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κ-Opioid receptor stimulation inhibits growth of neonatal rat ventricular myocytes

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Abstract

The effects of *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methanesulfonate salt (U50,488H), a selective κ-opioid receptor agonist, on growth in neonatal ventricular myocytes were determined. In 15% serum culture medium, U50,488H at 0.1–1 μM significantly reduced the protein content, [^{3}H]leucine uptake and cell size of the myocytes. The effect of U50,488H on protein content was abolished in the presence of 1 μM nor-binaltorphimine (nor-BNI), a selective κ-opioid receptor antagonist. In a 0.4% serum medium, U50,488H at 0.1–1 μM had no effect on myocyte growth. Interestingly, 1 μM U50,488H abolished the stimulatory effects of 1 μM norepinephrine on protein content, [^{3}H]leucine uptake and cell size of the myocytes in the low serum medium. The effect of U50,488H was abolished by 1 μM nor-BNI. With the exception of cell size, the effects of norepinephrine were completely abolished by blockade of both α- and β-adrenoceptors, but only partially blocked by blockade of either adrenoceptors. These results provide first evidence that κ-opioid receptor stimulation inhibits growth of the neonatal ventricular myocyte as a result of direct action as well as by inhibiting sympathetic stimulation of the heart. The stimulatory effects of sympathetic activity on growth occurs via both α- and β-adrenoceptors.

Keywords: U50,488H; Neonatal rat ventricular myocyte; Protein content; [^{3}H]Leucine; Cell size; Adrenoceptor

1. Introduction

Cardiac hypertrophy is an adaptation to increased hemodynamic workload. Hypertrophy is characterized by enlargement of cardiomyocytes and accumulation of both contractile and non-contractile proteins as a result of a positive protein synthesis to degradation ratio. Intensive studies have been performed to delineate the mechanisms. Opioid receptors have been shown to play an important role in growth (Hatzoglou et al., 1996). Administration of naloxone, a non-selective opioid receptor antagonist, throughout gestation increased both the DNA and protein content of the heart (McLaughlin et al., 2002), indicating inhibitory effects of opioid receptor activation on growth of the heart. Administration of met-enkephalin, a selective δ-opioid receptor agonist, inhibited the DNA synthesis of ventricular myocardial epicardial cells in 1-day-old rats and the effect was antagonised by another non-selective opioid receptor antagonist, naltrexone (McLaughlin, 1996). This result suggests a direct action of opioid peptides on growth via the opioid receptor in the heart. Receptor binding studies have demonstrated the presence of substantial κ-opioid receptor in the heart (Ventura et al., 1989; Tai et al., 1991; Jin et al., 1995; Zhang et al., 1996). It is therefore hypothesized that activation of κ-opioid receptor may inhibit growth/hypertrophy of the heart.

κ-Opioid receptor stimulation affects cardiac function as a result of direct action (Wong et al., 1990) as well as by inhibiting the actions of sympathetic stimulation (Yu et al., 1998) in the heart. It is likely, therefore, that if κ-

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opioid receptor stimulation indeed inhibits growth, this might result from direct action as well as inhibition of sympathetic stimulation. The purpose of the present study was twofold. Firstly, we tested the hypothesis that activation of κ-opioid receptor inhibits growth/hypertrophy of the heart. Secondly, we tested the hypothesis that the inhibitory effect of κ-opined receptor stimulation results, at least partly, from inhibiting the sympathetic stimulation via adrenoceptors. Two series of experiments were performed in ventricular myocytes from neonatal rats. In the first, we determined the effects of U50,488H, a selective κ-opioid receptor agonist, on myocyte growth in high serum medium, that enhances growth. In the second, we determined the effects of U50,488H at a concentration at which it had no effect in a low serum medium, on myocyte growth enhanced by norepinephrine. In addition, we determined the effects of blocking β- and/or α-adrenoceptors on norepinephrine -induced hypertrophy. Protein content, [^{3}H]leucine uptake and cell size were used as indices of growth. Results showed that κ-opioid receptor stimulation inhibits growth of neonatal rat cardiomyocytes via both direct action and inhibition of sympathetic stimulation. Norepinephrine-induced growth is mediated by both α- and β-adrenoceptors.

2. Materials and methods

2.1. Culture of neonatal rat ventricular myocytes

The study was approved by the Committee on the use of Live Animals for Teaching and Research, The University of Hong Kong. Sprague–Dawley rats, 1–3 days old, were killed and the hearts removed. The ventricle was separated from the atrium, trisected, and digested with trypsin (0.6 mg ml^{-1}, Sigma) for 20 min at 37 °C. Ventricular myocytes were cultured as described previously (Wang et al., 1995). The cell supernatant was collected by centrifugation, and the pellet was re-suspended in fetal bovine serum. The above steps were repeated 7–10 times until the ventricle was completely digested. The cell suspension was diluted to 1×10^6 ml^{-1} and placed in 24-well tissue culture plates in humidified 5% CO_2/95% air at 37 °C. The culture medium consisted of 15% heat-inactivated fetal bovine serum, 84% RPMI 1640 (Roswell Park Memorial Institute) medium and 1% penicillin–streptomycin solution, shown to enhance growth of cultured ventricular myocytes (Wang et al., 1995). In experiments involving norepinephrine, a low serum (0.4%) RPMI 1640 medium was used. The myocardial cells were made "quiescent" in low serum medium and did not grow (Berk et al., 1989).

2.2. Cellular protein content determination

Cells were cultured for 72 h in the presence of various compounds in the culture medium. Seventy-two hours was chosen because preliminary studies in our laboratory showed that maximum effects were obtained after 72 h. Dishes were washed rapidly three times with Hanks solution, the cells were dissolved in 1% sodium dodecylsulphate (SDS) and the protein content was measured by Lowry's method (Lowry et al., 1951).

2.3. [^{3}H]Leucine uptake

[^{3}H]Leucine uptake was used as an index of protein synthesis. The medium from cultured myocardial cells grown in 24-well plates was aspirated and replaced with a medium containing 1 μCi [^{3}H]leucine (Shanghai Nuclear Institute, China). Drugs were added and incubation continued for 48 h. The medium was then aspirated and cells were washed rapidly three times with cold Hanks solution. They were then lysed by addition of 1 ml/well 1% SDS. Lysates were collected and precipitated by addition of 1ml 5% trichloroacetic acid (TCA) and then applied to glassfibre GF/C filters. After washing with 5 ml Hanks solution three times, filters were dried and transferred to vials containing 4 ml scintillation fluid and the radioactivity was determined by the liquid scintillation method (Luo et al., 2001). The radioactivity, which represented the [^{3}H]leucine incorporated into newly synthesized protein, was expressed as cpm per 10^5 cells.

2.4. Measurement of cell size

The volume of ventricular myocytes was obtained by measuring cell diameter (Zheng et al., 1996). The medium was aspirated and cells were washed rapidly three times with D-Hanks solution. Cells were then treated with 0.3 ml/well 0.1% trypsin at 37 °C for 10 min and then the process was terminated with 10% fetal bovine serum (0.2 ml/well). Lysates were collected and measured on an inverted microscope. For cell size measurements, four or five fields were randomly chosen and photographed at high power (×400), and 75 individual cell areas were measured by a computer photograph analysis system.

2.5. Chemicals

Trans-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methanesulfonate salt (U50,488H), a selective κ-opioid receptor agonist (Zukin et al., 1988; Rothman et al., 1990), and nor-BNI, a selective κ-opioid receptor antagonist (Portoghese et al., 1987; Takemori et al., 1988; Tortella et al., 1989), were used to stimulate and block the κ-opioid receptor. Prazosin, an α_1-adrenoceptor-selective antagonist, and propranolol, a non-selective β-adrenoceptor antagonist, were used to suppress norepinephrine-induced cardiac hypertrophy via the α_1- and β_1-adrenoceptor pathways, respectively. The concentrations of U50,488H (Tai et al., 1992; Ventura et al., 1992; Sheng and Wong, 1996), nor-BNI (Tai et al.,

1992; Ventura et al., 1992; Sheng and Wong, 1996), norepinephrine (Xiao et al., 1997), prazosin (Amin et al., 2001) and propranolol (Amin et al., 2001) adopted were based on previous studies. All drugs were initially dissolved in distilled water and subsequently diluted in culture medium.

U50,488H, nor-BNI and norepinephrine were purchased from Tocris Cookson (USA). Prazosin and trypsin were purchased from Sigma. Propranolol was from Lisheng Pharmaceutical Factory (China). RPMI 1640 medium was from GIBCO BRL. Fetal bovine serum was obtained from SiJiQing Chemical (Hangzhou, China).

2.6. Statistical analysis

All data are expressed as mean±S.E.M. For effects of drugs at different concentrations; analysis of variance (one-way ANOVA) was used for comparison between control and treatment groups. The unpaired Student's *t*-test was used to test for differences between groups. *P*-value <0.05 was considered statistically significant.

3. Results

3.1. Effects of U50,488H on total protein content, [^{3}H]leucine uptake and cell size of cultured ventricular myocytes from neonatal rats

To determine whether κ-opioid receptor stimulation inhibited growth, U50,488H was added to cultured myocytes at final drug concentrations of 0.01, 0.1 and 1 μM. Incubation lasted for 48 h in 15% serum medium. As shown in Fig 1A, the protein content was reduced by 26% and 40% following treatment with 0.1 and 1 μM U50,488H, respectively. The reductions were statistically significant and concentration-dependent. The effect of 1 μM U50,488H was abolished in the presence of 1 μM nor-BNI (Fig 1A), confirming that the effect was mediated via the κ-opioid receptor. To further assess the effects of U50,488H on growth, we determined its effects on [^{3}H]leucine uptake and cell size. U50,488H at 0.1 and 1 μM inhibited [^{3}H]leucine uptake (Fig. 1B) and reduced cell size (Fig. 1C) concentration-dependently.

On the other hand, in 0.4% serum medium U50,488H at 0.01–1 μM did not significantly reduce the protein content (data not shown). Therefore, in subsequent studies to determine interactions between κ-opined receptor and adrenoceptors, the low serum medium was used.

3.2. Effects of U50,488H on norepinephrine-induced increase in protein content of cultured ventricular myocytes from neonatal rats

To investigate whether κ-opined receptor stimulation with U50,488H also inhibited growth induced by

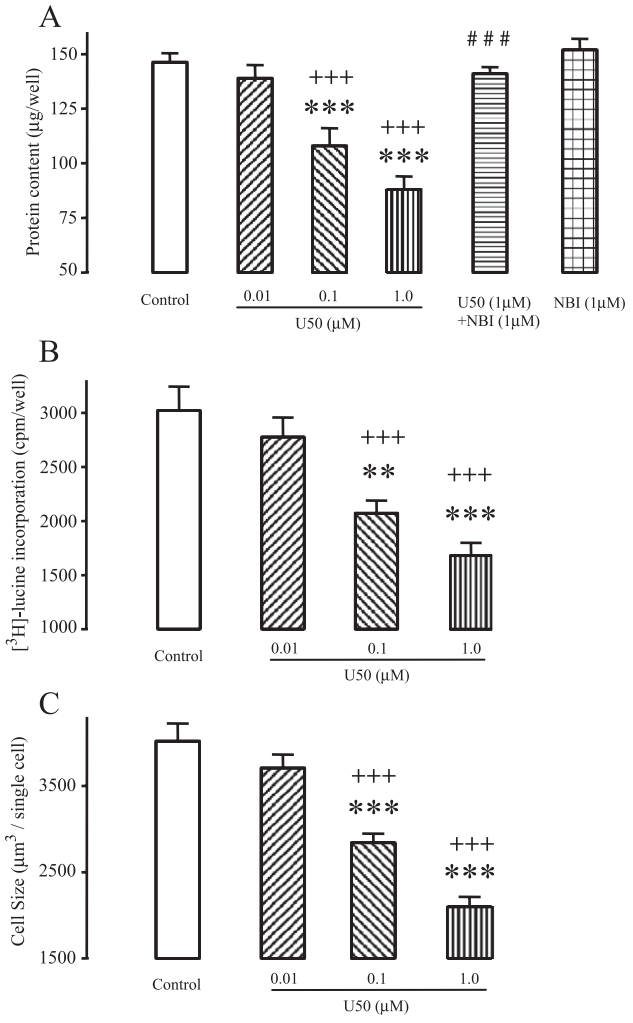

Fig. 1. (A) Effects of U50,488H (0.01–1 μM) on protein content of ventricular myocytes from neonatal rats cultured in normal serum medium. Values are mean±S.E.M., *N*=6 in each group. ***P<0.001 vs. control group; $^{\#\#\#}P$<0.001 vs. U50 1 μM; ^{+++}P<0.001 vs. the higher concentration of U50. U50: U50,488H; NBI: nor-BNI. (B) Effects of U50,488H (0.01–1 μM) on [^{3}H]leucine incorporation of ventricular myocytes from neonatal rats cultured in normal serum medium. Values are mean± S.E.M., *N*=6 in each group. $^{**,***}P$<0.01, 0.001 vs. control group; U50: U50,488H; ^{+++}P<0.001 vs. the higher concentration of U50. (C) Effects of U50,488H (0.01–1 μM) on cell size of ventricular myocytes from neonatal rats cultured in normal serum medium. Values are mean±S.E.M., *N*=60 in each group. ***P<0.001 vs. control group; ^{+++}P<0.001 vs. the higher concentration of U50. U50: U50,488H.

sympathetic stimulation, we first determined the effects of U50,488H at 0.01–1 μM on the protein content of myocytes treated with 1 μM norepinephrine. Norepinephrine (1 μM) significantly increased the protein content of myocytes cultured in 0.4% serum medium (Fig. 3). U50,488H at 0.01–1 μM, which itself had no significant effect, concentration-dependently reduced the protein content in the presence of norepinephrine; the effect of 1 μM U50,488H was abolished in the presence of 1 μM nor-BNI (Fig. 2).

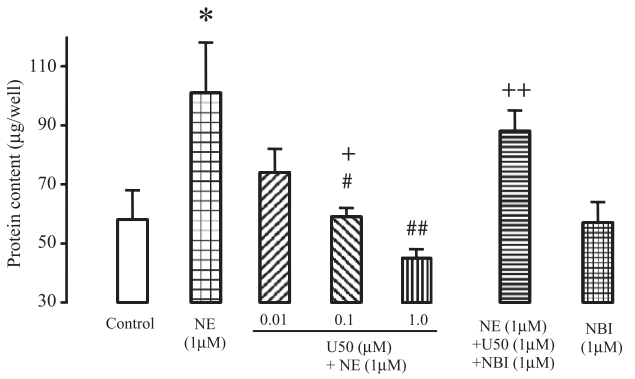

Fig. 2. Inhibitory effects of U50,488H (0.01–1 μM) on norepinephrine-induced increase in protein content in ventricular myocytes from neonatal rats cultured in low serum medium. Values are mean±S.E.M., N=6 in each group. *P<0.05 vs. control group; $^{\#,\#\#}P$<0.05, 0.01 vs. norepinephrine group; $^{+,++}P$<0.05, 0.01 vs. norepinephrine+U50488H 1 μM. NE: norepinephrine; U50: U50,488H; NBI: nor-BNI.

3.3. Effects of κ-opined receptor stimulation or blockade of adrenoceptors on norepinephrine-induced enhancement in protein content, [^{3}H]leucine uptake and cell size of cultured ventricular myocytes from neonatal rats

κ-Opined receptor stimulation with 1 μM U50,488H, or blockade of both α- and β-adrenoceptors with 0.1 μM prazosin and 2 μM propranolol, restored the protein content (Fig. 3A), [^{3}H]leucine uptake (Fig. 3B) and cell size (Fig. 3C) induced by norepinephrine to control levels. Blockade of either α- or β-adrenoceptor significantly reduced the enhancements in protein content (Fig. 3A), [^{3}H]leucine uptake (Fig. 3B) and cell size (Fig. 3C) by norepinephrine. With the exception of cell size, the levels after blockade of either adrenoceptor remained significantly greater than those of the control (Fig. 3).

4. Discussion

Previous studies (McLaughlin, 1994; McLaughlin, 1996; McLaughlin, 2002) have shown that naloxone and naltrexone, the non-selective opioid receptor agonists, enhance growth of the heart while met-enkephalin, a δ-opioid receptor agonist, inhibits the growth of cultured ventricular myocytes in a naloxone/naltrexone-reversible manner. These observations indicate that activation of opioid receptor inhibits growth of the heart. In the present study, we observed that U50,488H, a κ-opioid receptor agonist, inhibited the growth of cultured myocytes and the effect was abolished by a selective κ-opioid receptor antagonist, nor-BNI. This is the first evidence that the κ-opioid receptor is involved in growth of the heart. This is not in agreement with the previous observation (McLaughlin, 1996) that κ-opioid receptor agonists, dynorphin A-(1–13), U69593 and ethylketocyclazocine, had no effect on the growth of the 1-day-old rat heart. However, in that study no growth was experimentally induced in an in vitro organ culture, whereas in our study growth was experimentally induced by a 15% serum medium in vitro.

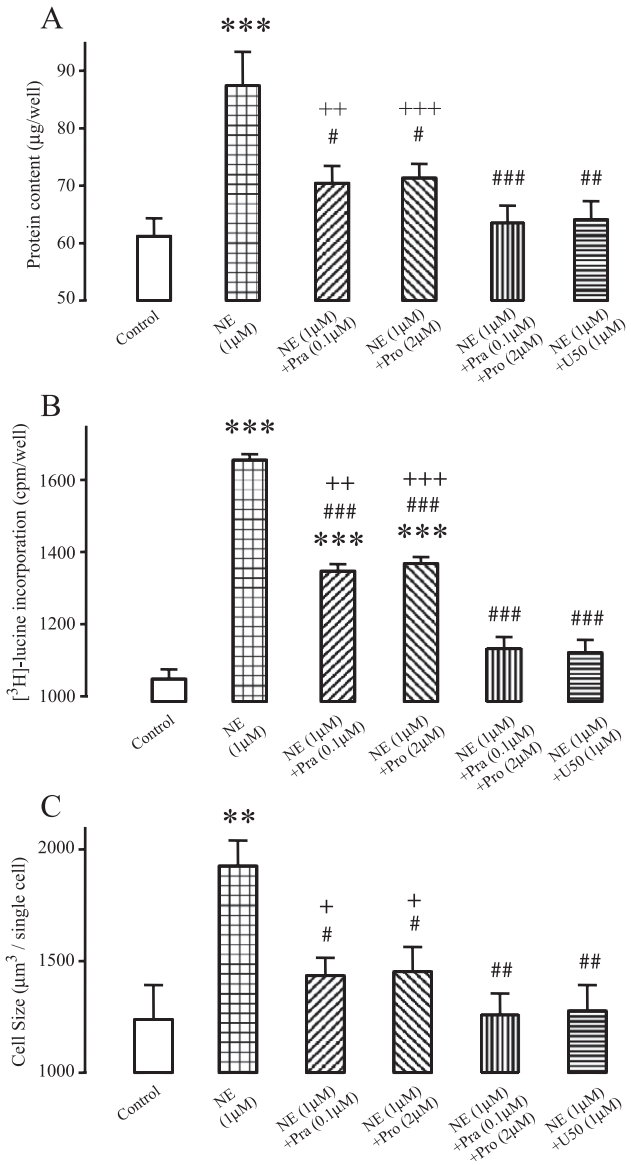

Fig 3. (A) A comparison of the effects of U50,488H, prazosin, and/or propranolol on norepinephrine-induced increase in protein content of cultured ventricular myocytes from neonatal rats. Values are mean±S.E.M., N=6 in each group. ***P<0.001 vs. control group; $^{\#,\#\#,\#\#\#}P$<0.05, 0.01, 0.001 vs. norepinephrine group; $^{++,+++}P$<0.01, 0.01 vs. norepinephrine+prazosin+propranolol. NE: norepinephrine; U50: U50,488H; Pra: prazosin; Pro: propranolol. (B) Effects of U50,488H, prazosin, and/or propranolol on the norepinephrine-induced increase in [^{3}H]leucine incorporation in cultured ventricular myocytes from neonatal rats. Values are mean±S.E.M., *N=8 in each group. ***P<0.001 vs. control group; $^{\#\#\#}$P<0.001 vs. norepinephrine group; $^{++,+++}$P<0.01, 0.001 vs. norepinephrine+prazosin+propranolol group. NE: norepinephrine; U50: U50,488H; Pra: prazosin; Pro: propranolol. (C) Effects of U50,488H, prazosin, and/or propranolol on the norepinephrine-induced increase in cell size in cultured ventricular myocytes from neonatal rats. Values are mean±S.E.M., N=80 in each group. **P<0.01 vs. control group; $^{\#,\#\#}$P<0.05, 0.01 vs. norepinephrine group. $^{+}$P<0.05 vs. norepinephrine+prazosin+propranolol. NE: norepinephrine; U50: U50,488H; Pra: prazosin; Pro: propranolol.*

An interesting observation in the present study is that U50,488H, at a concentration range which itself has no effect, inhibited the growth enhancement by norepinephrine in myocytes in low serum medium and this effect was abolished by blocking κ-opioid receptor with nor-BNI. So, in addition to a direct action, U50,488H inhibits growth by inhibiting the action of sympathetic activation. This is in agreement with our previous observation that κ-opioid receptor stimulation inhibits the action of norepinephrine on the heart (Yu et al., 1998). Results from both studies are evidence of cross-talk between κ-opioid receptor and adrenoceptors.

Previous functional (Zhang et al., 1999) and binding (Zhang et al., 1996) studies have shown that κ-opioid receptor is the predominant opioid receptor in the heart. In addition, mRNA of the precursor of dynorphin, a κ-opioid receptor agonist, is expressed in cultured myocytes (Ventura et al., 1994), indicating that the heart produces the peptide. The findings suggest that the endogenous κ-opioid peptides may participate in the regulation of growth/hypertrophy of the heart in an autocrine or paracrine manner.

It should be noted that nor-BNI itself had no effect, indicating that in the in vitro condition, there is no basal activity. This is in agreement with a previous finding that in cardiomyocytes, naloxone, an opioid receptor antagonist, has no effect (McLaughlin, 1996). On the other hand, naltrexone, another opioid receptor antagonist, administered throughout gestation alters postnatal heart development (McLaughlin, 2002), indicating that in vivo there is a basal release of opioid peptides.

Our previous studies showed that κ-opioid receptor stimulation suppresses the adenylyl cyclase/cyclic AMP cascade (Zhang and Wong, 1998). Furthermore, κ-opioid receptor stimulation inhibits the action of β-adrenoceptor activation on the heart, mainly as a result of cross-talk between the Gs-protein of the β-adrenoceptor signaling pathway and the Gi/o protein of the κ-opioid receptor signaling pathway (Shan et al., 2002). In both cases, κ-opioid receptor stimulation may lead to a reduction in protein kinase A (PKA), known to play an important role in growth and differentiation (Zolk et al., 2003). It is known that both PKA_1 and PKA_2 are involved and a balance between these two isoforms is essential in the control of growth and differentiation. Further studies are warranted to delineate how κ-opioid receptor stimulation affect these two isoforms, thus inhibiting growth in the heart.

It is well established that norepinephrine induces cardiac hypertrophy. There is evidence suggesting that the hypertrophic effect of norepinephrine is mediated via the α-adrenoceptor, which activates the Gq-protein (Adams and Brown, 2001), whereas activation of the Gs-coupled β-adrenoceptor induces apoptosis (Steinberg, 1999). However, in the present study, we found that the hypertrophic action of norepinephrine was eliminated when both α- and β-adrenoceptors were blocked, but merely reduced when either α- or β-adrenoceptor was blocked. This finding suggests that both α- and β-adrenoceptor may be involved in the norepinephrine-induced hypertrophy. Further studies are needed to clarify the role of these adrenoceptor types in growth/hypertrophy.

The mammalian myocardium expresses κ-opined receptor neonatally and continue to express in the adult (Zimlichman et al., 1996). On the other hand, both α- (Nakanishi et al., 1989) and β- (Fan and Banerjee, 1985) adrenoceptors decrease with age in the heart . In the present study, we used ventricular myocytes of neonatal rats. Therefore the direct effect of κ-opioid receptor stimulation on growth may still apply to the adult rat heart while the inhibition of norepinephrine induced growth by κ-opioid receptor may not be relevant to the adult rat heart.

In conclusion, for the first time the present study provides evidence that κ-opioid receptor inhibits growth/hypertrophy of the heart. The effect may result from direct action on the heart as well as by inhibiting the hypertrophic action of norepinephrine.

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