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Effects of C-type natriuretic peptide on ionic currents in mouse sinoatrial node: a role for the NPR-C receptor

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Rose, Robert A., Alan E. Lomax, Colleen S. Kondo, Madhu B. Anand-Srivastava, and Wayne R. Giles. Effects of C-type natriuretic peptide on ionic currents in mouse sinoatrial node: a role for the NPR-C receptor. *Am J Physiol Heart Circ Physiol* 286: H1970–H1977, 2004. First published January 2, 2004; 10.1152/ajpheart.00893.2003.—The effects of C-type natriuretic peptide (CNP) on heart rate and ionic currents were demonstrated by recording the ECG from adult mice and performing voltage-clamp experiments on single sinoatrial (SA) node cells isolated from mouse heart. The selective natriuretic peptide type C receptor (NPR-C) agonist cANF (10^{-7} M) significantly decreased heart rate in the presence of isoproterenol (5×10^{-9} M), as indicated by an increase in the R-R interval of ECGs obtained from Langendorff-perfused hearts. Voltage-clamp measurements in enzymatically isolated single pacemaker myocytes revealed that CNP (10^{-8} M) and cANF (10^{-8} M) significantly inhibited L-type Ca^{2+} current [$I_{Ca(L)}$]. These findings suggest that the CNP effect on this current is mediated by NPR-C. Further support for an NPR-C-mediated inhibition of $I_{Ca(L)}$ in SA node myocytes was obtained by altering the functional coupling between the G protein G_i and NPR-C. In these experiments, a "G_i-activator peptide," which consists of a 17-amino acid segment of NPR-C containing a specific G_i protein-activator sequence, was dialyzed into SA node myocytes. This peptide decreased $I_{Ca(L)}$ significantly, suggesting that NPR-C activation can result in a reduction in $I_{Ca(L)}$ when CNP is bound and the G_i protein pathway is activated. This effect of CNP appears to be selective for $I_{Ca(L)}$, because the hyperpolarization-activated current was unaffected by CNP or cANF. These results provide the first demonstration that CNP has a negative chronotropic effect on heart rate and suggest that this effect is mediated by selectively activating NPR-C and reducing $I_{Ca(L)}$ through coupling to G_i protein.

C receptor; calcium current; hyperpolarization-activated current

C-TYPE NATRIURETIC PEPTIDE (CNP) is one member of a group of peptide hormones that also includes atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). These peptides are produced in mammalian, including human, hearts (27, 51). Natriuretic peptides, including CNP, elicit vasodilator responses. In this way, they can modulate blood pressure in healthy humans and in a number of pathophysiological states such as congestive heart failure (6, 9, 40, 43). Natriuretic peptides elicit their physiological effects by binding to specific cell surface receptors, which have been denoted natriuretic peptide type A, B, and C receptors (NPR-A, NPR-B, and NPR-C) (28, 30). NPR-A preferentially binds ANP and BNP; whereas NPR-B is more selective for CNP. NPR-A and NPR-B include intracellular particulate guanylyl cyclase domains. After a natriuretic peptide binds to one of these receptors, intracellular cGMP levels increase (28). NPR-C has similar affinity for all three natriuretic peptides; however, this receptor subtype has no guanylyl cyclase domain. Most natriuretic peptide-mediated effects in the cardiovascular system have been attributed to occupancy of NPR-A or NPR-B and the resulting changes in intracellular cGMP levels (28).

NPR-C was originally referred to as a "clearance receptor"; however, in the heart and in gastrointestinal smooth muscle, NPR-C is known to be functionally linked to adenylyl cyclase via a pertussis toxin-sensitive G protein, G_i (3, 5, 37, 38). NPR-C receptors are disulfide-linked homodimers that have a single transmembrane domain. Therefore, they differ significantly from the traditional heptahelical receptor and heterotrimeric G_i protein complex (4, 39). Importantly, NPR-C includes a specific G_i-activator domain within the intracellular portion of the receptor, a motif that was first described for the insulin-like growth factor receptor (41). This G_i-activator sequence is characterized by the presence of two NH_2-terminal basic residues and a COOH-terminal BBXXB motif, where B and X are basic and nonbasic residues, respectively (41, 42). In NPR-C, a 17-amino acid sequence (R^{469}–R^{485}) within the 37-amino acid intracellular domain is responsible for activation of G_i (44, 52). A shorter 12-amino acid fragment of NPR-C containing a G_i-activator peptide sequence has been shown to be equipotent with the 17-amino acid fragment in terms of its ability to inhibit adenylyl cyclase (44). In this way, natriuretic peptides, when bound to NPR-C, are able to activate G_i proteins and decrease cAMP levels via the inhibition of adenylyl cyclase (3, 44).

Recently, Mangoni et al. (31) and Mangoni and Nargeot (32) described a procedure for isolating single myocytes from the sinoatrial (SA) node region of the mouse heart. The myogenic pacemaker function of these specialized myocytes is due to their ability to generate a slow diastolic depolarization, during which the membrane potential slowly approaches the threshold for eliciting an action potential (1). In the mammalian heart, several different ionic currents have been proposed to play a role in the generation of the slow diastolic depolarization. These include a delayed rectifier K^+ current and at least four voltage-dependent inward currents: the hyperpolarization-activated current (I_f), the sustained inward current, and T- and

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L-type Ca^{2+} currents [$I_{Ca(T)}$ and $I_{Ca(L)}$] (8, 11, 13, 23). Two of these inward currents in particular, $I_{Ca(L)}$ and I_f, are thought to contribute to the generation of the diastolic depolarization phase of the myogenic pacemaker activity (1, 31, 48).

Recently, we described a novel electrophysiological effect mediated by NPR-C in the heart (45). In this study on bullfrog single atrial myocytes, CNP, when bound to NPR-C, was found to markedly shorten the action potential by inhibiting $I_{Ca(L)}$. This inhibitory effect on $I_{Ca(L)}$ was mimicked by the NPR-C-selective agonist cANF (2) and maintained in the presence of the NPR-B antagonist HS-142-1 (33), thus demonstrating the involvement of NPR-C (34).

The main goal of the present study was to determine the ionic mechanism that is responsible for a natriuretic peptide effect on heart rate in the adult mouse. An initial focus was to determine whether CNP can modulate cAMP-sensitive currents in isolated mouse SA node myocytes. Our findings demonstrate that CNP has a negative chronotropic effect in adult mouse heart and provide strong evidence that this is mediated by NPR-C. In the isolated SA node myocytes, this negative chronotropic effect of CNP is mainly due to the selective inhibition of $I_{Ca(L)}$, because another cAMP-sensitive pacemaker current, I_f, was unaffected by CNP superfusion.

METHODS

ECG measurements. Adult male C57BLK6 mice (25–30 g) received heparin (200 IU ip) 10 min before they were anesthetized with methoxyflurane (Metafane; Janssen Pharmaceutica). The heart was excised quickly and placed into ice-cold Krebs solution containing (in mM) 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4 \cdot 7H_2O$, 1.25 $CaCl_2$, 25 $NaHCO_3$, and 11 glucose. Excess tissue was dissected away. The heart was secured to an apparatus for retrograde perfusion through the aorta on a Langendorff apparatus, during which warm Krebs solution (37°C), which was equilibrated to pH 7.4 with carbogen (95% O_2-5% CO_2), was perfused through the heart at a flow rate of 2 ml/min. The perfused heart was immersed into a water-jacketed organ bath and maintained at 37°C. During these experiments, a bipolar silver wire electrode (chlorided with $AgCl_3$) was placed on the left ventricle for recording electrograms. Data were recorded using a two-channel ECG high-fidelity low-noise amplifier (Anton Paar; Graz, Austria) (29) and collected using a National Instruments analog-to-digital board (models PCI-MIO-16XE-10 and NI-DAQ) using custom-designed acquisition software (29).

After the heart was allowed to stabilize for 15–20 min, baseline ECG recordings were made, and the effects of isoproterenol (Iso, 5×10^{-9} M) and cANF (10^{-7} M) on the ECG were recorded. The R-R interval (used as an indicator of heart rate) was monitored in real time as the experimental compounds were added to the temperature-controlled perfusate. In each experiment, Iso was added to mimic a level of adrenergic tone, which was maintained throughout the experiment. After a steady state was achieved in the presence of Iso, cANF was added to the perfusate.

Single cell preparation. Cells from the SA node region were isolated from the hearts of adult male C57BLK6 mice (25–30 g) according to the methods of Mangoni and Nargeot (32). Briefly, adult mice were anesthetized with methoxyflurane. The heart was excised and placed in Tyrode solution consisting of (in mM) 140 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 5.55 glucose, and 5 HEPES, with pH adjusted to 7.4 with NaOH, at 35°C. The SA node region of the heart was isolated by separating the atria from the ventricles, cutting open the superior and inferior venae cavae, and pinning the tissue so that the crista terminalis could be identified. The SA node area is demarcated by boundaries consisting of the crista terminalis, the interatrial septum, and the openings of the two great veins. This SA node region was cut into strips, which were transferred to a "low-Ca^{2+}-Mg^{2+}-free" solution containing 140 mM NaCl, 5.4 mM KCl, 1.2 mM KH_2PO_4, 0.2 mM $CaCl_2$, 50 mM taurine, 18.5 mM glucose, 5 mM HEPES, and 1 mg/ml BSA, with pH adjusted to 6.9 with NaOH. SA node tissue strips were digested in 5 ml of low-Ca^{2+}-Mg^{2+}-free solution containing 4.2 mg of collagenase (type II, Worthington; 268 U/mg), 80 μl of elastase (Worthington; 88 U/ml), and 65.2 μl of 1 mg/100 μl protease solution (type XIV, Sigma; 4.3 U/mg) for 20–23 min. Then the tissue was transferred to 5 ml of modified KB solution (in mM: 100 potassium glutamate, 10 potassium aspartate, 25 KCl, 10 KH_2PO_4, 2 $MgSO_4$, 20 taurine, 5 creatine, 0.5 EGTA, 20 glucose, and 5 HEPES, and 1% BSA, with pH adjusted to 7.2 with KOH), and digestion was continued for 5–9 min at 35°C under mechanical agitation with a wide-bore pipette. This procedure yielded a sufficient number of SA node myocytes with cellular automaticity that was recovered after readapting the cells to a physiological concentration of Ca^{2+} (by the addition of a solution containing 10 mM NaCl and 1.8 mM $CaCl_2$ and then a normal Tyrode solution containing 1 mg/ml BSA). Cells were confirmed to be from the SA node by their spontaneous beating and expression of I_f, which was demonstrated under voltage-clamp conditions. The University of Calgary Animal Resource Centre approved all experimental protocols and animal procedures used in this study.

Solutions and drugs. The recording chamber was superfused with a control Tyrode solution (22–23°C) containing (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, and 5 glucose, with pH adjusted to 7.4 with NaOH. This solution was used for recording SA node electrophysiological responses and for studying I_f. To record $I_{Ca(L)}$, the following external solution was used (in mM): 140 TEA-Cl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 5 glucose, with pH adjusted to 7.4 with CsOH. The pipette filling solution used for recording I_f consisted of (in mM) 135 KCl, 1 $CaCl_2$, 10 EGTA, 4 Mg-ATP, 1 $MgCl_2$, 6.6 Na-phosphocreatine, and 10 HEPES, with pH adjusted to 7.2 with KOH. For recording $I_{Ca(L)}$, the pipette contained (in mM) 135 CsCl, 10 EGTA, 4 Mg-ATP, 1 $MgCl_2$, 6.6 Na-phosphocreatine, and 10 HEPES, with pH adjusted to 7.2 with TEA-OH. In experiments examining the effects of the G_i-activator peptide on $I_{Ca(L)}$, 0.01 mM GTPγS was added to the recording pipette. GTPγS was included on the basis of the findings of Pagano and Anand-Srivastava (44), which demonstrated a more robust effect of this G_i-activator peptide in the presence of GTPγS. G_i-activator peptides have been shown to facilitate the binding of GTPγS to G_i protein (41).

Iso and cAMP were purchased from Sigma (St. Louis, MO). CNP and cANF, an NPR-C-selective agonist that has no effect on the NPR-B-cGMP pathway (2), were purchased from Peninsula Laboratories (San Carlos, CA). The G_i-activator peptide (provided by M. B. Anand-Srivastava) (44) was stored in solution at −70°C and synthesized by standard solid-phase techniques and purified (95–99%) by high-performance liquid chromatography (Peninsula Laboratories).

Electrophysiological protocols. The whole cell configuration of the patch-clamp technique (17) was used for voltage-clamp studies of single SA node myocytes. Micropipettes were pulled from borosilicate glass (with filament, 1.5 mm OD, 0.86 mm ID; Sutter Instrument, Novato, CA) using a Flaming/Brown pipette puller (model p-87, Sutter Instrument). The resistance of these pipettes was 5–10 MΩ when they were filled with recording solution.

Microelectrodes were positioned with a hydraulic micromanipulator (SD Instruments) mounted on the stage of an inverted microscope (Nikon Diaphot). Seal resistances were 2–15 GΩ. Rupturing the sarcolemma in the patch resulted in access resistances of 5–15 MΩ. Series resistance compensation was usually not employed because of the small capacitance of SA node myocytes (typically 15–30 pF) and low current densities. The calculated voltage error in the absence of series resistance compensation was 3–5 mV and therefore was ignored. When it was used, series resistance compensation averaged 80–85% when a Multiclamp 700A or Axopatch 200B amplifier (Axon Instruments, Foster City, CA) was used. There was no significant

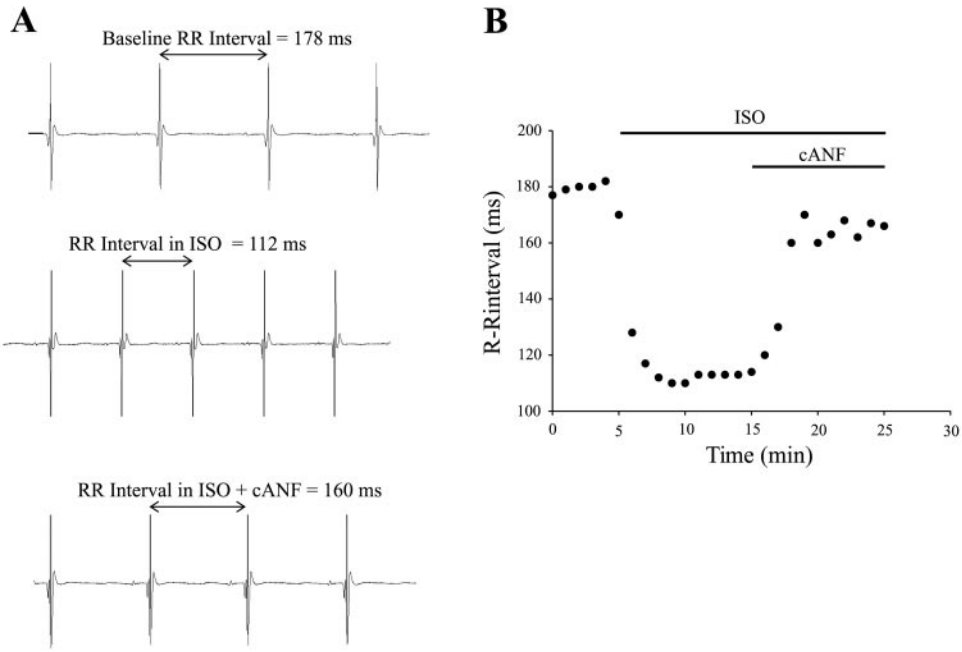

Fig. 1. Effect of 10^{-7} M cANF on the R-R interval determined from ECGs of Langendorff-perfused mouse hearts. *A*: representative ECG traces illustrating changes in the R-R interval during superfusion with 5×10^{-9} M isoproterenol (Iso) and 10^{-7} M cANF + Iso. Under control conditions, the baseline R-R interval was 178 ms; Iso reduced this value to 112 ms. After application of cANF + Iso, the R-R interval increased to 160 ms. *B*: representative data illustrating the time course of these changes in heart rate. As indicated by horizontal bars, the heart was perfused with 5×10^{-9} M Iso at 5 min and then with 10^{-7} M cANF at 15 min. Application of Iso decreased the R-R interval from 180 to ~110 ms. Subsequent application of cANF increased the R-R interval to ~170 ms.

difference between currents recorded with and those recorded without series resistance compensation. These sets of results were therefore combined. Voltage-clamp command waveforms and resulting capacitative and ionic current changes were digitized using a Digidata 1322A interfaced with pCLAMP 8 software (Axon Instruments). Data were stored on computer for offline analysis.

Current-voltage (*I-V*) relations for I_f were generated by applying a series of 2-s voltage-clamp steps in 10-mV increments from −35 to −135 mV from a holding potential of −60 mV. Peak $I_{Ca(L)}$ was recorded by first applying a depolarizing step from −60 to −40 mV to inactivate the Na^+ current (18). Immediately after this prepulse, 250-ms voltage-clamp steps were applied from −50 to +70 mV in 10-mV increments. This voltage-clamp protocol allows the measurement of $I_{Ca(L)}$ carried by the $Ca_V1.2$ (α_{1C}) family of channels. The peak inward current was measured, and *I-V* relations were plotted. When the effects of acetylcholine (ACh) on I_f were tested, Ba^{2+} (10^{-5} M) was included in the external solution to block the inwardly rectifying ACh-sensitive K^+ current (26).

Statistical analysis. Summary data are presented as means ± SE. The data were analyzed using an ANOVA with Dunnett's multiple comparison procedure (in most cases) or a paired Student's *t*-test (Fig. 1) to identify significant differences. In all instances, $P < 0.05$ was considered significant.

RESULTS

Effects of cANF on heart rate and ECG intervals. In the initial experiments, effects of cANF on the R-R interval of the Langendorff-perfused mouse heart were measured to determine whether activation of NPR-C could alter heart rate. On the basis of our previous work and that of others (44), which show that NPR-C-mediated effects are more robust in the presence of β-adrenergic stimulation, Iso was added initially, and then cANF was added to selectively activate NPR-C in the presence of "adrenergic tone."

Figure 1*A* shows representative ECG data for which the R-R interval (used as an indicator of heart rate) is labeled in control conditions, after the addition of Iso (5×10^{-9} M), and in the presence of Iso + cANF (10^{-7} M). In this experiment, Iso decreased the R-R interval from 178 to 112 ms. Subsequent application of cANF resulted in an R-R interval increase to 160 ms. The averaged effects of Iso and cANF on the R-R, P-R, and Q-T intervals are summarized in Table 1. As expected, the R-R and P-R intervals were significantly decreased by Iso application. Subsequent application of cANF significantly increased both of these ECG parameters.

Effects of CNP on $I_{Ca(L)}$. To explore the ionic mechanism(s) of these NPR-C-mediated effects on heart rate, voltage-clamp measurements of $I_{Ca(L)}$ were made on single SA node myocytes (32). Our previous work demonstrated that CNP can inhibit basal $I_{Ca(L)}$ and also showed that the effect is more pronounced in the presence of β-adrenergic receptor stimulation (45). Therefore, the following experiments are conducted in the presence of Iso (5×10^{-9} M). Representative raw data are illustrated in Fig. 2*A*. On average, peak $I_{Ca(L)}$ was about −9 pA/pF in the presence of Iso (5×10^{-9} M). Application of CNP (10^{-7} M) in this setting significantly decreased peak current to approximately −4 pA/pF (Fig. 2*B*).

We have previously demonstrated that, in bullfrog atrial myocytes, CNP can inhibit $I_{Ca(L)}$ by binding to NPR-C (45). Accordingly, the next series of experiments were designed to evaluate this hypothesis in mouse SA node myocytes. Figure 3 illustrates the effects of cANF (10^{-8} M), which is a selective agonist of NPR-C with no capacity to bind to NPR-B or activate the cGMP second messenger pathway (2). The raw

Table 1. *Effects of Iso and cANF on ECG intervals in Langendorff-perfused mouse heart*

Interval	Control	Iso (5×10^{-9} M)	Iso (5×10^{-9} M) + cANF (10^{-7} M)
R-R	168.8 ± 3.4	114.2 ± 2.4*	148.2 ± 8.3†
P-R	38.6 ± 1.8	25.5 ± 1.6*	33.3 ± 2.2†
Q-T	50.1 ± 2.4	43.2 ± 3.6	44.1 ± 3.1

Values are means ± SE in ms (n = 5–7 hearts). Iso, isoproterenol; cANF, NPR-C-selective agonist. *Significantly different from control. †Significantly different from Iso.

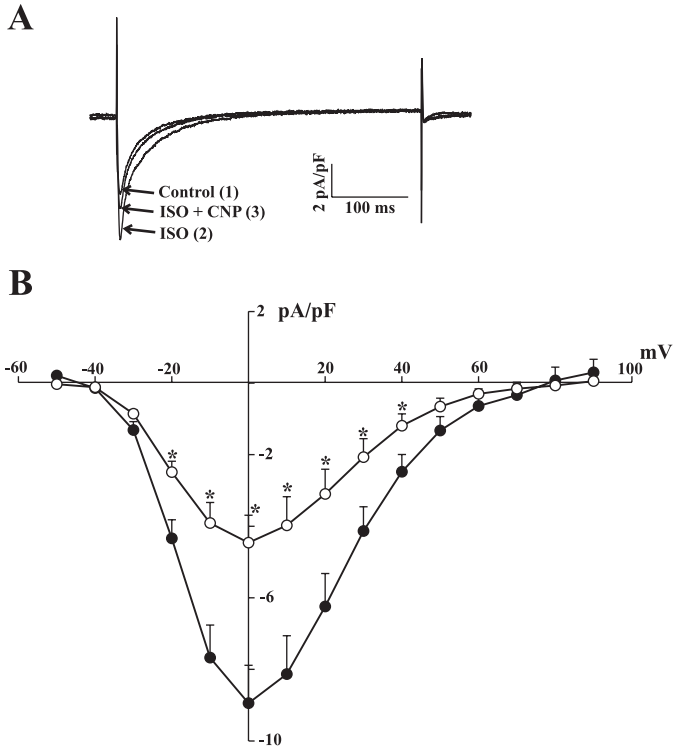

Fig. 2. Effect of 10^{-8} M C-type natriuretic peptide (CNP) on L-type Ca^{2+} current [$I_{Ca(L)}$] in the presence of 10^{-7} M Iso in adult mouse sinoatrial (SA) node myocytes. *A*: representative current traces demonstrate the effect of CNP on peak $I_{Ca(L)}$ recordings elicited by depolarizing voltage-clamp steps to 0 mV. Peak $I_{Ca(L)}$ was approximately −4 pA/pF in control conditions (*1*). Addition of Iso (*2*) increased this value to approximately −8 pA/pF, and subsequent addition of CNP (*3*) decreased peak $I_{Ca(L)}$ to −5 pA/pF. *B*: summary current-voltage (*I-V*) curve illustrating the effect of CNP (○) on Iso-stimulated $I_{Ca(L)}$ (●). Values are means ± SE of 7 cells. *Significant inhibition of $I_{Ca(L)}$ by CNP.

data in Fig. 3*A* show that Iso approximately doubles peak $I_{Ca(L)}$ from −5 to −10 pA/pF. After the addition of cANF, peak $I_{Ca(L)}$ is reduced to about −6 pA/pF. On average, cANF decreased peak $I_{Ca(L)}$ from −13 to about −6 pA/pF. In summary, these electrophysiological findings (Figs. 2 and 3) suggest that the effects of CNP on $I_{Ca(L)}$ are mediated by NPR-C.

Effects of G_i-activator peptide on $I_{Ca(L)}$. Recently, two independent research groups published results that demonstrate that NPR-C contains a specific G_i-activator sequence (44, 52). These studies showed that amino acids 469–485, which are located in the middle region of the intracellular domain of NPR-C, are necessary and sufficient for activation of G_i proteins in the heart.

This important finding was used in our experimental design to provide further evidence for an NPR-C-mediated effect on $I_{Ca(L)}$. This G_i-activator peptide (the 17-amino acid fragment of NPR-C containing the G_i-activator sequence) was applied intracellularly to single SA node myocytes by including it in the micropipette filling solution. The time course of the effects of the G_i-activator peptide is illustrated in Fig. 4*A*. Myocytes were first exposed to Iso (10^{-7} M) for ~3 min. During the subsequent impalement of the cell, the G_i-activator peptide (10^{-7} M), which was included in the recording pipette, was able to diffuse into the cell under conventional whole cell recording conditions. In the presence of Iso, peak $I_{Ca(L)}$ was approximately −12 pA/pF, and it was decreased significantly as the G_i-activator peptide entered the cell over the course of ~200 s to about −3 pA/pF.

NPR-C-mediated effects have been shown to result from activation of the G_i family of G proteins, which are known to inhibit adenylyl cyclase activity (3, 44). Accordingly, we reasoned that inhibition of $I_{Ca(L)}$ by the G_i-activator peptide should be antagonized in the presence of elevated cAMP levels. To test this hypothesis, the G_i-activator peptide (10^{-7} M) and cAMP (10^{-5} M) were added to the recording pipette with the expectation that each compound could enter the myocyte under whole cell recording conditions. Figure 4*B* illustrates that when a high concentration of cAMP is in the micropipette, peak $I_{Ca(L)}$ increased from −4 to −15 pA/pF. The time course and magnitude of this effect of cAMP on $I_{Ca(L)}$, in the presence of the G_i-activator peptide, are similar to the increase in $I_{Ca(L)}$ that has been observed in the presence of cAMP alone (16, 45). These findings demonstrate that this effect of cAMP developed fully in the presence of the inhibitory G_i-activator peptide.

Effects of CNP on I_f. To evaluate the specificity of these inhibitory effects of CNP on $I_{Ca(L)}$ in mouse SA node myocytes, I_f was measured. I_f is strongly modulated by intracellular cAMP levels (1, 32). Accordingly, CNP, which activates G_i

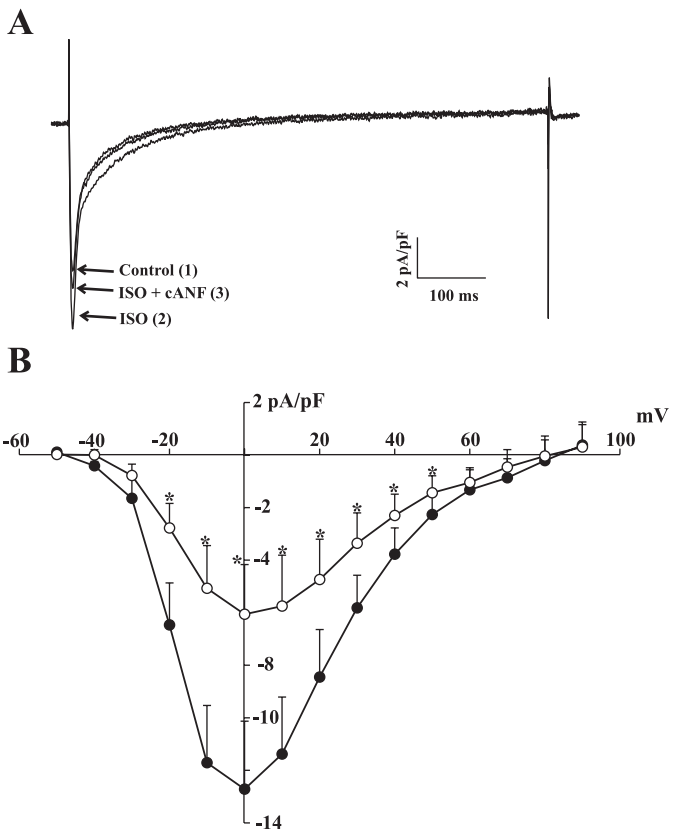

Fig. 3. Effect of the natriuretic peptide type C receptor (NPR-C)-specific agonist cANF (10^{-8} M) on $I_{Ca(L)}$ in the presence of 10^{-7} M Iso in mouse SA node myocytes. Note that application of cANF selectively activates NPR-C without stimulating the guanylyl cyclase-linked NPR-B. *A*: raw current traces. Control peak $I_{Ca(L)}$ (*1*) increased from −5 to −10 pA/pF after addition of Iso (*2*). Subsequent application of cANF (*3*) decreased $I_{Ca(L)}$ to approximately −6 pA/pF. *B*: *I-V* curve summarizing these effects of cANF (○) on Iso-stimulated $I_{Ca(L)}$ (●). Values are means ± SE of 6 cells. *Significant inhibition of $I_{Ca(L)}$ by cANF.

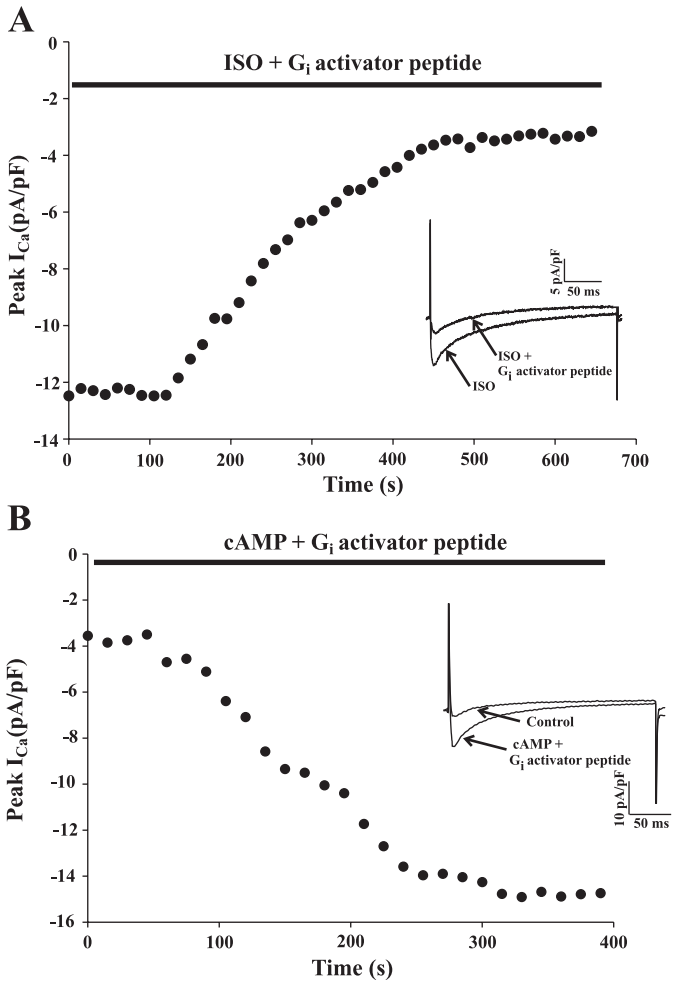

Fig. 4. Effects of intracellular application of G_i-activator peptide on $I_{Ca(L)}$ after application of 10^{-7} M Iso or in conjunction with elevated cAMP (10^{-5} M) in mouse SA node myocytes. G_i-activator peptide (10^{-7} M) was added to the recording pipette and entered the myocyte by diffusion under conventional whole cell recording conditions. *A*: electrophysiological recordings were begun after Iso had been superfused for ≥3 min. As shown in raw current traces (*inset*), peak $I_{Ca(L)}$ increased to a steady-state level of approximately −12 pA/pF after stimulation with Iso. After the G_i-activator peptide entered this myocyte, peak $I_{Ca(L)}$ decreased to about −3 pA/pF. This inhibitory effect is apparent in the time-course experiment. As indicated by the horizontal bar, Iso was present throughout the experiment. The G_i-activator peptide, which began to diffuse into the myocyte at *time 0*, started to elicit an inhibitory effect at ~100 s. Data are representative of measurements made on 10 cells from 4 hearts. *B*: $I_{Ca(L)}$ measurements in the presence of G_i-activator peptide and cAMP, both of which are included in the recording pipette. *Inset*: raw current traces. Control $I_{Ca(L)}$ is approximately −5 pA/pF. After G_i-activator peptide and cAMP entered the myocyte, the current increased to a maximum of −15 pA/pF. As illustrated in the time-course experiment, peak $I_{Ca(L)}$ showed a nearly 4-fold increase over the course of 200 s of exposure to G_i-activator peptide and cAMP. Data are representative of measurements on 4 cells from 2 hearts.

protein, inhibits adenylyl cyclase, and decreases cAMP levels, may inhibit I_f.

Raw data and plots of the time course of the effects of Iso (10^{-7} M) and cANF (10^{-8} M) on I_f in SA node myocytes are illustrated in Fig. 5, *A* and *B*, respectively. As expected, Iso significantly increased I_f from −38 to −50 pA/pF (Fig. 5*B*). Note, however, that cANF failed to significantly inhibit this current. The *I-V* curves that summarize this data set show that neither cANF (to selectively activate NPR-C; Fig. 5*C*) nor CNP (Fig. 5*D*) has any significant effect on I_f in the presence of Iso.

To gain further insight into this negative result, ACh (5×10^{-6} M) was applied to SA node myocytes. SA node myocytes were stimulated with Iso (10^{-7} M) and then with CNP (10^{-8} M). ACh was added in the presence of Iso and CNP. Figure 6*A* illustrates representative measurements of I_f. CNP caused a very small reduction in current, whereas ACh, in combination with CNP, markedly reduced I_f. As shown in the *I-V* relation that summarizes these findings (Fig. 6*B*), the effects of CNP on I_f were not significant. In contrast, the effects of ACh on this current were more pronounced and consistent. These results confirm that I_f is sensitive to cAMP levels and that ACh can inhibit this current (1, 32) but that CNP has no effect on I_f in these same myocytes.

DISCUSSION

CNP effects on the heart. CNP is known to mediate vasodilatory and natriuretic effects in mammals (27, 51); however, very few studies have examined the effects of this peptide on heart rate. Most effects of natriuretic peptides on heart rate are attributed to the guanylyl cyclase-linked NPR-A and NPR-B. Recently, Herring et al. (19) reported that CNP induces bradycardia as a result of presynaptic facilitation of vagal neurotransmission. This effect could be antagonized by the particulate guanylyl cyclase blocker HS-142-1 (33, 34) and, thus, was suggested to be mediated by NPR-B.

Our results show that CNP can have a negative chronotropic effect on the Langendorff-perfused mouse heart that is mediated by NPR-C. The data in Fig. 1 were obtained using the selective NPR-C agonist cANF, which has no capacity to activate the NPR-B-cGMP pathway but strongly inhibits adenylyl cyclase after binding to NPR-C (2). CNP elicited a negative chronotropic effect very similar to that shown for cANF in Fig. 1 (data not shown). In addition to this effect on heart rate (R-R interval), cANF also significantly increased the P-R interval (Table 1). The P-R interval is an indicator of conduction across the atria and through the atrioventricular node (35). The increased P-R interval in the presence of cANF indicates that conduction has slowed within the atrioventricular conduction system. Because NPR-C has approximately the same affinity for all natriuretic peptides, we suggest that ANP and BNP could have effects on heart rate and ECG intervals similar to those identified for cANF and CNP in the present study.

After the effects of CNP on heart rate were defined, the ionic mechanism(s) for these CNP-induced changes in heart rate was examined in voltage-clamp experiments using single SA node myocytes from the primary pacemaker region of the adult mouse heart. The SA node expresses at least two cAMP-sensitive currents, $I_{Ca(L)}$ and I_f, which can contribute to generation of diastolic depolarization or the pacemaker phase of the action potential (1, 31). $I_{Ca(L)}$ current density increases dramatically when the channel is phosphorylated by PKA (20, 22). The PKA-mediated phosphorylation of $I_{Ca(L)}$ increases or decreases when cAMP levels in the cell increase or decrease, respectively (22). I_f current density is also altered by changing levels of intracellular cAMP (1); however, in contrast to $I_{Ca(L)}$, this effect appears not to be mediated by PKA. Instead, cAMP

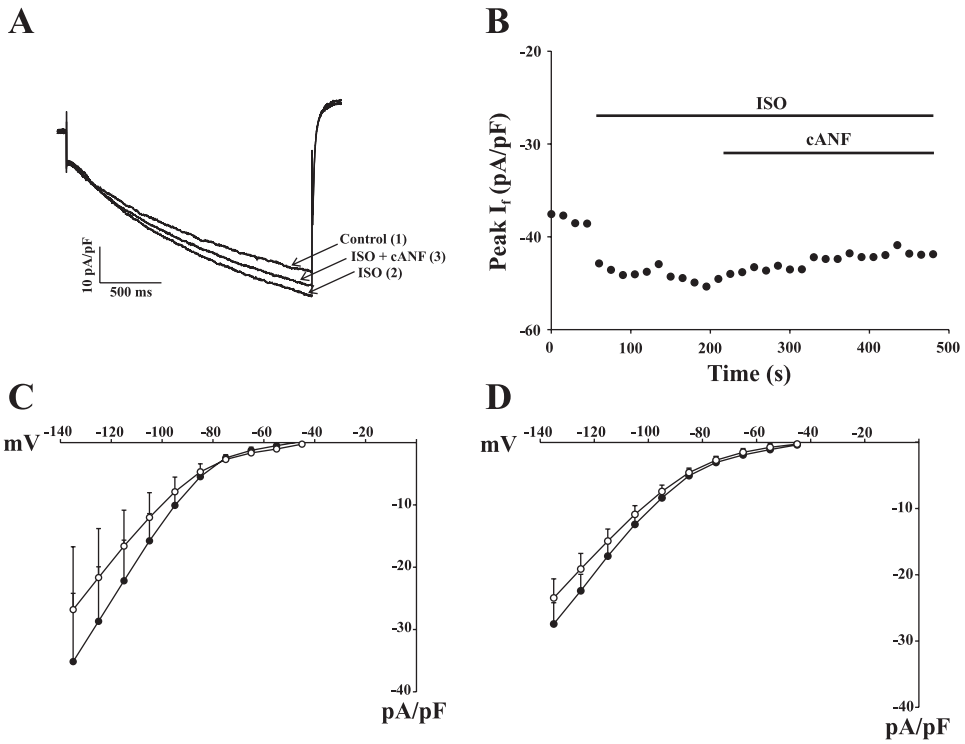

Fig. 5. Effects of 10^{-8} M CNP and 10^{-8} cANF on hyperpolarization-activated current (I_f) in mouse SA node myocytes recorded in the presence of 10^{-7} M Iso. *A*: representative I_f traces recorded during a hyperpolarizing voltage-clamp step to −135 mV illustrating control (*1*), Iso (*2*), and Iso + cANF (*3*). Iso significantly increased I_f. Subsequent addition of cANF caused no significant change in I_f. *B*: time course of Iso and cANF effects on I_f. Horizontal bars (*top*) indicate when each compound is added. Iso increased I_f (measured at the end of a 2-s voltage-clamp step to −135 mV) from −38 to −50 pA/pF. I_f was not significantly altered after superfusion of cANF. *C* and *D*: summary isochronal *I-V* relations for effects of cANF (○, *C*) or CNP (○, *D*) on Iso-stimulated I_f (●, *C* and *D*). Neither cANF nor CNP had a significant effect on I_f. Values are means ± SE of 6 cells for *C* and 9 cells for *D*.

binds directly to the intracellular COOH-terminal portion of the HCN channel subunits and alters its biophysical properties (14, 23). As described previously, NPR-C includes a G_i-activator sequence, which inhibits adenylyl cyclase and, thus, decreases cAMP levels in the presence of natriuretic peptides (3, 44, 52). We anticipated that $I_{Ca(L)}$ and I_f would be inhibited by CNP because we previously showed that CNP can inhibit L-type Ca^{2+} current in isolated bullfrog atrial cells (45).

Our results demonstrate that CNP potently inhibits $I_{Ca(L)}$ in single SA node myocytes (Fig. 2). The involvement of NPR-C was confirmed by using cANF, which is a selective NPR-C agonist (Fig. 3). It is important that the G_i-activator peptide (Fig. 4), which consists of the specific 17-amino acid segment of NPR-C known to inhibit adenylyl cyclase activity (44, 52), also substantially decreased $I_{Ca(L)}$. This effect of CNP is selective, inasmuch as I_f was not significantly changed by CNP (Fig. 5). Although CNP had no effect on I_f, ACh was able to significantly inhibit this current (Fig. 6), a result that provides a useful positive control for the responsiveness of I_f to cAMP levels in the cell (1). Altering cAMP level in SA node myocytes may result in a significant shift in the activation curve of I_f (1). It is theoretically possible that CNP could alter the activation kinetics of this current without significantly altering the magnitude of I_f. Until the effects of CNP on I_f activation are measured experimentally, this possibility cannot be completely ruled out. However, in the present study, we show that the magnitude of I_f was increased in the presence of Iso and decreased in the presence of ACh, whereas CNP had no effect on the magnitude of I_f. On the basis of these electrophysiological findings, we conclude that the major effect of CNP in the mouse SA node is a selective inhibition of $I_{Ca(L)}$ due to binding to NPR-C with no significant change in another cAMP-sensitive current, I_f.

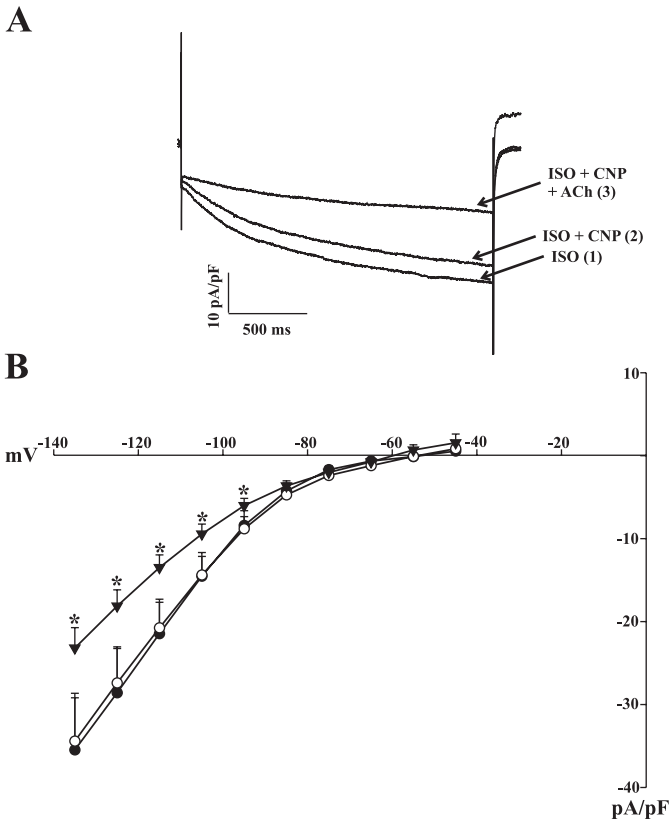

Fig. 6. Effects of 10^{-8} M CNP and 5×10^{-6} M ACh on I_f in the presence of 10^{-7} M Iso in mouse SA node myocytes. *A*: representative recordings of I_f in response to a hyperpolarizing voltage-clamp step from −60 to −135 mV in Iso (*1*), Iso + CNP (2), and Iso + CNP + ACh (*3*). Note that application of CNP did not significantly decrease I_f. In contrast, and as expected, subsequent addition of ACh resulted in a significant reduction in I_f. *B*: summary isochronal *I-V* relation showing CNP data (○) and effects of subsequent addition of ACh + CNP (▼) on Iso-stimulated I_f (●). CNP had no significant effect on I_f. Values are means ± SE of 6 cells. *Significant decrease in I_f by ACh.

Selectivity of CNP effects. Our initial working hypothesis was that $I_{Ca(L)}$ and I_f would be inhibited by CNP, because the biophysical properties of both are modulated by cAMP levels in these pacemaker myocytes. However, this was not the case in the mouse SA node. The ability of CNP to selectively inhibit $I_{Ca(L)}$ without affecting I_f suggests that the HCN channels that are responsible for I_f are functionally isolated from the pool of intracellular cAMP that is modulated by the CNP-NPR-C signaling pathway and mediates the inhibition of $I_{Ca(L)}$. Intracellular compartmentation of cAMP has been described previously in the mammalian heart (7, 46, 47). For example, it is known that although β_1-adrenergic receptor activation in ventricular myocytes leads to cAMP-mediated phosphorylation of Ca^{2+} channels, phospholamban, troponin I, and ryanodine receptors, β_2-adrenergic receptor activation results in the exclusive phosphorylation of L-type Ca^{2+} channels (25). Thus activation of β_1-adrenergic receptors leads to an increase in cAMP levels throughout the cell, whereas β_2-adrenergic signaling results in an increase in cAMP levels that is spatially restricted and is sometimes described as occurring in microdomains. One key factor leading to the compartmentation of β_2-adrenergic receptor-mediated cAMP signaling may be the functional coupling of the receptor to G_s and G_i proteins (10, 24). In this way, activation of G_i prevents an increase in cAMP levels throughout the entire myoplasm. Conversely, β_1-adrenergic receptors are coupled only to G_s proteins and, therefore, can result in a more global rise in intracellular cAMP levels (7). Other factors that may be involved in this compartmentation phenomenon are spatially localized phosphatase (24) and/or phosphodiesterase activity (21). It is not known whether NPR-C is colocalized with L-type Ca^{2+} channels or whether compartmentation is responsible for the ability of CNP to inhibit $I_{Ca(L)}$ but not I_f.

Significance of CNP effects. CNP is present and sarcolemmal receptors for it are expressed in the heart of all mammals that have been examined, including mouse, rat, pig, and human (4, 6, 9, 39, 40, 49). NPR-C appears to be the receptor most prominently expressed in cardiac myocytes, whereas the guanylyl cyclase-linked NPR-B may be restricted to the nonmyocyte population of cells in the heart (15). Thus it is anticipated that the effects of CNP on the mouse SA node described in the present study will apply to other mammals.

The levels of all natriuretic peptides in the heart, including CNP, increase substantially during heart failure (50). Plasma BNP levels are often used as an indicator of systolic function and the onset of heart failure (12, 36, 43). In situations of increased contractile force and/or stretch in the heart (such as those that occur in normal physiology or during pathophysiological conditions such as heart failure), the release of natriuretic peptides, including CNP, is augmented. Under these circumstances, the NPR-C-mediated inhibition of $I_{Ca(L)}$ may produce a negative chronotropic effect in the SA node.

In summary, our results identify a novel negative chronotropic effect of CNP in the mouse heart. In SA node myocytes this response is mediated by NPR-C and is due to a strong inhibition of $I_{Ca(L)}$. In contrast, I_f is unaltered by CNP.

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