Inhibition of L-type Ca\(^{2+}\) current by C-type natriuretic peptide in bullfrog atrial myocytes: an NPR-C-mediated effect

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Natriuretic peptides are a family of molecules that are involved in the regulation of blood pressure and natriuresis. Atrial natriuretic peptide (ANP), which is synthesized and secreted into the circulation from the atria of the heart, was the first member of the family to be discovered (16, 19). This natriuretic peptide family now includes ANP, brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP).

Three separate natriuretic peptide receptors (NPR-A, NPR-B, and NPR-C) have been identified. NPR-A preferentially binds ANP and BNP, whereas NPR-B is more selective for CNP. NPR-A and NPR-B are both membrane bound and include particulate guanylyl cyclase domains. An increase in cGMP occurs when a ligand binds to these receptors. NPR-C has no guanylyl cyclase domain. This receptor subtype has approximately equal affinity for all three natriuretic peptides (6, 12).

NPR-C has traditionally been referred to as a “clearance” receptor. It is thought to function mainly as a buffer system that can modulate concentration of natriuretic peptides in the circulation without activating intracellular second messenger cascades (36, 38). However, in the rat heart and in the gastrointestinal smooth muscle, NPR-C is functionally linked to an adenylyl cyclase enzyme via an inhibitory G (Gi) protein (1, 3, 42, 51, 56). Recent studies in the rat heart have identified a small cytoplasmic domain of NPR-C, which is responsible for the interaction with Gi. Activation of Gi results in the inhibition of adenylyl cyclase (51).

The original experimental work suggested that natriuretic peptides act by binding to NPR-A and NPR-B and then altering intracellular cGMP levels. ANP and CNP can increase cGMP in atrium and ventricle of mammals, including humans (28, 33, 34, 55). CNP is known to have potent negative inotropic effects on the mammalian heart (45) and has been shown to facilitate presynaptic vagal neurotransmission (25). In vascular tissue, CNP is a potent endothelium-independent vasodilator, and it has been shown to have direct effects on canine coronary arteries (12). CNP can also inhibit norepinephrine-induced contractions of both atrial and ventricular strips of heart muscle from the bullfrog (57).

The amino acid sequence and two- and three-dimensional structure (24) of both CNP and its receptors are highly conserved among vertebrate species. For example, there is 96% homology between rat and human forms of CNP (47); and bullfrog CNP has only four amino acid differences from that of the rat (6, 62). CNP was first identified in the brain, but is now known to be present in mammalian heart (61). A variety of techniques have localized this peptide to both the atria and the ventricles (58, 59). NPR-C is also expressed in many tissues (6, 46). In fact, NPR-C makes up ~90% of the total population of NPRs in heart and vascular smooth muscle (5, 37).
The goal of the present study was to determine whether CNP can modulate electrophysiological responses in isolated bullfrog atrial myocytes. Our findings show that CNP, at physiological concentrations, can strongly inhibit L-type $\text{Ca}^{2+}$ current in atrial myocytes from bullfrog hearts and provide the first evidence that this effect is mediated by the C-type receptor NPR-C.

METHODS

Single cell preparation. Single myocytes were isolated from bullfrog atrium using methods described previously (31). In brief, adult bullfrogs, *Rana catesbeiana*, were double pithed, and the heart was excised and placed in normal Ringer solution composed of (in mM) 110 NaCl, 2.5 KCl, 5 MgCl$_2$, 2.5 CaCl$_2$, 10 glucose, and 5 HEPES, and buffered to a pH of 7.4 with NaOH. The atrium was removed, cut into 1-mm$^3$ pieces, and subjected to a series of enzymatic dissociation steps. In the first step, pieces of atrial tissue were placed in 5 ml of nominally $\text{Ca}^{2+}$-free Ringer solution (same composition as normal Ringer solution, except that CaCl$_2$ was $6 \times 10^{-8}$ M) containing 200 IU/ml of collagenase (type I from *clostridium histolyticum*, Sigma) combined with 21,000 IU/ml of trypsin (type III from bovine pancreas, Sigma) and gently stirred for 45 min at room temperature. Next, this atrial tissue was transferred into 5 ml of nominally $\text{Ca}^{2+}$-free Ringer solution containing 1 mg/ml bovine serum albumin (Sigma) for 5 min. Finally, the tissue was incubated in a solution of 350 IU/ml of collagenase for an additional 40–60 min. On completion of the final enzyme treatment, the tissue was gently triturated with a large bore (3 mm diameter) pipette to mechanically dissociate it, yielding a population of single atrial myocytes, which were stored in nominally $\text{Ca}^{2+}$-free Ringer solution at 4°C. During the experiment, aliquots of the solution containing the single cells were transferred to the recording chamber, which was superfused with normal frog Ringer solution at room temperature ($22^\circ$–$23^\circ$C). The University of Calgary Animal Resource Center approved all experimental protocols and animal procedures used in this study.

Solutions and drugs. The filling solution for the recording microelectrodes contained the following (in mM): 90 K$^+$ aspartate, 15 KCl, 5 NaCl, 1 MgCl$_2$, 1 EGTA, 3 ATP (dipotassium salt), and 10 HEPES; pH was adjusted to 7.2 with KOH. In some experiments, cAMP was added to the recording pipette at a concentration of $10^{-8}$ M. The superfuse consisted of normal frog Ringer solution.

The drugs and chemicals used in this study included tetradotoxin (TTX), isoproterenol (Iso), and cAMP. All were purchased from Sigma (St. Louis, MO). CNP and NPR-C agonist (cANF) were purchased from Peninsula Laboratories (San Carlos, CA). HS-142-1 was obtained from Kyowa Hakko Kogyo.

Electrophysiological methods. The whole cell configuration of the patch clamp technique (22, 30) was employed to study single cells under both current clamp and voltage clamp conditions. Micropipettes were pulled using a Flaming/ Brown pipette puller (model p-87, Sutter Instrument; Novato, CA) from borosilicate glass tubing (1.5 mm outer diameter, World Precision Instruments; Sarasota, FL). The resistance of these pipettes was between 1 and 5 MΩ when filled with recording solution.

Microelectrodes were positioned with a hydraulic micromanipulator (SD Instruments), mounted on the stage of an inverted microscope (Nikon Diaphot). Acceptable seal resistances varied between 2 and 10 GΩ. When the membrane under the pipette was ruptured, the series resistance was typically 5–10 MΩ. This was compensated (80–85%) using an Axopatch 200 amplifier (Axon Instruments; Foster City, CA). Cell capacitances were 70–100 pF.

Current clamp and voltage clamp signals were digitized using a Digidata 1322A interfaced with pCLAMP 8 software (Axon Instruments). Data were stored on a computer for analysis offline.

Action potentials were recorded from current-clamped atrial myocytes by applying 10-ms depolarizing pulses of $\approx 0.5$ nA. These stimuli were applied at a frequency of 0.16 Hz. The peak amplitude as well as the 50% repolarization time (APD$_{50}$) of these action potentials were measured. Peak inward $\text{Ca}^{2+}$ current ($I_{\text{Ca}}$) in frog atrial myocytes was identified and recorded after blocking the sodium current with TTX ($5 \times 10^{-8}$ M) (10, 13). $I_{\text{Ca}}$ was measured as the difference between the peak inward current and the point at which the current reached steady state (the end of a 200-ms voltage step from a holding level of $-80$ to $+20$ mV). It is important to note that the activation kinetics of the delayed rectifier K$^+$ current ($I_{\text{K}}$) are very slow in bullfrog atrial myocytes, so that its contribution to net current changes is negligible during the first 100–200 ms (10). $I_{\text{Ca}}$ is the only repolarizing K$^+$ conductance activated at depolarized potentials in bullfrog atrium, which means it is not necessary to replace K$^+$ when measuring $I_{\text{Ca}}$ in this preparation. Thus our measurements of $I_{\text{Ca}}$ during a 200 ms voltage step in the presence of TTX represent “pure” $I_{\text{Ca}}$ (10). Current voltage ($I$-$V$) relationships for peak $I_{\text{Ca}}$ were generated by applying a series of 10-mV steps between $-120$ and $+80$ mV from a holding potential of $-80$ mV. Isochronal $I$-$V$ curves for the inward rectifier K$^+$ current ($I_{\text{K}}$) were plotted from measurements taken at the end of a 200-ms voltage step in the range of $-100$ to $-60$ mV (the threshold of activation of $I_{\text{K}}$).

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

RESULTS

Effects of CNP on atrial action potentials. In the initial experiments, CNP was added to the superfusate at physiological concentrations (21, 59) so that its effects on the action potential of single myocytes could be evaluated. On the basis of the results from the rat heart, which showed that ANP can inhibit adenylyl cyclase (51), isoproterenol was added at the start of each experiment to stimulate adenylyl cyclase and thus maximize the probability of obtaining consistent CNP effects.

Figure 1A shows representative effects of Iso ($10^{-7}$ M), followed by CNP ($10^{-8}$ M) on the action potential (stimulated at 0.16 Hz) of an atrial myocyte from bullfrog heart. As expected, Iso lengthened the action potential and increased its amplitude. Both of these effects were markedly reduced by the addition of CNP ($10^{-8}$ M). These changes, expressed in terms of APD$_{50}$ and peak amplitude, are shown in Fig. 1B. Iso increased APD$_{50}$ and peak amplitude by ~100% and 18%, respectively. The subsequent addition of CNP significantly decreased APD$_{50}$ (~45%) and caused a small reduction in peak amplitude (~5%). Both of these CNP effects were statistically significant.
Effects of CNP on I_{Ca}. To explore the ionic mechanism(s) of these CNP effects on the action potential, voltage clamp measurements of I_{Ca} were made as described previously (50). The effects of CNP were first measured on basal (unstimulated) I_{Ca}. Under these conditions, CNP (10^{-8} M) caused a small reduction in peak I_{Ca} from ~140 to 80 pA (refer to summary IV curve in Fig. 2). The effects of CNP on I_{Ca} were also measured after I_{Ca} was stimulated with Iso (10^{-7} M). Under this condition, I_{Ca} increased about five- to sixfold (compare control curve in Fig. 2 with Iso-stimulated curve in Figs. 3–5). Application of CNP (10^{-8} M) in the presence of Iso produced a significant fivefold reduction in I_{Ca} (Fig. 3). All subsequent measurements on I_{Ca} were made in the presence of Iso.

In the next series of experiments, an attempt was made to identify the NPR(s) that mediate this electrophysiological effect. cANF was used to test the hypothesis that CNP causes electrophysiological changes by binding to the C receptor. After application of Iso (10^{-7} M), cANF (10^{-8} M) was applied and I_{Ca} was significantly reduced by about threefold (Fig. 4). Because cANF binds only to NPR-C [and therefore has no capacity to increase cGMP levels (1)], these electrophysiological findings (Figs. 3 and 4) suggest an NPR-C-mediated effect.

As an indication of the selectivity of these effects on I_{Ca}, I_{K1} was also measured in the voltage range of −100 to −50 mV (the threshold of activation of I_{Ca}). Neither CNP nor cANF caused any significant changes in this K^+ conductance (Figs. 2–4), confirming that the effects are mainly on Ca^{2+} influx. This finding is also consistent with the current-clamp experiments in which neither compound affected the resting potential (Fig. 1). It was very difficult to reverse the effects of either CNP or cANF. Even after 20–30 min, during which the myocytes were superfused with normal Ringer solution, I_{Ca} remained stable but significantly reduced in peak amplitude.

Fig. 1. Effect of C-type natriuretic peptide (CNP; 10^{-8} M) on bullfrog atrial myocyte action potentials recorded in the presence of isoproterenol (Iso; 10^{-7} M). A: representative data showing control action potentials and the effects of Iso (10^{-7} M) and CNP (10^{-8} M). Action potentials (elicited at 0.16 Hz, room temperature, 22°C) were recorded as the compounds were added. Iso was added first, and after this CNP was added in combination with Iso. B: average effects of Iso and CNP on 50% repolarization time (APD_{50}) (solid bars) and peak amplitude (open bars). *P < 0.05, values in Iso are significantly greater than control; **P < 0.05, values in Iso + CNP are significantly less than those in Iso alone (n = 7 cells).

Fig. 2. Effect of CNP (10^{-8} M) on bullfrog atrial myocyte Ca^{2+} current (I_{Ca}) in basal conditions. A: two representative recordings of I_{Ca} elicited by a voltage clamp step to +20 mV in control conditions (1) and after the addition of CNP (2). CNP reduced the peak of the current from ~275 to 175 pA (mean ± SE, n = 11 cells). The membrane voltages at which CNP significantly inhibited I_{Ca}.
CNP INHIBITS $I_{\text{Ca}}$ VIA NPR-C

Effects of blocking NPR-A and NPR-B with HS-142-1. Additional independent evidence for an NPR-C-mediated effect on $I_{\text{Ca}}$ was obtained by applying the competitive NPR-A and NPR-B receptor antagonist HS-142-1 (10^{-6} M). This compound binds to both of these guanylyl cyclase-linked receptors, thereby blocking the ability of CNP to increase cGMP levels (39). Previous studies (39, 40) have shown that HS-142-1, used at this same concentration, abolished the ability of nanomolar concentrations of CNP to increase cGMP levels.

The raw data and summary $I-V$ curves in Fig. 5 show that HS-142-1 has no significant effect on $I_{\text{Ca}}$ in the presence of Iso. After 5–10 min of superfusion of HS-142-1, CNP was added, $I_{\text{Ca}}$ was inhibited by $\sim$50%, and $I_{K_1}$ was unaffected. These findings provide further evidence that CNP inhibits $I_{\text{Ca}}$ by binding to NPR-C.

CNP effects in presence of elevated intracellular cAMP. Results from biochemical and molecular studies on rat heart strongly suggest that the C receptor is functionally linked to the inhibition of adenyllyl cyclase, and that this inhibitory interaction follows the activation of $G_i$ (51). To evaluate this possibility in bullfrog atrium, cAMP ($10^{-5}$ M) was added to the pipette to increase $I_{\text{Ca}}$. Because cAMP production is downstream from the activation of both the G protein and adenyllyl cyclase, this maneuver could provide insight into the part of the biochemical pathway that is modulated by CNP. In these experiments, after the cAMP-induced increase in $I_{\text{Ca}}$ had reached steady state, cANF was added to the superfusate to selectively activate NPR-C. The data in Fig. 6A show that intracellular elevation of cAMP ($10^{-5}$ M) increased $I_{\text{Ca}}$ significantly.
CNP INHIBITS $I_{Ca}$ VIA NPR-C

**DISCUSSION**

*Natriuretic peptide effects on the heart.* Both CNP and the NPR-C receptor have been shown by immunohistochemistry to be present in the heart of mammals, including humans (46, 58), and CNP has a prominent negative inotropic effect on the rat heart (45). CNP can influence heart rate by acting on either presynaptic or postsynaptic sites (25). However, few studies (21, 33, 55) have examined the electrophysiological effects of natriuretic peptides in cardiac tissue, and most of the published data have been obtained using only ANP. A consistent finding is that ANP causes a decrease in $I_{Ca}$. However, all of these results have been previously interpreted in terms of the peptide activating a guanylyl cyclase-linked receptor, with a resulting increase in cGMP.

Our results provide the first electrophysiological evidence that CNP, at physiological concentrations, can inhibit $I_{Ca}$. In addition, we have shown that this effect is mediated via the C receptor (NPR-C) in the heart. Our findings also conclusively demonstrate that NPR-C mediates important physiological functions via the cAMP second messenger system in the heart. Thus, both cANF (a NPR-C-specific agonist), and CNP applied in the presence of HS-142-1 (an NPR-A and NPR-B antagonist) were able to significantly inhibit $I_{Ca}$ (Figs. 4 and 5). In combination, these two data sets demonstrate that the inhibition of $I_{Ca}$ is mediated by NPR-C. On the basis of these findings, it is no longer appropriate to denote NPR-C as a “clearance receptor” in the heart. Previously, physiological roles for NPR-C have been demonstrated in a variety of cultured cell lines (35, 43, 56). Our conclusion that CNP inhibits $I_{Ca}$ by binding to NPR-C relies on the specificity of the agonists and antagonists used in this study. In previous studies (2, 42, 43), cANF has been shown to have no capacity to bind NPR-B or increase cGMP levels and therefore it is used as an NPR-C agonist. Similarly, HS-142-1, at the concentration used in this study, blocks NPR-B and prevents the accumulation of cGMP in the presence of CNP (39, 40). Data obtained with HS-142-1 provide consistent evidence that it is a specific blocker of NPR-B in many tissues, including the heart and vascular smooth muscle (11, 32, 53).

It is noteworthy that CNP caused a small, but significant, reduction in basal Ca$^{2+}$ current (Fig. 2). This is consistent with the supposition that there is some turnover of the enzyme adenylyl cyclase under basal conditions (26). Application of CNP can, therefore, activate $G_i$ and reduce basal adenylyl cyclase activity that is normally present in cardiac myocytes (20).

Gisbert and Fischmeister (21) first reported that atrial natriuretic factor (as ANP is sometimes referred to) can strongly inhibit $I_{Ca}$ in frog ventricular myocytes; however, they did not specifically examine which NPR subtype was involved. Their papers also showed that including cAMP in the recording pipette can prevent the inhibitory effect of atrial natriuretic factor. For these reasons, they concluded that atrial natriuretic factor acted by binding to NPR-B, increasing...
cGMP levels, and activating a cGMP-dependent phosphodiesterase, which would be expected to decrease cAMP levels (21, 29). The reductions in $I_{\text{Ca}}$ we have illustrated in Figs. 4 and 5 (which were obtained by blocking any possible contribution from the NPR-B/cGMP pathway) were slightly smaller than the effect illustrated in Fig. 3 (where both NPR-B and NPR-C could have been stimulated). It is therefore possible that activation of NPR-B could make a small contribution to the reduction in $I_{\text{Ca}}$. Nevertheless, our results show that NPR-C is the main receptor responsible for decreasing $I_{\text{Ca}}$. Available cell signaling and molecular data suggest that this occurs after activation of Gi and the inhibition of adenylyl cyclase as opposed to the well-known NPR-B-mediated increase in intracellular cGMP (51).

A recent study by Doyle et al. (17) on NPR expression in the rat heart has shown that NPR-B (the main guanylyl cyclase-linked CNP receptor) is primarily expressed in a nonmyocyte population of cells. In this study, anti-receptor antibody staining was detected in the smooth muscle of the vasculature and interstitial cells, but not the myocytes themselves. The same antibody detected NPR-B in immunoblots of protein extracts from nonmyocytes and recognized an equivalent protein in cardiac fibroblasts. Thus it appears that expression of NPR-B in the heart is localized mainly to fibroblasts (17).

Intracellular signaling pathway associated with NPR-C. An elegant series of papers examining the effects of natriuretic peptides on isolated myocardial preparations from rat heart (3, 4, 51) allow our findings to be put into the context of well-defined biochemical cascades in mammalian heart. These biochemical studies demonstrated that ANP significantly inhibited adenylyl cyclase in a dose-dependent fashion in cultured atrial and ventricular myocytes from neonatal rats (1). The inhibitory effect of ANP on adenylyl cyclase was attenuated by pertussis toxin (4), suggesting that a Gi protein was responsible for the ANP-mediated inhibition of adenylyl cyclase.

More recent molecular investigations (3, 51) have demonstrated that adenylyl cyclase and NPR-C can interact in mammalian heart. Anand-Srivastava and co-workers (3) raised polyclonal rabbit antisera against the 37 amino acid sequence corresponding to the cytoplasmic domain of bovine NPR-C and showed that application of this antibody markedly suppressed the ANP-mediated inhibition of adenylyl cyclase. This result implicates the cytoplasmic domain of NPR-C in the inhibition of adenylyl cyclase. As a more direct test, a synthetic peptide corresponding to the 37-amino acid sequence of NPR-C was used. This peptide alone significantly reduced adenylyl cyclase activity, an effect that was completely abolished by pertussis toxin. Pagan and Anand-Srivastava (51) subsequently demon-
strated that the cytoplasmic domain of NPR-C contains specific Gι activator sequences that can directly inhibit adenylyl cyclase, as first described by Okamoto and Nishimoto (48). Most recently, the precise 17-amino acid sequence of the intracellular domain of NPR-C that is responsible for the direct activation of Gι has been identified with the use of site-directed mutagenesis techniques (63). Thus the cytoplasmic domain of NPR-C can directly activate the Gι protein and inhibit adenylyl cyclase.

In our experiments, when Iso (which increases cAMP via a stimulatory G protein) was used to increase ICa, both CNP and cANF inhibited the Ca2+ current by at least 50%. However, including cAMP in the pipette (which bypasses adenylyl cyclase and provides the cell with a continuous supply of cAMP) resulted in an increase in ICa that was sustained during subsequent application of cANF. These results suggest that CNP acts via the Gι protein and adenylyl cyclase, rather than through CGMP-dependent phosphodiesterase.

Our observations and previous studies provide a basis for identifying some of the intracellular effector proteins involved in this pathway (refer to Fig. 7). The production of cAMP is dependent on adenylyl cyclase activity. The adenylyl cyclase V and VI isoforms are expressed at high levels in the heart (23, 54). Inhibition of adenylyl cyclase occurs via the Gι proteins (Gι1, Gια2, and Gια3), and Gια2 and Gια3 are expressed in the heart (3). Thus our working hypothesis is that in cardiac myocytes CNP binds to NPR-C and then activates either Gια2 or Gια3, thus inhibiting adenylyl cyclase V or VI. Inhibition of adenylyl cyclase results in a decrease in the level of phosphorylation of the ICa by protein kinase A, which decreases Ca2+ influx (27). Significance of our electrophysiological findings. It is intriguing that the effects of CNP observed in this study are similar to the effects of acetylcholine (18, 20, 44). Acetylcholine causes well-characterized negative inotropic effects that are mediated via a sarcolemmal muscarinic (M2) receptor, which is distinct from that used by CNP. In a variety of cardiac cells, including murine atrial cells, it has been clearly demonstrated that acetylcholine decreases cAMP levels that have been previously increased with catecholamines (8, 9). Many previous studies have established that acetylcholine, when bound to the M2 muscarinic receptor, stimulates Gι protein and inhibits adenylyl cyclase, which reduces cAMP levels (27). The similarity of CNP and acetylcholine effects illustrates a level of redundancy in signaling pathways within the heart. Multiple hormones and neurotransmitters activate very similar biochemical cascades and mediate the same electrophysiological effects. Furthermore, recent findings (7) clearly demonstrate that cAMP signaling occurs in distinct intracellular compartments. This suggests that different G protein-coupled receptor-signaling pathways may utilize different pools or sources of intracellular second messengers such as cAMP (7). The NPR-C effects described in this study may involve this phenomenon of intracellular compartmentalization.

Acetylcholine is also known to bind to the M2 muscarinic receptor and activate an inwardly rectifying K+ conductance via the release of the βγ-subunits of the heterotrimeric G protein (60). CNP is unlikely to mimic this acetylcholine effect because NPR-C is not a traditional heterotrimeric G protein and is not known to elicit any G protein-mediated βγ-subunit effects. Rather, NPR-C contains specific Gι activator domains, which directly modulate Gι and the enzyme adenylyl cyclase (51, 63).

In humans, pathological conditions such as congestive heart failure can result in significant increases in the release of all natriuretic peptides (59). Elevated BNP levels, which are documented after acute myocardial infarction (41), are considered to be a strong risk factor for mortality (14, 49). Accordingly, plasma BNP levels are now used as a diagnostic tool for patients with myocardial infarctions and congestive heart failure (15, 52). NPR-C binds to all of the natriuretic peptides with similar affinity, therefore it is anticipated that ANP and BNP would also decrease ICa in the same way we have demonstrated for CNP.

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DISCLOSURES

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