Natriuretic peptides stimulate the cardiac sodium pump via NPR-C-coupled NOS activation

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William M, Hamilton EJ, Garcia A, Bundgaard H, Chia KK, Figtree GA, Rasmussen HH. Natriuretic peptides stimulate the cardiac sodium pump via NPR-C coupled NOS activation. Am J Physiol Cell Physiol 2008;294:C1067–C1073. First published February 13, 2008; doi:10.1152/ajpcell.00243.2007.—Natriuretic peptides (NPs) and their receptors (NPRs) are expressed in the heart, but their effects on myocyte function are poorly understood. Because NPRs are coupled to synthesis of cGMP, an activator of the sarcolemmal Na+-K+ pump, we examined whether atrial natriuretic peptide (ANP) regulates the pump. We voltage clamped rabbit ventricular myocytes and identified electrogenic Na+-K+ pump current (arising from the 3:2 Na+:K+ exchange and normalized for membrane capacitance) as the shift in membrane current induced by 100 μmol/l ouabain. Ten nanomoles per liter ANP stimulated the Na+-K+ pump when the intracellular compartment was perfused with pipette solutions containing 10 mmol/l Na+ but had no effect when the pump was at near maximal activation with 80 mmol/l Na+ in the pipette solution. Stimulation was abolished by inhibition of cGMP-activated protein kinase with KT-5823, nitric oxide (NO)-activated guanylyl cyclase with ODQ sensitive, we exposed myocytes to AP-811, a highly selective ligand for the NPR-C “clearance” receptor. It abolished ANP-induced pump stimulation. Conversely, the selective NPR-C agonist ANP(4-23) reproduced stimulation. The stimulation was blocked by l-NAME. To examine NO production in response to ANP(4-23), we loaded myocytes with the NO-sensitive fluorescent dye diacylated diaminofluorescein-2 and examined them by confocal microscopy. ANP(4-23) induced a significant increase in fluorescence, which was abolished by l-NAME. We conclude that NPs stimulate the Na+-K+ pump via an NPR-C and NO-dependent pathway.

cyclic guanosine 3',5'-cyclic monophosphate-activated kinase; nitric oxide synthase; congestive cardiac failure; atrial natriuretic peptide

NATRIURETIC PEPTIDES, synthesized and secreted in the heart, were originally found to provide a humoral link to the kidneys and the vasculature. However, subsequent demonstration of cardiac natriuretic peptide receptors (NPRs) suggested that the peptides also have direct effects on the myocardium (11, 24). There are three known NPRs: NPR-A, NPR-B, and NPR-C. NPR-A mediates most known effects of natriuretic peptides. Both NPR-A and NPR-B function as membrane guanylyl cyclases, also referred to as “particulate” guanylyl cyclases (pGCs), and activate downstream cGMP-dependent processes (29). NPR-C does not have a catalytic intracellular domain, and its main function is thought to be the clearance of natriuretic peptides from the extracellular compartment through receptor-mediated internalization and degradation (29). However, NPR-C has also been reported to be linked to intracellular messenger pathways. Coupling to a G_i protein mediates a reduction in Ca^2+ channel currents in mouse sinoatrial node cells (30). It is also coupled to a nitric oxide (NO) synthase (NOS)-dependent pathway in various noncardiac cells (2). NO activates “soluble” guanylyl cyclase (sGC) that mediates synthesis of cGMP, the same messenger molecule as that synthesized by the catalytic subunit of NPR-A and NPR-B.

We have previously demonstrated cGMP-induced Na+-K+ pump activation in isolated ventricular myocytes. Pump activation was seen with pharmacological activation of sGC and with direct perfusion of the intracellular compartment with a cGMP analog or cGMP-dependent protein kinase (PKG) (39). Given that natriuretic peptides activate cGMP production, our previous study suggests that they may stimulate the Na+-K+ pump. However, effects of natriuretic peptides on the pump cannot be assumed to be similar to that elicited with the experimental activation of cGMP-dependent pathways in our previous study (39) since signaling by cGMP is likely to be restricted to microdomains that differ for NPR-A/B and sGC. In agreement with this reservation, cellular effects of cGMP synthesized by pGC and sGC can differ in cardiac myocytes (40). We have now examined the effect of NPR activation on the sarcolemmal Na+-K+ pump in ventricular myocytes.

MATERIALS AND METHODS

Cells. Single ventricular myocytes were isolated from male White New Zealand rabbits. Details of anesthesia, excision of the heart, and cell isolation techniques have been described previously (18). The institutional review committee for animal research had approved the experimental protocols. The myocytes were used on the day of isolation only and were stored at room temperature in Krebs-Henseleit buffer solution.

Measurement of electrogenic Na+-K+ pump current. We used the whole cell patch-clamp technique to measure electrogenic Na+-K+ pump current (I_p, arising from the 3:2 Na+:K+ exchange ratio). Myocytes were suspended in a tissue bath mounted on an inverted microscope for experimentation. While we established the whole cell configuration, we perfused the bath with modified Tyrode’s solution, which contained (in mmol/l) 140 NaCl, 5.6 KCl, 2.16 CaCl_2, 1 MgCl_2, 10 glucose, 0.44 NaH_2PO_4, and 10 N-2-hydroxyethyl piper-
azine-N'-2-ethene-sulfonic acid (HEPES). It was titrated to a pH of 7.40 at 35°C with NaOH. Within 2–3 min of establishing the whole cell configuration, we switched to a superfusate that was identical except that it was nominally Ca\(^{2+}\) free and contained 0.2 mmol/l CdCl\(_2\) to reduce Na\(^{+}\)/Ca\(^{2+}\) exchange and prevent myocyte Ca\(^{2+}\) overload and contracture during subsequent Na\(^{+}\)-K\(^{+}\) pump inhibition. The Ca\(^{2+}\)-free superfusate included 2 mmol/l BaCl\(_2\) to block K\(^{+}\) channels. It also included atrial natriuretic peptide (ANP) or ANP(4–23) when indicated. Myocytes were exposed to these solutions for 10–12 min before \(I_p\) was measured. In some experiments, we modified this solution by replacing Na\(^{+}\)-containing compounds with N-methyl-D-glucamine (NMG.Cl) (16).

We used wide-tipped patch pipettes (4 μm) filled with solution containing (in mmol/l) 5 HEPES, 2 MgATP, 5 ethylene glycol-bis(β-aminoethy1 ether)-N,N,N',N'-tetraacetic acid (EGTA), 70 potassium glutamate, 10 sodium glutamate, and 80 tetramethylammonium chloride (TMA.Cl). It was titrated to a pH of 7.20 at 35°C with 1 mol/l KOH. We eliminated Na\(^{+}\)-containing compounds in some experiments (replaced with TMA.Cl), whereas the Na\(^{+}\) concentration was increased to 80 mmol/l others (osmotic balance was maintained by adjusting the concentration of TMA.Cl). Patch pipettes filled with these solutions had resistances of 0.8–1.1 MΩ. The series resistance after formation of the whole cell configuration was ≤2.8 MΩ, satisfying criteria that we have previously defined as essential for experimental validity (39).

\(I_p\) was identified at a holding potential of ~40 mV as the difference between stable plateaus of holding current before and after Na\(^{+}\)-K\(^{+}\) pump blockade with 100 mmol/l ouabain 10–15 min after establishing the whole cell configuration. A stable current was identified when no drift could be identified on the digital display of the voltage-clamp amplifier for at least 50 s. The plateaus were defined by the means of five samples of the holding current obtained with an electronic cursor taken at approximately 5-s intervals before and after exposure to ouabain. Sampling rate for all recordings was 1 s/1. We used the continuous single-electrode mode of Axoclamp 2A or 2B amplifiers supported by AxoTape and pCLAMP software (Axon Instruments, Foster City, CA) for measurements. We report \(I_p\) normalized for membrane capacitance and hence cell size. Since the effect of ouabain on \(I_p\) is not reversible within the time frame that ensures stable holding currents (10, 39), separate myocytes were used for measurements of \(I_p\) under different experimental conditions.

**Imaging of intracellular NO production by diacetylated diaminofluorescein-2.** To detect NO production, we loaded myocytes with diacetylated diaminofluorescein-2 (DAF-2 DA). This membrane-permeable dye is hydrolyzed intracellularly by cystosolic esterases releasing diaminofluorescein-2 (DAF-2), which is converted in the presence of NO into a fluorescent product, DAF-2 triazole (21). Some myocytes were preincubated with the NOS inhibitor N\(^{\text{G}}\)-nitro-L-arginine methyl ester (L-NAME; 1 mM) for 15 min before loading with DAF-2 DA (1 μmol/l) for 30 min in the dark at 37°C. They were then exposed to solution containing ANP(4–23) or no ANP(4–23) for 10 min before fixation (2% paraformaldehyde in ice for 4 min). The myocytes were washed and then mounted on poly-L-lysine-coated glass slides in Vectashield and examined under a laser-scanning confocal microscope (Nikon Cl). The excitation wavelength was 488 nm and the emission wavelength 530 nm. The fluorescence images were obtained using constant settings of scanning speed, pinhole diameter, and voltage gain. Myocytes (≥6) representative of each experimental condition were selected randomly for measurement of fluorescence intensity (Photoshop, Adobe). Only myocytes with clear striations and rodlike shape were included in the analysis. The average of each experiment was normalized against its control [DAF-loaded, not exposed to ANP(4–23)] after subtraction of baseline (fluorescence intensity in non-DAF-loaded myocytes).

**Chemicals and reagents.** TMA.Cl and NMG.Cl were purum grade and were obtained from Fluka Chemicals. All other chemicals used in Tyrode’s solution were analytical grade and were obtained from BDH (Sydney, Australia). Ouabain, heat shock protein 90 (HSP90), ANP(4–23), L-NAME, and DAF-2 DA were obtained from Sigma Chemical (St. Louis, MO). ANP (human) was supplied by Clinalfa (Darmstadt, Germany). ODQ, KT-5823, okadaic acid (OA), and methyl okadaic acid were supplied by Calbiochem (La Jolla, CA). AP-811 was supplied by California Peptide Research (Nappa, CA). Vectashield was obtained from Vector Laboratories (Burlingame, CA).

**Statistical analysis.** Results are expressed as means ± SE. Students t-test for unpaired data was used for statistical comparisons. We used Dunnett’s test when the same control group was used for more than one comparison. \(P < 0.05\) was regarded as significant in all comparisons. Student’s t-test for paired data, with single-tail distribution, was used for analysis of differences in DAF-2-DA fluorescence intensity.

**RESULTS**

**ANP stimulates \(I_p\)** We first examined the effect of ANP on the Na\(^{+}\)-K\(^{+}\) pump. We used ANP in a concentration of 10 mmol/l, the lowest concentration at which binding of ANP to cardiac sarcolemmal membranes is nearly saturated in vitro (32). Control myocytes and myocytes exposed to ANP were maintained in the whole cell configuration for 10–15 min before they were exposed to ouabain. The experimental protocol is illustrated in Fig. 1A. In initial experiments, we used 10 mmol/l Na\(^{+}\) in patch pipette solutions, and a standard Na\(^{+}\) containing superfusate. The ouabain-induced shift in holding current was larger for myocytes exposed to ANP than for the control myocytes, as exemplified in the top two traces of Fig. 1B. The mean \(I_p\) for control myocytes and myocytes exposed to ANP are shown in Fig. 1C. ANP induced a statistically significant increase in \(I_p\) at physiological levels of intra- and extra-cellular Na\(^{+}\).

The ANP-induced increase in \(I_p\) could be due to an increase in intrinsic Na\(^{+}\)-K\(^{+}\) pump activity or it could be due to ANP-induced influx of Na\(^{+}\) into myocytes and secondary pump stimulation. To distinguish between direct and indirect pump stimulation, we initially used nominally Na\(^{+}\)-free patch pipette solutions and a Na\(^{+}\)-containing superfusate. Holding currents of a control myocyte and a myocyte exposed to ANP are shown in the two middle traces of Fig. 1B. In the absence of Na\(^{+}\) in pipette solutions, the ouabain-induced shift in holding current was barely detectable in the control myocytes or in the myocytes exposed to ANP. The mean \(I_p\) for control myocytes and myocytes exposed to ANP are shown in Fig. 1C. \(I_p\) was very low for both groups of myocytes, and there was no significant difference between mean \(I_p\) for control myocytes and myocytes exposed to ANP (14).

To eliminate ANP-induced Na\(^{+}\) influx as a possible cause of Na\(^{+}\)-K\(^{+}\) pump stimulation, we used a superfusate that was nominally Na\(^{+}\) free. Patch pipette solutions contained 10 mmol/l Na\(^{+}\). Holding currents of a control myocyte and a myocyte exposed to ANP are shown in the two lower traces of Fig. 1B. Despite the lack of Na\(^{+}\) in the superfusate, the ouabain-induced shift in holding current was larger for the myocyte exposed to ANP than for the control myocyte. The mean \(I_p\) for control myocytes and myocytes exposed to ANP are shown in Fig. 1C. ANP induced a
statistically significant increase in \( I_p \) in the absence of extracellular \( \text{Na}^+ \).

The mean levels of normalized holding currents recorded after exposure of myocytes to ouabain using all protocols exemplified in Fig. 1B were similar, indicating that ANP had no effect on leak current in the absence of ouabain, i.e., the peptide did not induce a detectable change in nonpump membrane current under the experimental conditions we used. ANP induced an increase in ouabain-sensitive membrane current that was dependent on the presence of \( \text{Na}^+ \) in pipette solutions and could be demonstrated in the absence of extracellular \( \text{Na}^+ \). We conclude that the increase is due to direct stimulation of the \( \text{Na}^+\text{-K}^+ \) pump.

To examine whether ANP induces an increase in maximal \( \text{Na}^+\text{-K}^+ \) pump turnover rate, we patch clamped myocytes using 80 mmol/l \( \text{Na}^+ \) in pipette solutions, a concentration expected to nearly maximally stimulate the pump at intracellular sites. Superfusates contained \( \text{Na}^+ \). Holding currents of a control myocyte and a myocyte exposed to ANP are shown in Fig. 2. Ouabain-induced shifts in currents were much larger...
than those recorded using 10 mmol/l Na⁺ in pipette solutions (Fig. 1), and they were similar for a myocyte exposed or not exposed to ANP. The mean \( I_p \) for experiments performed using 80 mmol/l Na⁺ in pipette solution are presented in Fig. 2. There was no significant difference between mean \( I_p \) of control myocytes and myocytes exposed to ANP, suggesting that ANP does not stimulate maximal pump turnover.

ANP stimulates \( I_p \) via NOS/sGC/PKG. Since effects of ANP in target tissues usually are meditated by cGMP, we examined the effect of inhibition of PKG. We included 0.5 μmol/l KT-5823 in the patch pipette solution perfusing the intracellular compartment. This compound inhibits PKG with a \( K_i \) of \( \sim 0.2 \) μmol/l and protein kinases A (PKA) and -C (PKC) with a \( K_i \) of \( \sim 10 \) and \( \sim 4 \) μmol/l. The Na⁺ concentration in the pipette solutions was 10 mmol/l and the superfusate contained Na⁺ and ANP or was ANP free. The mean \( I_p \) of these experiments are presented in Fig. 3. KT-5823 abolished the ANP-induced Na⁺-K⁺ pump stimulation. It is unlikely that this was due to inhibition of PKC since PKC mediates a decrease in \( I_p \) in our model (8, 17). To examine whether inhibition of PKA could account for the effect of KT-5823 on the ANP-induced pump stimulation, we included 0.5 μmol/l H-89 rather than KT-5823 in pipette solutions. This caused no decrease in ANP-induced pump stimulation (data not shown).

This is in accordance with the absence of any effect of H-89 on an increase in \( I_p \) induced by inclusion of a cGMP analog in pipette solutions (39). We conclude that the effect of KT-5823 to abolish the ANP-induced Na⁺-K⁺ pump stimulation implicates PKG in the mechanism for the stimulation.

PKG is activated by cGMP. This may be synthesized by a receptor membrane guanylyl cyclase or sGC. We included 10 μmol/l ODQ in pipette solutions to inhibit sGC. The superfusate contained ANP or was ANP free. As shown in Fig. 3, ODQ abolished the ANP-induced pump stimulation. This implicates sGC and hence NO in the stimulation because ODQ is a specific inhibitor of NO-activated sGC. To support a role for NO, we examined the effect of inhibiting NOS with \( \mu \)-NAME. We included 10 μmol/l \( \mu \)-NAME in patch pipette solutions. As shown in Fig. 3, \( \mu \)-NAME abolished ANP-induced pump stimulation.

Since the molecular chaperone HSP90 plays an important role in NOS activation (28), we examined the effect of including 10 μmol/l of the HSP90 inhibitor radicicol (28) in pipette solutions. The superfusate contained ANP or was ANP free. As shown in Fig. 3, radicicol abolished ANP-induced Na⁺-K⁺ pump stimulation, implicating HSP90 in NOS activation. To further support this conclusion, we included 50 mmol/l recombinant HSP90 in the pipette solution. Myocytes were exposed to an ANP-free superfusate. The introduction of recombinant HSP90 into three myocytes induced an \( I_p \) similar to that of myocytes exposed to ANP (mean \( I_p = 0.52 \pm 0.07 \) pA/pF).

If PKG-mediated phosphorylation of pump molecules were the final step in the pathway linking ANP to the pump, inhibition of dephosphorylation would be expected to enhance pump stimulation. We examined the effect of including the protein phosphatase inhibitor OA in patch pipette solutions. Figure 4 summarizes \( I_p \) recorded using pipette solutions that included 5, 10, or 100 nmol/l OA. OA induced a decrease rather than an increase in the ANP-induced pump stimulation, whereas the inactive OA analog, methyl okadaic acid, had no effect. Inclusion of membrane-permeable substances in the patch pipette may not result in uniform distribution throughout a patch-clamped myocyte (for discussion see Ref. 39). Since OA is membrane permeable, we also included it in the superfusate to examine whether an OA in the low concentration of 5 nmol/l had an effect on the ANP-induced pump stimulation. The superfusate used both during establishment of the whole cell configuration and subsequently during measurement of \( I_p \) contained 5 nmol/l OA. Results are included in Fig. 4. OA abolished the ANP-induced Na⁺-K⁺ pump stimulation.

NPR-C mediates stimulation of \( I_p \). The dependence of ANP-induced stimulation of \( I_p \) on NO/sGC is inconsistent with activation of NPR-A/B. However, NPR-C has been reported to be coupled to NOS in noncardiac tissues. To examine whether NPR-C mediates pump stimulation, we used two highly selective NPR-C ligands, AP-811 and ANP(4–23). ANP(4–23) can elicit a cellular response and is hence regarded as an NPR-C agonist (34). While AP-811 is a highly selective NPR-C ligand (19, 38), it is not known whether it is an agonist or an antagonist.

In one series of experiments, we exposed myocytes to 100 nmol/l AP-811, included in all superfusates before and after the whole cell configuration was established. Results are shown in Fig. 5. Mean \( I_p \) of myocytes exposed to AP-811 was similar to mean \( I_p \) of controls. We next examined whether AP-811 blocked the effect of ANP by also including 10 nmol/l ANP in superfusates after the whole cell configuration was established according to the protocol shown in Fig. 1A. Results are included in Fig. 5. AP-811 abolished the increase in \( I_p \) that was induced by ANP alone (shown in Fig. 1), i.e., AP-811 was an antagonist in the experimental model used in the present study. We also exposed myocytes to 200 nmol/l ANP(4–23), using the same protocol as that illustrated for ANP in Fig. 1A. Results are included in Fig. 5. ANP(4–23) induced an increase in \( I_p \), similar to that induced by ANP. The increase was abolished by inclusion of \( \mu \)-NAME in patch pipette solutions.

NPR-C activates NOS. The functional Na⁺-K⁺ pump studies suggest that ANP stimulates the pump via an NO-mediated pathway. Since NPR-C has been reported to activate NOS, we

Fig. 3. Dependence of ANP-induced Na⁺-K⁺ pump stimulation on PKG, soluble guanylyl cyclase (sGC), nitric oxide (NO) synthase (NOS), and heat shock protein 90 (HSP90). Myocytes were superfused with control solutions or solutions containing ANP. Where indicated, patch pipettes were filled with solutions containing the PKG inhibitor KT-5823 (0.5 μmol/l), the sGC inhibitor H-1-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μmol/l), the NOS inhibitor \( \mu \)-nitro-L-arginine methyl ester (\( \mu \)-NAME; 10 μmol/l), or the HSP90 inhibitor radicicol (10 μmol/l). *Significant difference versus control.
examined whether ANP(4–23) induces an increase in fluorescence of the NO-sensitive dye DAF-2 DA. Myocytes were loaded with DAF-2 DA and examined by fluorescence confocal microscopy. Representative micrographs are shown in Fig. 6A. Unstimulated cells emitted a moderate degree of fluorescence over the background autofluorescence (no DAF-2 DA). Exposure to 200 nmol/l ANP(4–23) for 10 min induced augmentation of DAF-2 DA fluorescence. The augmentation was blocked by preincubation with the NOS inhibitor L-NAME (1 mmol/l). The mean fluorescence intensity for each condition, after the subtraction of baseline fluorescence, is shown in Fig. 6B. ANP(4–23) induced a significant increase in DAF-2 DA fluorescence compared with control. The increase was abolished by L-NAME.

**DISCUSSION**

Exposure of patch-clamped cardiac myocytes to ANP induced an increase in the ouabain-induced shift in holding current. This can only be attributed to a direct effect of ANP on the Na⁺-K⁺ pump if we can exclude secondary pump stimulation arising from transsarcolemmal Na⁺ influx. Both the Na⁺-K⁺-2Cl⁻ cotransporter (9) and the Na⁺/H⁺ exchanger (36) are sensitive to ANP in cardiac myocytes. However, they are inhibited rather than activated by ANP. Any contribution of these or other transporters or channels to ANP-induced Na⁺-K⁺ pump stimulation secondary to enhanced Na⁺ influx is ruled out by the dependence of the stimulation on Na⁺ in pipette solutions and the persistence of stimulation when Na⁺-free superfusates were used (Fig. 1).

The conclusion that ANP directly stimulates the sarcolemmal Na⁺-K⁺ pump is at odds with the effect of ANP in renal tissue. In cells permeabilized to induce a high intracellular Na⁺ concentration, ANP caused a decrease in activity of Na⁺-K⁺-ATPase attributed to an effect on maximal turnover of the enzyme (7, 35), whereas we observed pump stimulation when the Na⁺ concentration in pipette solutions was near physiological intracellular levels with no effect on maximal turnover. The difference in response of the pump to ANP may be due to tissue-dependent differences in cell signaling. In renal tissue, the effect of ANP on Na⁺-K⁺-ATPase activity was attributed...
to recruitment on silent dopamine receptors from the interior of tubular cells to the cell membrane and possible activation of adenyl cyclase (7), whereas adenyl cyclase activation did not account for the effect of ANP in our study. In agreement with our study, NO-dependent and PKG-dependent signaling stimulates Na\(^+-\)K\(^+\)-ATPase activity in cerebellar tissue (25).

Effects of ANP are usually attributed to the intrinsic guanylyl cyclase activity of NPR-A-mediating synthesis of cGMP (20, 22). The effect of KT-5823 to inhibit ANP-induced pump stimulation supports the role of cGMP-activated protein kinase. However, pump stimulation was abolished by ODQ, a selective inhibitor of the alternative cellular source of cGMP, sGC (33). Even at a concentration 10-fold higher than used in our study, ODQ has no effect on pGC activity (15) that is expected to include NPR-A. The effect of ODQ therefore implicates sGC and hence an NO-mediated pathway. The role of NO in ANP-induced pump stimulation is supported by \(\pi\)-NAME and radicicol blocking stimulation and HSP90 reproducing it. To our knowledge, NPR-A is not coupled to a NO/sGC/HSP90-mediated pathway, whereas NPR-C is linked to activation of NOS and NO in noncardiac cells (1, 5, 26). The highly selective NPR-C ligand AP-811 (19, 38) abolished ANP-induced pump stimulation, whereas the NPR-C agonist ANP(4–23), in a concentration not expected to have any effect on NPR-A (19, 38), reproduced it. ANP(4–23) also induced an increase in myocyte DAF fluorescence that was abolished by \(\pi\)-NAME. While the fluorescence can detect species other than NO (31), it is not expected to detect the product of NPR-A activation, cGMP. We conclude that the NPR-C receptor, coupled to NOS, mediates Na\(^+-\)K\(^+\) pump stimulation. The complete blockade of ANP-induced pump stimulation by AP-811 or by the interruption of NO/sGC pathways implies that cGMP synthesized by NPR-A guanylyl cyclase is not coupled to Na\(^+-\)K\(^+\) pump activation. Consistent with our findings, others have found ANP activation of cGMP-dependent protein kinase in cardiac myocytes is completely abolished by \(\pi\)-NAME (37).

The NOS1 and NOS3 isoforms constitutively expressed in cardiac myocytes are regarded as Ca\(^{2+}\)-dependent, yet NOS-dependent pathways were activated when we used a nominally Ca\(^{2+}\)-free superfuse and perfused the intracellular compartment of myocytes with EGTA-containing solution. However, the change in superfusates is not instantaneous, and myocytes would have been exposed to extracellular Ca\(^{2+}\), albeit in a diminishing concentration, during a transitional phase when ANP was washed in (see Fig. 1A). Other sources of Ca\(^{2+}\) include cellular storage pools (12), Ca\(^{2+}\)-rich submembranous microdomains, or the large pool of Ca\(^{2+}\) bound to proteins at the intracellular membrane surface of cardiac myocytes (3). Na\(^+-\)K\(^+\) pumps and NOS3 colocalize with caveolin in cardiac myocytes (13, 23), and caveolae play a particularly important role in Ca\(^{2+}\)-dependent signaling with Ca\(^{2+}\)-mobilized from localized pools (6). In the period immediately after a stimulus, the patch pipette solution may not buffer Ca\(^{2+}\) in signaling microdomains, and once NOS is activated, an association of NOS with HSP90 maintains its activity independent of intracellular Ca\(^{2+}\) (27).

The molecular mechanism that couples the NPR-C/NOS/sGC pathway to the Na\(^+-\)K\(^+\) pump molecule cannot be identified from our study. While the pump molecule can be nitrosylated (12), it is unlikely that the ANP-induced pump stimulation is due to a direct effect of NO since stimulation was abolished when sGC or PKG were inhibited. However, if pump stimulation was mediated by direct phosphorylation of the pump molecule or an associated protein, stimulation should have been enhanced rather than abolished by OA. A dephosphorylation step is implicated in ANP-induced pump stimulation, but the specific step cannot be identified. The sensitivity to OA of pump stimulation induced by other stimuli has been reported previously (4, 16).

ANP-induced Na\(^+-\)K\(^+\) pump stimulation shown in our study is expected to decrease cardiac contractility because of the known positive relationship between intracellular Na\(^+\) and contractility in most species. This prediction has been confirmed by a study using experimental protocols closely reflecting ours. Exposure of isolated rabbit ventricular myocytes to 10 nmol/l ANP reduced contractility. The messenger pathways implicated were also in good agreement with our study since ANP doubled intracellular cGMP levels and inhibition of PKG abolished the decrease in contractility (41).

The NPR-C has traditionally been regarded as a clearance receptor, and only a few downstream physiological events regulated by NPR-C have been identified. The present study demonstrates that the NPR-C is coupled to activation of the membrane Na–K pump in the heart via a NOS/NO/cGMP-dependent pathway. In view of the pivotal role of the pump in cardiac myocyte function, this is likely to have important physiological and pathophysiological implications. Our study also has important implications for the experimental investigation of cGMP signaling in cardiac myocytes. The common practice of exposing myocytes to ANP to selectively activate pGC should be reconsidered.

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