Apelin, the Novel Endogenous Ligand of the Orphan Receptor APJ, Regulates Cardiac Contractility

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Abstract—The orphan receptor APJ and its recently identified endogenous ligand, apelin, exhibit high levels of mRNA expression in the heart. However, the functional importance of apelin in the cardiovascular system is not known. In isolated perfused rat hearts, infusion of apelin (0.01 to 10 nmol/L) induced a dose-dependent positive inotropic effect (EC50: 33.1±1.5 pmol/L). Moreover, preload-induced increase in dP/dt max significantly augmented (P<0.05) in the presence of apelin. Inhibition of phospholipase C (PLC) with U-73122 and suppression of protein kinase C (PKC) with staurosporine and GF-109203X markedly attenuated the apelin-induced inotropic effect (P<0.001). In addition, zoniporide, a selective inhibitor of Na+–H+ exchange (NHE) isoform-1, and KB-R7943, a potent inhibitor of the reverse mode Na+-Ca2+ exchange (NCX), significantly suppressed the response to apelin (P<0.001). Perforated patch-clamp recordings showed that apelin did not modulate L-type Ca2+ current or voltage-activated K+ currents in isolated adult rat ventricular myocytes. Apelin mRNA was markedly downregulated in cultured neonatal rat ventricular myocytes subjected to mechanical stretch and in vivo in two models of chronic ventricular pressure overload. The present study provides the first evidence for the physiological significance of apelin in the heart. Our results show that apelin is one of the most potent endogenous positive inotropic substances yet identified and that the inotropic response to apelin may involve activation of PLC, PKC, and sarcolemmal NHE and NCX. (Circ Res. 2002;91:434-440.)

Key Words: apelin • contractility • signal transduction • gene expression

Although apelin and APJ mRNA have been found to be ubiquitously expressed in peripheral tissues as well as various regions of the central nervous system,4–7 the exact function of apelin has not yet been established. Interestingly, sequence analysis of the mature apelin peptide revealed identity, albeit limited, to angiotensin II.5 In addition, APJ and AT1 as well as apelin and angiotensinogen showed significant similarity in tissue distribution,5 suggesting that apelin and angiotensin II may affect the same biological processes. Indeed, intraperitoneal administration of apelin resulted in short-term increases in drinking behavior in rats,5 in parallel with the thirst-promoting effect of angiotensin II. In contrast to the well-established vasopressor effect of angiotensin II, intravenous injection of apelin lowered blood pressure in anesthetized rats.5,8 Based on these preliminary results, one can anticipate that apelin, like angiotensin II, may have an important role in the regulation of cardiovascular homeostasis.

In the peripheral rat tissues, high levels of apelin mRNA4–6 and moderate levels of APJ mRNA6,7 were detected in the heart. Furthermore, quantitative autoradiography revealed the presence of specific APJ binding sites in human and rat...
myocardium with a comparable receptor density to AT1.9 However, to date, there is no information available regarding the functional significance of the APJ-apelin system in the myocardium. Therefore, the objective of the present study was to characterize the direct cardiac effects of apelin as well as the underlying signaling pathways in vitro by using isolated perfused rat heart preparation and perforated patch-clamp recordings. Moreover, to test the potential pathophysiological importance of apelin, we studied the gene expression of apelin and APJ in vitro in cultured neonatal rat ventricular myocytes (NRVMs) subjected to mechanical stretch and in vivo in two models of chronic ventricular pressure overload.

Materials and Methods

Isolated Perfused Rat Heart Preparation

All protocols were reviewed and approved by the Animal Use and Care Committee of the University of Oulu. Male 7-week-old Sprague-Dawley (SD) rats (n=230) from the Center for Experimental Animals at the University of Oulu were used. Rats were decapitated and hearts were quickly removed and arranged for retrograde perfusion by the Langendorff technique as described previously.10,11 The hearts were perfused with a modified Krebs-Henseleit bicarbonate buffer, pH 7.40, equilibrated with 95% O2/5% CO2 at 37°C. Hearts were perfused at a constant flow rate of 5.5 mL/min with a peristaltic pump (Minipuls 3, model 312). Heart rate was maintained constant (304±1 bpm) by atrial pacing using a Grass stimulator (model S88, 11 V, 0.5 ms). Contractile force (apicobasal displacement) was obtained by connecting a force displacement transducer (Grass Instruments, FT03) to the apex of the heart at an initial preload stretch of 2 g. In another set of experiments, left ventricular contractility was assessed by measuring isovolumic left ventricular pressure. Details of the methods and the experimental protocols are provided in an expanded Materials and Methods section, which can be found in the online data supplement available at http://www.circresaha.org.

Perforated Patch-Clamp Recordings

The whole-cell membrane currents were recorded from enzymatically isolated single adult ventricular myocytes by the Amphotericin B–perforated patch-clamp method12 (for details see the online data supplement).

Mechanical Stretch of Neonatal Rat Ventricular Myocytes

NRVMs were subjected to cyclic stretch by means of the Flexercell computer-driven vacuum system13 (for details see the online data supplement).

Isolation of Cytoplasmic RNA and Northern Blot Analysis

Total RNA isolation and atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and 18S RNA Northern blot analysis were performed as previously described.14 Real-Time Quantitative RT-PCR Analysis

Rat apelin, APJ receptor, and 18S RNA levels were measured by real-time quantitative RT-PCR analysis using Taqman chemistry on an ABI 7700 Genetic Analyzer (Applied Biosystems) as described previously.15 The sequences of the forward (F) and reverse (R) primers and probes (P) for RNA detection were as follows: apelin (F) 5'-CAAGGATCCCTTTGGCCC-3', (R) 5'-AGGAGAAGCTGGG-TCTCCAA-3', (P) 5'-Fam-TCTTCTGCAACTCTTGGACTGC-Tamra-3'; APJ (F) 5'-CCTGGTGGAACATCTGAGTGG-3', (R) 5'-AGGCGGATGCAAAATTT-3', (P) 5'-Fam-TGACCTTTGCCCT-GTGTCGGATGC-Tamra-3'; 18S (F) 5'-TGGTGTCAAAAGCTGA-AACTTAAG-3', (R) 5'-AGTCAAAATAGCCGCAGGC-3', (P) 5'-Vic-CCTGGTGGAACATCTGAGTGG-Tamra-3'.

Statistical Analysis

Results are presented as mean±SEM. Data were analyzed with repeated measures- or 1-way ANOVA followed by Bonferroni post hoc test. Differences were considered statistically significant at the level of P<0.05.

Results

Effect of Apelin on Contractility in Isolated Rat Hearts

Administration of apelin-16 (0.01 to 10 nmol/L) induced a dose-dependent increase in developed tension in the isolated rat heart preparation (Figure 1A; see also online Table 1, which can be found in the online data supplement available at http://www.circresaha.org). Maximal response to apelin was observed at the concentration of 1 nmol/L and the half-maximal effective concentration (EC50) of apelin was 33.1±1.5 pmol/L. As shown in Figure 1B, the elevation of developed tension in response to apelin was gradual (F=72.1, P<0.001, apelin versus vehicle; for drug and time interaction, repeated measures ANOVA). A significant increase in contractility was observed 2 minutes after the start of apelin infusion, and the maximal increase was seen at approximately 24 minutes. The time-course of the effect of apelin was similar to those of the potent inotropic agents endothelin-11,16,17 and adrenomedullin10,11; however, it was markedly different from the rapidly developing, short-lived effect of the β-adrenergic receptor agonist isoproterenol (Figure 1B). The maximal responses to apelin, endothelin-1, and adrenomedullin were equal at the concentration of 1 nmol/L. Furthermore, the increase in developed tension induced by apelin was 69% of the maximal response to isoproterenol (10 μmol/L) (Figure 1B, online Table 1).

The apelin-induced increase in developed tension was not associated with significant changes in time-to-peak tension (before and after 1 nmol/L apelin: 57.0±2.6 versus 56.3±2.4 ms; P=NS) or time to 50% relaxation (40.9±1.0 versus 39.8±0.6 ms; P=NS). The resting tension (2.0±0.01 g) of the perfused hearts was not significantly affected by apelin (0.01 to 1 nmol/L), except that it induced a slight increase (2.2±0.03 g; P<0.05) at the highest concentration (10 nmol/L). Overall, changes in perfusion pressure induced by apelin (0.03 to 10 nmol/L) were small: eg, 1 nmol/L apelin slightly decreased the perfusion pressure from 32.4±1.6 to 30.6±1.4 mm Hg (P<0.001).

Effect of Preload on Apelin-Induced Positive Inotropic Effect

Next, we tested the effect of apelin on contractility at different levels of preload in isolated isovolumic rat hearts. The maximal derivative of isovolumic left ventricular pressure (dP/dtmax) was similar in the presence and absence of apelin (1 nmol/L) at a left ventricular end-diastolic pressure (LVEDP) of 1 or 5 mm Hg. However, when LVEDP was increased to 10 or 15 mm Hg in the presence of apelin, dP/dtmax was increased by 33% (P<0.05) and 35% (P<0.05) versus respective control values (Figure 1C). In contrast to the enhanced contractility, the diastolic function was not
affected by apelin even at the highest level of LVEDP. The minimal derivative of isovolumic pressure (dP/dt min: 83 ± 2 versus 80 ± 3 mm Hg/s; P<0.05) and time-from-peak systolic pressure to 60% relaxation (48.6 ± 5.8 versus 50.3 ± 2.4 ms; P<0.05) did not differ significantly between vehicle and apelin-infused hearts at 15 mm Hg of LVEDP.

Specificity of the Apelin-Induced Positive Inotropic Effect

Because the APJ receptor shares sequence homology with AT₁ receptor, we tested if the effect of apelin was mediated via angiotensin receptors. Infusion of CV-11974 (10 nmol/L), an AT₁ receptor antagonist, had no influence on developed tension (P=NS) and did not alter the positive inotropic response to 1 nmol/L apelin (P=NS; Figure 2A, online Table 2). To further characterize the specificity of the effect of apelin, we infused the peptide in the presence or absence of bosentan (1 μmol/L), an ET₁/ET₂ endothelin receptor antagonist, propranolol (1 μmol/L), a β-adrenergic receptor blocker, and prazosin (0.1 μmol/L), an α-adrenergic receptor blocker. As shown in Figure 2, the apelin-induced increase in developed tension was not attenuated by the receptor antagonists (P=NS; online Table 2). None of the various antagonists affected baseline contractility (P=NS, Figure 2, online Table 2). Recently, apelin has been reported to lower blood pressure via a nitric oxide–dependent mechanism. Because low concentrations of nitric oxide can increase cardiac contractility, we tested the effect of inhibition of myocardial nitric oxide synthase on apelin-induced positive inotropic response. Infusion of L-NAME (300 μmol/L) did not alter the apelin-induced increase in developed tension (43 ± 2% versus 46 ± 2%, apelin plus L-NAME versus apelin, n=4; P=NS).

Phospholipase C, Protein Kinase C, and Apelin-Induced Positive Inotropic Effect

An important mechanism for the regulation of cellular processes in cardiac myocytes involves phospholipase Cβ–induced phosphoinositide hydrolysis with subsequent activation of protein kinase C. To assess the involvement of phospholipase C (PLC) in the positive inotropic effect of apelin, we used U-73122, a potent inhibitor of this enzyme. Infusion of U-73122 (100 nmol/L) alone had no effect on contractile force (P=NS, Figure 3A, online Table 3). When U-73122 was infused in combination with apelin (1 nmol/L), it significantly decreased the apelin-induced inotropic effect throughout the entire experimental period, the maximal reduction being 68% at the end of 30 minutes of infusion time (F=18.6, P<0.001; Figure 3A, online Table 3). To examine whether activation of protein kinase C (PKC) contributes to
the positive inotropic action of apelin, we evaluated the effects of the broad spectrum protein kinase inhibitor staurosporine, along with the specific PKC inhibitor GF-109203X. When apelin (1 nmol/L) was infused in the presence of staurosporine (10 nmol/L) or GF-109203X (90 nmol/L), the inotropic response was attenuated maximally by 77% (F = 27.9; P < 0.001) and 70% (F = 28.1; P < 0.01; Figure 3A, online Table 3), respectively. Infusion of staurosporine or GF-109203X alone had no influence on developed tension (P = NS; Figure 3A, online Table 3).

**Na\(^+\)-H\(^+\) Exchange, Na\(^+\)-Ca\(^{2+}\) Exchange, and Apelin-Induced Positive Inotropic Effect**

Activation of PKC leads to phosphorylation of various cellular proteins including the sarcolemmal Na\(^+\)-H\(^+\) exchanger. To assess the contribution of Na\(^+\)-H\(^+\) exchange (NHE) to the effect of apelin, we used MIA, an inhibitor of NHE. Infusion of MIA (1 \(\mu\)mol/L) alone had no effect on contractile force (P = NS; online Table 3). When given together with apelin (1 nmol/L), MIA significantly attenuated the overall apelin-induced positive inotropic effect, the maximal reduction being 58% (F = 13.8; P < 0.001; Figure 3B, online Table 3). Next, we examined the effect of simultaneous inhibition of NHE and NCX on the inotropic effect of apelin. When given together with apelin (1 nmol/L), zoniporide (1 \(\mu\)mol/L) and KB-R7943 (250 nmol/L) significantly reduced the overall apelin-induced positive inotropic effect, the maximal reduction being 58% (F = 13.8; P < 0.001; Figure 3B, online Table 3). Infusion of zoniporide in combination with KB-R7943 had no effect on developed tension (P = NS; Figure 3B, online Table 3).

**Effect of Apelin on Ca\(^{2+}\) and K\(^+\) Currents**

We studied the effect of apelin on voltage-activated Ca\(^{2+}\) and K\(^+\) currents by performing amphotericin B–perforated patch-clamp recordings in isolated adult rat ventricular myocytes. Figure 4 shows the current-voltage relations established for the L-type Ca\(^{2+}\) current (I_{Ca,L}), the transient outward (I_{to}), and the sustained K\(^+\) current (I_{K,sus}) from the holding potential of −60 mV. These recordings showed that apelin (10 nmol/L) did not modulate I_{Ca,L}, I_{to}, or I_{K,sus}. The present study provides the first evidence for the functional importance of apelin, a putative ligand for the APJ orphan receptor in the heart. Among the peripheral rat tissues,
high levels of apelin mRNA\textsuperscript{4–6} and considerable levels of APJ mRNA\textsuperscript{6,7} have been found in the myocardium. Our results show that both apelin and APJ are mainly expressed in cardiac myocytes, indicating the existence of a potential autocrine/paracrine regulatory loop in the myocardium. Indeed, our results demonstrate that apelin exerts a potent, dose-dependent positive inotropic effect in vitro, in the isolated perfused rat heart preparation. On a molar basis, endothelin-1\textsuperscript{16} and adrenomedullin\textsuperscript{10} have been considered previously to be the most potent stimulators of cardiac contractility with EC\textsubscript{50} values of 50 pmol/L. Because apelin was active in the subnanomolar range, with an EC\textsubscript{50} value of 33 pmol/L, apelin appears to be among the most potent endogenous positive inotropic substances yet identified. Because plasma concentrations of apelin are approximately 23 pmol/L in rats\textsuperscript{4} and local levels may be even higher because of active synthesis, circulating or locally produced apelin may play an important role in the regulation of cardiac function. Notably, the apelin-induced increase in developed tension was 69% of the maximal inotropic response to isoproterenol and it was comparable to the effect of endothelin-1 and adrenomedullin in isolated perfused rat hearts. Of particular interest was our finding that apelin possessed a slowly developing but sustained inotropic response that clearly differed from the classical β-adrenergic effect, which develops rapidly, usually over a matter of seconds, suggesting that apelin may modulate the inotropic responsiveness of the heart over a different time frame (eg, from minutes to hours).

APJ receptor shares modest sequence homology with AT\textsubscript{1} receptor.\textsuperscript{2} However, it is unlikely that the effect of apelin is

**Figure 4.** Current-voltage relationship of $I_{\text{Ca}}$ (A) in the absence and presence of apelin (10 nmol/L). Current was elicited by test pulses stepping from $-40$ mV to $+40$ mV for 200 ms in 20-mV increments from the holding potential of $-60$ mV. Original current tracings before and after superfusion with apelin (from $-60$ mV to 0 mV) are shown in the inset. Current-voltage relationship for $I_{\text{Ca}}$ (B) and $I_{\text{K,sus}}$ (C) in the absence and presence apelin (10 nmol/L). Current was elicited by test pulses stepping from $-40$ mV to $+80$ mV for 400 ms in 10-mV increments from the holding potential of $-60$ mV. Inset shows original current records (from $-60$ mV to $+80$ mV) before and after superfusion with apelin. Data are mean\textpm SEM (n=4).

**Figure 5.** Effect of mechanical stretch on apelin (A) and BNP (B) gene expression in cultured NRVMs. Left ventricular levels of apelin (C) and ANP mRNA (D) in SD, dTG, WKY, and SHR animals. mRNA values are expressed as the ratio of apelin, BNP, or ANP mRNA to 18S as determined by quantitative reverse transcription-PCR analysis or Northern blot analysis, respectively. Data are mean\textpm SEM (n=6 to 7). *P<0.05, †P<0.01, and ‡P<0.001 vs control by unpaired Student’s t test.
mediated via angiotensin receptors, because CV-11947, a specific AT₁ receptor antagonist, failed to attenuate the inotropic response to apelin. Moreover, the effect of apelin remained unchanged in the presence of an ET₄/ET₆ endothelin receptor antagonist, α- and β-adrenergic receptor blockers, and a nitric oxide synthase inhibitor. Thus, it appears that apelin can bind and activate its own receptors in the heart specifically, independent of release of endogenous angiotensin II, endothelin, catecholamines or nitric oxide.

Prolonged activation of PKC by diacylglycerol, a product of PLCβ-induced phosphoinositide hydrolysis, has been considered to be an important pathway in cellular responses in cardiac myocytes.²⁰ Our results suggest that activation of PLC and PKC are involved in the positive inotropic effect of apelin, because the apelin-induced increase in developed tension was markedly attenuated by U-73122, a PLC inhibitor, and staurosporine and GF-109203X, a nonselective and a specific inhibitor of PKC, respectively. Activated PKC can phosphorylate a wide spectrum of cellular proteins including the sarcolemmal NHE.²³ Previously, the apelin-induced promotion of extracellular acidification rate in Chinese hamster ovary cells expressing the APJ receptor was suppressed by MIA, a nonspecific inhibitor of NHE.²⁷ In our experiments, MIA and zoniporide, a highly selective inhibitor of NHE-1, significantly attenuated the inotropic response to apelin, suggesting that activation of NHE, at least in part, contributes to the effect of apelin. Stimulation of the NHE can lead to intracellular alkalization and sensitization of cardiac myofilaments to intracellular Ca²⁺. On the other hand, NHE-mediated accumulation of intracellular Na⁺ can indirectly promote a rise in intracellular levels of Ca²⁺ via reverse mode NCX.²⁶

In the present study, KB-R7943, a selective inhibitor of the reverse mode NCX markedly reduced the apelin-induced increase in developed tension. Based on the concentration of KB-R7943 (250 nmol/L), it is likely that the compound acted selectively, because it inhibits the voltage-gated Na⁺ current, Ca²⁺ current, and the inward rectifier K⁺ current with IC₅₀ values of 14, 8, and 7 μmol/L,²⁷ respectively, and has no effect on NHE up to a concentration of 30 μmol/L.²⁷ Our observation that simultaneous administration of zoniporide and KB-R7943 could not attenuate further the inotropic response to apelin may suggest that NHE and NCX are proximal and distal components, respectively, of a contiguous signaling pathway. It is noteworthy that both mechanisms have been shown previously to play an important role in the positive inotropic effect of endothelin-1.²⁸,²⁹ Activation of protein kinase A leads to a robust increase in Iακ, which plays a critical role in the positive inotropic response to β-adrenergic stimulation. In contrast, the effect of stimulation of PKC on L-type Ca²⁺ channels is controversial. Iακ has been reported to be either modestly increased, decreased, or unchanged by ET-1, angiotensin II, and α₁-adrenergic agonists.³² In perforated patch-clamp experiments, we did not find any evidence for modulation of Iακ by apelin. In addition, apelin did not alter voltage-activated K⁺ currents (Iκs and Iκmax). These results suggest that activation of NHE and NCX contributes to the inotropic effect of apelin, whereas voltage-activated Ca²⁺ and K⁺ currents are not involved. The finding that ~40% of the apelin-induced positive inotropic effect remained unaffected even after combined inhibition of NHE and NCX indicates the existence of additional signaling mechanisms. Apelin may also affect the properties of more downstream elements of the contractile machinery such as the Ca²⁺ affinity of troponin C or the actomyosin crossbridge cycling rate.³³

Previously, it has been reported that the APJ receptor expressed in Chinese hamster ovary cells is coupled to pertussis toxin–sensitive G proteins (G₁₁ or G₁₄ protein).³⁴,³⁵ Our preliminary results showed that pertussis toxin pretreatment (25 μg/kg IP, 48 hours before the experiment)³² partly reduced the apelin-induced positive inotropic response (26.8±4.4% versus 46.3±1.9%, apelin with and without pertussis toxin pretreatment, n=4; P<0.05). Because the PLC-PKC pathway is coupled to pertussis toxin–insensitive G proteins (G₁₁),³⁶ the signaling mechanisms activated by apelin may involve both pertussis toxin–insensitive and –sensitive G proteins.

Taking into account the mechanism of action, one may speculate a potential pathophysiological relevance of apelin. An abrupt increase in hemodynamic load triggers a series of adaptive mechanisms in the myocardium.³⁷ Stretch of cardiac muscle generates a biphasic force response: an initial change that occurs almost immediately and a second slowly developing phase. The first phase has been attributed to increased myofilament Ca²⁺ responsiveness, whereas stretch-induced activation of NHE and NCX are likely to mediate the slow-force response.²⁶,³⁷,³⁸ It is intriguing that the time-course and the signaling pathways underlying the effect of apelin show similarities to the slow-force response. When stretch persists for longer periods, genes are switched on and off that eventually lead to cardiac hypertrophy and failure.³⁸ Notably, apelin gene expression was markedly downregulated in cultured ventricular myocytes subjected to mechanical stretch and in vivo in 2 models of chronic ventricular pressure overload. Thus, as a feedback mechanism, the cardiac effects of apelin may be offset by its decreased synthesis, and in the long run, it can contribute to the deterioration of cardiac function. Further studies are required to test the hypothesis that restoration of myocardial apelin synthesis can rescue the failing heart.

In summary, this is the first report showing that apelin exerts a potent positive inotropic effect in vitro. Our results suggest that the inotropic response to apelin may involve activation of PLC, PKC, sarcolemmal NHE, and NCX.

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