 on HR. Basal level, before administration of preparations (*1*); dalargin, 130 nmol/liter (*2*); and (±)-U-50,488, 100 nmol/liter (*3*). *$p<0.02$ and **$p<0.01$ compared to the basal level.

on rats with low basal HR (333 bpm) [6]. These animals exhibited an increase in HR after administration of dalargin. Therefore, D-Ala$^2$,Leu$^5$,Arg$^6$-enkephalin produces an "averaging effect" on HR. A similar reaction was observed in studying the regulatory role of OR in hormone secretion [3]. Dalargin increased the concentrations of glucocorticoids and vasopressin in the plasma from intact rats. However, dalargin decreased plasma hormone concentration in stressed animals with high levels of vasopressin and cortisol.

Our study on the isolated denervated heart showed that bradycardia results from activation of cardiac κ-receptors. Under these conditions circulating humoral factors do not modulate cardiac activity. In these animals dalargin reduced HR. This hypothesis is confirmed by the results of experiments with hexamethonium. This agent *in vivo* induced short-term denervation of the heart, which resulted in the development of persistent bradycardia in response to dalargin administration. This opioid *in vivo* (without hexamethonium) had a positive chronotropic effect in some rats with low basal HR. It was associated with the modulatory effect of D-Ala$^2$,Leu$^5$,Arg$^6$-enkephalin on the autonomic nervous system. This peptide cannot cross BBB. Therefore, the effects of D-Ala$^2$,Leu$^5$,Arg$^6$-enkephalin are associated with modulation of the peripheral autonomic nervous system. Opioids interact with presynaptic OR and inhibit the release of norepinephrine from sympathetic nerve terminals innervating the heart [3,4]. These changes led to bradycardia. Opioids can inhibit the release of acetylcholine via binding to presynaptic OR on *n. vagus* terminals innervating the heart. This leads to an increase in HR [3,4]. It can be hypothesized that the increase in HR after dalargin injection is associated with the reduction of acetylcholine release from *n. vagus* terminals due to activation of presynaptic κ-receptors on these terminals.

Our results show that selective δ-agonists *in vivo* exhibit properties of partial OR agonists. The positive and negative chronotropic effects of dalargin are associated with activation of peripheral κ-OR. The negative chronotropic effect of D-Ala$^2$,Leu$^5$,Arg$^6$-enkephalin results from its direct influence on the heart. The positive chronotropic effect of dalargin is related to modulation of the autonomic nervous system.

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