Atrial natriuretic peptide stimulates Na^+-dependent Ca^{2+} efflux from freshly isolated adult rat cardiomyocytes

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Received 26 September 1997; revised version received 12 November 1997

Abstract In the present study, we examined the effect of atrial natriuretic peptide (ANP) on Ca^{2+} efflux from freshly isolated adult rat cardiomyocytes. Rat ANP(1–28) stimulated the efflux of $^{45}Ca^{2+}$ from the cells in a concentration-dependent manner (10^{-8} M to 10^{-6} M). The $^{45}Ca^{2+}$ efflux was not affected by removal of extracellular Ca^{2+}, but was dependent on the presence of extracellular Na^+. In addition, rat ANP(1–28) caused $^{22}Na^+$ influx into the cells. The $^{45}Ca^{2+}$ efflux was also stimulated by C-type natriuretic peptide-22 (CNP-22), but not by rat brain natriuretic peptide-45 (BNP-45). It was also observed that both rat ANP(1–28) and CNP-22 stimulated guanosine 3′,5′-cyclic monophosphate production within the cells. These results indicate that ANP stimulates Na^+-dependent $^{45}Ca^{2+}$ efflux from freshly isolated adult rat cardiomyocytes, probably through Na^+/Ca^{2+} exchange, and that the stimulatory effect of ANP on Ca^{2+} efflux may be mediated via the natriuretic peptide receptor which has been shown to couple to guanylate cyclase. Since it is reported that Na^+/Ca^{2+} exchange is important in calcium homeostasis within cells, ANP may play a role in the extrusion of intracellular Ca^{2+} from isolated adult rat cardiomyocytes.

Key words: Atrial natriuretic peptide; Ca^{2+} efflux; Na^+/Ca^{2+} exchange; Guanosine 3′,5′-cyclic monophosphate; Natriuretic peptide receptor; Cardiomyocyte

1. Introduction

Atrial natriuretic peptide (ANP) was originally identified as a natriuretic substance from human atrium [1]. It has also been reported that this peptide affects water balance [2] and vascular tone [3] in many species. In cardiac tissues, ANP has been shown to cause a negative inotropic effect [4] and cell proliferation [5] in an autocrine/paracrine manner. The negative inotropic effect of ANP on the heart has been implicated to the inhibition of voltage-dependent Ca^{2+} channels in the cardiac cell membrane [6]. On the other hand, it has been reported that ANP reduces basal cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) without affecting voltage-gated Ca^{2+} currents in guinea-pig cardiomyocytes [7]. Therefore, the effect of ANP on intracellular Ca^{2+} homeostasis in cardiomyocytes is currently a topic of controversy. The exact mechanism of ANP on $[Ca^{2+}]_i$ regulation in cardiomyocytes still remains to be elucidated.

It is well known that several physiological stimuli cause a transient rise in $[Ca^{2+}]_i$ [8,9] which leads to contraction of cardiomyocytes. Following stimulation, increased $[Ca^{2+}]_i$ must rapidly return to resting level in order to elicit a response to subsequent stimulation. This decrease in $[Ca^{2+}]_i$ may be attributed mainly to the mechanism of Na^+/Ca^{2+} exchange at the plasma membrane and Ca^{2+} uptake into the sarcoplasmic reticulum [10]. However, little is known about the precise mechanism of this decrease. We have thus hypothesized that ANP is involved in the reduction of $[Ca^{2+}]_i$ in cardiomyocytes. In the present study, we focused on the mechanism of the decrease in $[Ca^{2+}]_i$ and examined the effect of rat ANP(1–28) itself on Ca^{2+} efflux from freshly isolated, quiescent adult rat cardiomyocytes. Moreover, the effect of rat ANP(1–28) on Ca^{2+} efflux was compared to that of rat brain natriuretic peptide-45 (BNP-45) and C-type natriuretic peptide-22 (CNP-22).

2. Materials and methods

2.1. Preparation of adult rat cardiomyocytes

Treatment of animals was based on the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised in 1985). Isolation of adult rat cardiomyocytes was performed by the method of Mitra and Morad with minor modifications [11]. Male Sprague-Dawley rats weighing 250–300 g were used. The rats were anesthetized by inhalation of saturated diethylether gas and were administered heparin (1000 U/kg) intravenously. The heart was excised and the aorta cannulated in a Langendorff perfusion apparatus[12]. The heart was then perfused for 3 min with calcium-free buffer medium (medium A: 135 mM NaCl, 5.6 mM KCl, 1.0 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 10.0 mM HEPES, 0.2% bovine serum albumin (BSA), adjusted with NaOH to pH 7.30). Thereafter, the perfusion medium was changed to a cell dispersing medium, medium B (medium A supplemented with 0.2% collagenase I and 0.04% protease XIV) for 12 min. After digestion, these enzymes were washed out with medium C (medium A containing 220 μM of $CaCl_2$) for 3 min. After the completion of perfusion, the heart was removed from the cannula and placed in a Petri dish containing 25 ml of medium D (medium C supplemented with 2% BSA). Small pieces of tissue (3×3 mm) were cut from the ventricle and gently shaken in 25 ml of medium D to release the dispersed cells. The cells were resuspended once in 25 ml of a calcium-containing medium to prevent Ca^{2+} paradox [13] (0.44 mM of $CaCl_2$ with medium D, to increase Ca^{2+} concentration stepwise) and centrifuged at 15×*g* for 30 s. Afterwards, the cells were washed once with modified Krebs-Henseleit bicarbonate buffer solution (K-H solution) (135 mM NaCl, 5.6 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 10 mM glucose, adjusted with HCl to pH 7.40) oxygenated with 95% of O_2 and 5% of CO_2 gas

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Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; cGMP, guanosine 3′,5′-cyclic monophosphate; $[Ca^{2+}]_i$, cytosolic free calcium concentration; K-H solution, Krebs-Henseleit bicarbonate buffer solution; NPR, natriuretic peptide receptor

PII S0014-5793(97)01470-1

mixture. Finally, the isolated cardiomyocytes were resuspended in 5 ml of K-H solution. The counted number of intact, rod-shaped cells using a hemocytometer was approximately 1.0×10^6 cells/ml, and these cells were quiescent when observed by microscope.

2.2. $^{45}Ca^{2+}$ efflux assay

The method for measurement of $^{45}Ca^{2+}$ efflux from the cells was as described previously [14]. Briefly, the isolated adult rat cardiomyocytes were incubated with 5 ml of K-H solution containing $^{45}CaCl_2$ (4 μCi/ml) for one hour at 37°C. Then 1.5-ml volumes of the cell suspension (ca. 1.5×10^6 cells) were resuspended and centrifuged twice with 10 ml of K-H solution and were applied to a custom-made superfusion column consisting of 20 μm pore-size filter. This column was cone-shaped, 5 cm long and 1 cm in diameter, and the cells were stratified on the filter. K-H solution was dropped onto the cells to keep the medium volume constant (ca. 1 ml), and the effluent from the column was collected. The cells were then superfused with K-H solution for 15 min to remove the unincorporated $^{45}Ca^{2+}$ (flow rate of approximately 1 ml/min). Afterwards, the effluent was collected 15 times at intervals of 30 s (ca. 1 ml/min) to determine the basal efflux level. The cells were then superfused with the reaction mixture with or without test agents, and the effluent was again collected 15× at intervals of 30 s to determine the agonist-stimulated $^{45}Ca^{2+}$ efflux levels. After agonist stimulation, the cells in the column were collected and dissolved in 1 ml of 1% Triton X-100 solution to determine the residual $^{45}Ca^{2+}$ in the cells. For experiments with Ca^{2+} or Na^+ removed from the medium, Ca^{2+}-free K-H solution or Na^+-deficient medium which was prepared with sucrose instead of Na^+ was used. Extracellular rat ANP(1–28) administered at 10^{-6} M did not cause contraction of the cardiomyocytes, as confirmed by microscopic observation. Samples were counted in 10 ml of liquid scintillation fluid for a 2-min period. The total radioactivity of $^{45}Ca^{2+}$ in the cells was determined as the sum of the radioactivity in each fraction and the residual radioactivity, and this value was used to calculate the fractional release of Ca^{2+} from the cells in each period.

2.3. Measurement of $^{22}Na^+$ influx into the cells

The isolated adult rat cardiomyocytes were incubated with 1 ml of K-H solution containing $^{22}Na^+$ (3 μCi/ml) in the presence or absence of test agents for 5 min at 37°C. After incubation, the medium was discarded and the cells were washed 3× with 1 ml of ice-cold K-H solution. The intracellular $^{22}Na^+$ was then extracted with 1 ml of 1% Triton X-100 and measured with a gamma counter as described previously [15].

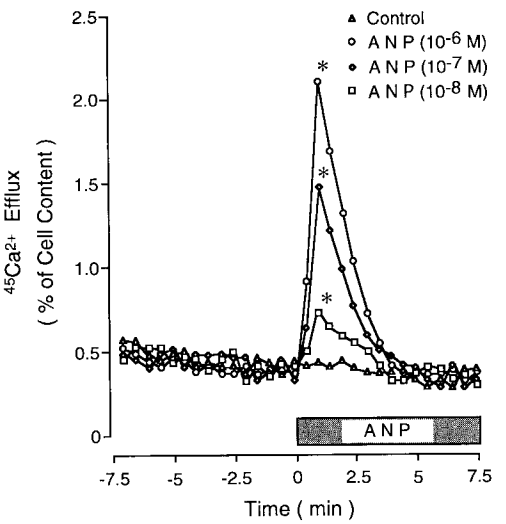

Fig. 1. Effects of various concentrations of rat ANP(1–28) on $^{45}Ca^{2+}$ efflux from freshly isolated adult rat cardiomyocytes. Data are means for 5 separate experiments. The maximal standard error was 8.1% of the mean value on ordinate at peak. To avoid complicating the figure, error bars are omitted. All peak levels with rat ANP(1–28) at 10^{-8} M to 10^{-6} M were significantly greater than the control level (*$P<0.05$).

2.4. Measurement of guanosine 3′,5′-cyclic monophosphate (cGMP) production within the cells

Rat ANP(1–28)-induced guanosine 3′,5′-cyclic monophosphate (cGMP) production was measured using a commercially available enzyme immunoassay kit [16]. Briefly, 1 ml of cell suspension (1×10^6 cells) was preincubated at 37°C for 20 min with 5×10^{-4} M 3-isobutyl-1-methylxantine (IBMX) to facilitate the measurement of cGMP production. Then, the cells were incubated at 37°C for 10 min with or without test agents, and the reaction was stopped by the addition of 100 ml of 100% trichloroacetic acid. To sediment the precipitate, the test tube was centrifuged at $2000\times g$ for 10 min and the resultant cGMP in the supernatant was assayed with the cyclic GMP enzyme immunoassay system. Results were expressed as pmol/10^6 cells.

2.5. Statistics

One-way ANOVA was used to determine the significance among groups, after which modified *t*-test with Bonferroni correction was used for comparison between individual groups. A value of $P<0.05$ was considered to be statistically significant.

2.6. Chemicals

$^{45}CaCl_2$ and the cyclic GMP enzyme immunoassay system were obtained from Amersham Corp. (Tokyo, Japan). Rat ANP(1–28), rat BNP-45 and CNP-22 were purchased from Peptide Institute Inc. (Osaka, Japan). Sodium nitroprusside was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were commercial products of reagent grade.

3. Results

3.1. Effect of ANP on $^{45}Ca^{2+}$ efflux from freshly isolated adult rat cardiomyocytes

The stimulatory effect of rat ANP(1–28) on $^{45}Ca^{2+}$ efflux was dose-dependent at concentrations of 10^{-8} to 10^{-6} M (Fig. 1). The efflux of $^{45}Ca^{2+}$ increased to a peak value within approximately 1 min after ANP addition. The peak value with 10^{-6} M rat ANP(1–28) was 2.1 ± 0.17% of total $^{45}Ca^{2+}$ within the cells. After the peak, the efflux level decreased rapidly within the next 5 min. The efflux then returned to almost prestimulation levels.

3.2. Influence of removal of extracellular Ca^{2+} or Na^+ on ANP-stimulated $^{45}Ca^{2+}$ efflux

Next, we determined whether ANP-induced $^{45}Ca^{2+}$ efflux is dependent on the presence of extracellular Ca^{2+} or Na^+. We carried out a series of experiments in the absence of extracellular Ca^{2+} or Na^+. As shown in Fig. 2, rat ANP(1–28)-induced $^{45}Ca^{2+}$ efflux was not influenced by the absence of extracellular Ca^{2+}. However, Na^+-free medium which was completely replaced by sucrose significantly inhibited rat ANP(1–28)-induced $^{45}Ca^{2+}$ efflux from the cells. From these results, it is assumed that the effect of ANP in stimulating Ca^{2+} efflux across the plasma membrane may be mediated by an extracellular Na^+-dependent mechanism in isolated rat cardiomyocytes, presumably Na^+/Ca^{2+} exchange mechanism.

3.3. Effect of ANP on $^{22}Na^+$ influx into the cells

To evaluate the possibility that ANP-induced $^{45}Ca^{2+}$ efflux from the cells is mediated by the Na^+/Ca^{2+} exchange mechanism, we examined the effect of rat ANP(1–28) on $^{22}Na^+$ influx into the cardiomyocytes. Experiments were performed using normal medium and Ca^{2+}-free medium. $^{22}Na^+$ influx into the cells was induced by ANP (control, 20.2 ± 1.7 nmol/10^6 cells; 10^{-6} M rat ANP(1–28), 32.1 ± 2.8 nmol/10^6 cells; 10^{-6} M rat ANP(1–28) in Ca^{2+} medium, 29.5 ± 2.1 nmol/10^6

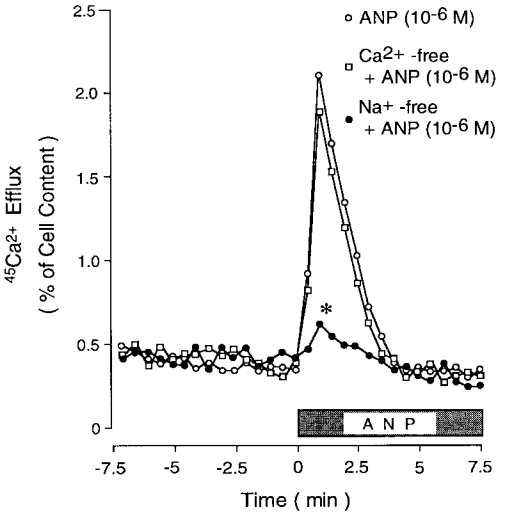

Fig. 2. Influence of Ca^{2+} or Na^+ removal from the medium on rat ANP(1–28)-induced $^{45}Ca^{2+}$ efflux from freshly isolated adult rat cardiomyocytes. The medium was changed to Ca^{2+}- or Na^+-free medium 5 min prior to rat ANP(1–28) administration. Na^+-free medium was prepared using sucrose instead of Na^+. Data are means for 5 separate experiments. The maximal standard error was 9.5% of the mean value on ordinate at peak. To avoid complicating the figure, error bars are omitted. The peak level with rat ANP(1–28) in Na^+-deficient medium was significantly less than that with rat ANP(1–28) in normal medium (*$P < 0.05$).

cells). Therefore, ANP-induced $^{45}Ca^{2+}$ efflux from the cells, which is mediated by the Na^+/Ca^{2+} exchange mechanism, is suggested.

3.4. Effects of rat BNP-45 and CNP-22 on $^{45}Ca^{2+}$ efflux from the cells and comparison with that of rat ANP(1–28)

To determine whether ANP specifically stimulates $^{45}Ca^{2+}$ efflux from the cells, we examined the effects of other types of natriuretic peptides on $^{45}Ca^{2+}$ efflux. As shown in Table 1, the efflux of $^{45}Ca^{2+}$ from the cells was stimulated by CNP-22 (10^{-6} M) to almost the same extent as by rat ANP(1–28) (10^{-6} M). However, rat BNP-45 (10^{-6} M) failed to cause $^{45}Ca^{2+}$ efflux from the cardiomyocytes.

3.5. Effects of rat ANP(1–28), rat BNP-45 and CNP-22 on cGMP production in isolated adult rat cardiomyocytes and comparison with that of sodium nitroprusside

As shown in Table 2, application of 10^{-6} M rat ANP(1–28) caused a significant increase in cGMP production during the 10 min incubation. The rat BNP-45 (10^{-6} M)-induced cGMP production was less pronounced than that of ANP and there was no statistical significance from the control value. CNP-22 (10^{-6} M) also caused a significant rise in cGMP accumulation to almost the same extent as ANP. Sodium nitroprusside (10^{-4} M), which is known to be a potent stimulator of guanylate cyclase, caused approximately a 10-fold increase in cGMP production over the basal value.

Table 1
Effects of rat ANP(1–28), rat BNP-45 and CNP-22 on $^{45}Ca^{2+}$ efflux from freshly isolated adult rat cardiomyocytes

	Peak of $^{45}Ca^{2+}$ efflux (% of cell content)
Control	0.41 ± 0.06
Rat ANP(1–28) (10^{-6} M)	2.1 ± 0.17*
Rat BNP-45 (10^{-6} M)	0.62 ± 0.08
CNP-22 (10^{-6} M)	2.0 ± 0.18*

Cells were preloaded with $^{45}CaCl_2$ as described in the text. After the stabilization of basal efflux level, rat ANP(1–28) or rat BNP-45 or CNP-22 was added at the final concentration of 10^{-6} M, then the cells were superfused for 7.5 min. Data are means ± S.E. for 5 separate experiments. The asterisk (*) represents the statistical significance from the control value ($P < 0.05$).

Table 2
Effects of rat ANP(1–28), rat BNP-45 and CNP-22 on cGMP production in isolated adult rat cardiomyocytes

	pmol/10^6 cells
Control	0.16 ± 0.03
Rat ANP(1–28) (10^{-6} M)	0.81 ± 0.16*
Rat BNP-45 (10^{-6} M)	0.21 ± 0.05
CNP-22 (10^{-6} M)	0.97 ± 0.18*
Sodium nitroprusside (10^{-4} M)	1.61 ± 0.32*

Cells were preincubated with IBMX (5×10^{-4} M) as described in the text. Then, rat ANP(1–28) or rat BNP-45 or CNP-22 was added at the final concentration of 10^{-6} M and incubated for 10 min. Data are means ± S.E. for 3 separate experiments. The asterisk (*) represents the statistical significance from the control value ($P < 0.05$).

4. Discussion

The effect of ANP on $[Ca^{2+}]_i$ homeostasis in cardiomyocytes is a controversial issue. It has been reported that ANP decreases isoprenaline-induced Ca^{2+} channel current without affecting basal Ca^{2+} channel current [17]. On the other hand, one study has shown a reduction of basal $[Ca^{2+}]_i$ caused by ANP in guinea pig cardiomyocytes [7]. It has also been reported that ANP reduces basal $[Ca^{2+}]_i$ through the activation of a cGMP-mediated process in cardiac myocytes [18]. However, no investigation has directly measured the ANP-induced Ca^{2+} efflux from cardiomyocytes. We have hypothesized that ANP may cause Ca^{2+} efflux from the cells. In the present study, we thus examined the mechanism by which rat ANP(1–28) stimulates Ca^{2+} efflux from adult rat cardiomyocytes preloaded with $^{45}CaCl_2$.

Results shown in Fig. 1 revealed that rat ANP(1–28) caused a significant Ca^{2+} efflux from cardiomyocytes in a concentration-dependent manner (10^{-8} M to 10^{-6} M). The efflux of $^{45}Ca^{2+}$ increased to a peak value within approximately 1 min after ANP addition, and decreased rapidly within the next 5 min. This ability of ANP to mobilize Ca^{2+} outside the plasma membrane has not been previously identified, since earlier reports have concentrated on $[Ca^{2+}]_i$ measurement [7,18]. Our method of superfusion detected, for the first time to our knowledge, an ANP-induced Ca^{2+} efflux from cardiomyocytes preloaded with $^{45}CaCl_2$.

To investigate the intracellular mechanism of ANP-induced Ca^{2+} efflux from cardiomyocytes, we examined the influence of Ca^{2+} or Na^+ deprivation from the medium on the efflux. It has been reported that the decline in $[Ca^{2+}]_i$ may be primarily due to both the mechanism of Na^+/Ca^{2+} exchange at the plasma membrane and Ca^{2+} uptake into the sarcoplasmic reticulum [10]. As shown in Fig. 2, rat ANP(1–28)-induced Ca^{2+} efflux from cardiomyocytes was dependent on the presence of extracellular Na^+, but not on Ca^{2+}. The removal of Na^+ from the incubation medium essentially inhibits Na^+/Ca^{2+} exchange to extrude intracellular Ca^{2+} outside the cells, which in turn allows Na^+ influx. These results suggest that ANP stimulates Ca^{2+} efflux from cardiomyocytes through the

mechanism of Na^+/Ca^{2+} exchange. This suggestion is also supported by the finding that rat ANP(1–28) caused a significant $^{22}Na^+$ influx into the cells. It has been reported that the Na^+/Ca^{2+} exchanger is activated in the failing heart [19], and that cardiac ANP content and its secretion are increased in congestive heart failure [20,21]. Considering these findings, it is reasonable to speculate that ANP may activate the Na^+/Ca^{2+} exchanger concomitantly with the progress of heart failure. Irrespective of this, it is assumed that the acceleration of Na^+/Ca^{2+} exchange through the plasma membrane plays a key role in ANP-induced Ca^{2+} efflux from cardiomyocytes.

As the existence of natriuretic peptide receptors (NPR) in cardiomyocytes has been reported [22], ANP may affect these receptors in the cells. These NPRs can be classified into three major subgroups, i.e., NPR-A, NPR-B, and NPR-C [22]. It is indicated that NPR-A, which is stimulated by ANP and BNP, and NPR-B, which is recognized by CNP, both couple to guanylate cyclase to produce cGMP in cardiomyocytes [23]. However, recently identified NPR-C, which is lacking guanylate cyclase, is thought to participate in peptide clearance [24]. We then examined the effects of rat BNP-45 and CNP-22 on Ca^{2+} efflux from the cardiomyocytes. Although rat CNP-22 is identical to human CNP-22, rat BNP-45 was employed for our experiments because it is the major circulating form of rat BNP which shows hypotensive and natriuretic properties [25]. As shown in Table 1, Ca^{2+} efflux from the cells was stimulated by CNP-22 to almost the same extent as by rat ANP(1–28), but not by rat BNP-45. Although the reason why BNP failed to induce Ca^{2+} efflux is unclear at present, it can be explained in part by the lower affinity of BNP to NPR-A in rat tissues [26]. As an alternative explanation, less potency of BNP than of ANP in activating guanylate cyclase may be involved [23]. These possibilities were confirmed in the present study by the finding that cGMP production was enhanced by rat ANP(1–28) and by CNP-22, but not by rat BNP-45 (Table 2). Nevertheless, it may be assumed that the stimulatory effects of ANP and CNP on Ca^{2+} efflux from cardiomyocytes is mediated by NPR-A and NPR-B, respectively. Since it has been reported that stimulation of these two receptors causes an increase in cGMP within the cells [23], it is conceivable that the stimulatory effects of these peptides on Ca^{2+} efflux from the cardiomyocytes occurs through the activation of guanylate cyclase. We have previously reported that dibutyryl cGMP and sodium nitroprusside, which are known as potent activators of guanylate cyclase, induce Ca^{2+} efflux from bovine adrenal chromaffin cells through the Na^+/Ca^{2+} exchange mechanism [27]. From these findings it may be reasonable to speculate that ANP-induced Ca^{2+} efflux from adult rat cardiomyocytes may be mediated by the mechanism of Na^+/Ca^{2+} exchange, and this in turn may accelerate Ca^{2+} efflux from the cardiomyocytes, due to the increase in cGMP that results from activation of NPRs coupled to guanylate cyclase. However, the exact mechanism which causes Ca^{2+} efflux from cardiomyocytes is still unclear. We are further investigating the intracellular mechanism involved in ANP-induced Ca^{2+} efflux from freshly isolated adult rat cardiomyocytes.

In conclusion, ANP stimulates Ca^{2+} efflux from adult rat cardiomyocytes, probably through NPR. The underlying mechanism which causes Ca^{2+} efflux from the cells is still not entirely understood; however, it can be explained at least in part by a Na^+/Ca^{2+} exchange mechanism mediated by an increase in cGMP via guanylate cyclase activation.

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