Arginine Vasopressin-mediated Cardiac Differentiation

INSIGHTS INTO THE ROLE OF ITS RECEPTORS AND NITRIC OXIDE SIGNALING*

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Despite the existence of a functional arginine vasopressin (AVP) system in the adult heart and evidence that AVP induces myogenesis, its significance in cardiomyogenesis is currently unknown. In the present study, we hypothesized a role for AVP in cardiac differentiation of D3 and lineage-specific embryonic stem (ES) cells expressing green fluorescent protein under the control of atrial natriuretic peptide (ANP) or myosin light chain-2V (Mlc-2V) promoters. Furthermore, we investigated the nitric oxide (NO) involvement in AVP-mediated pathways. AVP exposure increased the number of beating embryoid bodies, fluorescent cells, and expression of Gata-4 and other cardiac genes. V1a and V2 receptors (V1aR and V2R) differentially mediated these effects in transgenic ES cells, and exhibited a distinct developmentally regulated mRNA expression pattern. A NO synthase inhibitor, L-NAME, powerfully antagonized the AVP-induced effects on cardiogenic differentiation, implicating NO signaling in AVP-mediated pathways. Indeed, AVP elevated the mRNA and protein levels of endothelial NO synthase (eNOS) through V2R stimulation. Remarkably, increased beating activity was found in AVP-treated ES cells with down-regulated eNOS expression, indicating the significant involvement of additional pathways in cardiomyogenic effects of AVP. Finally, patch clamp recordings revealed specific AVP-induced changes of action potentials and increased L-type Ca²⁺ (ICa,L) current densities in differentiated ventricular phenotypes. Thus, AVP promotes cardiomyocyte differentiation of ES cells and involves Gata-4 and NO signaling. AVP-induced action potential prolongation appears likely to be linked to the increased ICa,L current in ventricular cells. In conclusion, this report provides new evidence for the essential role of the AVP system in ES cell-derived cardiomyogenesis.

The neurohypophyseal hormone arginine vasopressin (AVP)² is essential for cardiovascular homeostasis. AVP is involved in the regulation of body fluid osmolality, blood volume and pressure via the stimulation of specific receptors with distinct pharmacological profiles and intracellular second messengers (1, 2). AVP activity is mediated by at least 3 different G-protein-coupled receptors, called V1a, V1b (also known as V3), and V2. The V1a receptor (V1aR), abundantly expressed in vascular smooth muscle cells, hepatocytes, and platelets, and V1b receptor (V1bR), predominantly found in the anterior pituitary, are linked to the phosphoinositol signaling pathway with intracellular calcium as second messenger. In contrast, the V2 receptor (V2R) is coupled to the adenylate cyclase signaling with intracellular cAMP as the second messenger. V2R has long been recognized to be exclusively expressed in kidney (3), but there is increasing evidence of extrarenal V2R expression (4—6).

Recently, a role was suggested for AVP as a novel myogenesis-promoting factor (7, 8). AVP was shown to regulate the expression of early and late myogenic differentiation markers in cultured myogenic cell lines. In addition, studies on endothelial and osteoblast-like cells indicated a potential involvement of nitric oxide (NO) in proliferation pathways mediated by AVP (6, 9). NO is a universal signaling molecule implicated in many physiological and pathophysiological functions. In the adult heart, NO is involved in the regulation of heart rate, contractility, and coronary perfusion. Moreover, increasing evidence indicates an important role of NO in cardiac differentiation (10).

In a previous report, we addressed mechanisms promoting differentiation of the mammalian cardiac conduction system in murine embryonic stem (ES) cells (11). Cardiomyogenic differentiation in ES cells is manifested by the appearance of spontaneously and rhythmically beating cardiomyocytes within developing embryoid bodies (EBs). Differentiation of ES cells to the cardiac lineage provides a model for the exploration of mechanisms and genes involved in the earliest steps of cardiac development.

Therefore, the aim of this study was to undertake a detailed analysis of the cardiomyogenic actions of AVP during ES cell differentiation and the role of NO in AVP-mediated pathways. To determine whether AVP specifically affects the differentiation of atrial or ventricular phenotypes, we used ES cell clones expressing the green fluorescent protein (GFP) gene under the control of cardiac-restricted atrial natriuretic peptide (ANP) or FACS, fluorescence-activated cell sorter; RT, reverse transcriptase; eNOS, endothelial nitric-oxide synthase; siRNA, small interfering RNA.
myosin light chain-2V (Mlc-2V) promoters and a D3 ES cell line as a wild type counterpart. In addition, we examined the effects of AVP on the action potential (AP) profile and expression of the L-type Ca2+/H11001 (I\textsubscript{Ca,L}) channel in ES cell-derived cardiomyocytes.

In this study, we demonstrate that AVP promotes ES cell-derived cardiomyogenic differentiation through stimulation of its receptors and involves NO signaling. Moreover, a unique developmentally regulated pattern of AVP receptor expression was found throughout the differentiation period of ES cells. We further show specific alterations of the AP pattern and increased I\textsubscript{Ca,L} expression in ES cell-derived ventricular phenotypes in response to AVP. This report provides new evidence for the essential role of the AVP system in cardiomyogenesis, as determined by the application of combined molecular-biological, flow cytometric, and electrophysiological studies.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—To generate ventricular-specific ES cells, the CMV\textsubscript{enh}/MLC-2V/GFP construct (a generous gift from Dr. C. L. Mummery, Hubrecht Laboratory, University Medical Center, Utrecht, The Netherlands) was stably transfected into D3 ES cells with a FuGENE 6 kit (Roche) according to the manufacturer’s protocol. The transfected ES cells were cultured for 10 days in the presence of neomycin (G418), and resistant clones were further selected. The ANP-EGFP expressing ES cell line has been described previously (11).

ES cells were grown feeder-independent in Dulbecco’s modified Eagle’s medium supplemented with 1,000 units/ml leukemia inhibitory factor (Chemicon International Inc., Temecula, CA), 15% fetal calf serum, penicillin/streptomycin, non-essential amino acids, glutamax, and -mercaptoethanol (Invitrogen). For differentiation, about 400 cells were allowed to aggregate in hanging drops to form EBs, which were rinsed off after 2 days and subsequently propagated in suspension (Dulbecco’s modified Eagle’s medium, 20% fetal calf serum) for additional 4 days. Finally, at day 6, EBs were plated separately on gelatin-coated 24-well plates and cultivated in the presence or absence of AVP for 14 days as indicated. All experiments were performed on EBs of 3 different clones in each cell line at days 14–15 after plating, unless the developmental stage is specified otherwise.

AVP concentration was determined in preliminary studies where spontaneously beating activity of developing EBs was assessed in the presence of increasing AVP concentrations (10\textsuperscript{−9} to 10\textsuperscript{−5} M) (Bachem Bioscience, PA). Because a maximal response was observed for treatment with 10\textsuperscript{−7} M AVP, this concentration was used throughout the study. Selective 10\textsuperscript{−7} M V1aR (\(\beta\)-mercapto-\(\beta\)-\textsubscript{3}cyclopentamethylene-propionyl\(\beta\)-O-Me-Tyr\textsuperscript{2}, [Arg\textsuperscript{8}]vasopressin), V2R (adamantaneacetyl\(\beta\)-O-Et-D-Tyr\textsuperscript{2}, Val\textsuperscript{4}, aminobutyryl\textsuperscript{6}, [Arg\textsuperscript{8},9]vasopressin), or oxytocin receptor (OTR) (\(\beta\)-mercapto-\(\beta\)-\textsubscript{3}cyclopentamethylene-propionyl-Tyr (Me)\textsuperscript{2}-Ile-Thr-Asn-Cys-Pro-Orn-Tyr-NH\textsubscript{2}) antagonists were
added to the AVP containing differentiation media. When appropriate, EBs were also cultured in the presence of 10^{-4} M l-NAME (N,G-nitro-l-arginine-methyl ester, Sigma) with or without AVP supplementation.

**Flow Cytometry Analysis (FACS)**—ANP-EGFP and MLC-2V-GFP expressing EB outgrowths at early (designated as days 2–4 after plating), intermediate (days 6–8), and late (days 12–14) developmental stages were rinsed with phosphate-buffered saline and harvested after treatment with Accutase (Innovative Cell Technologies Inc., CA). The cells were suspended in phosphate-buffered saline containing Ca^{2+} (1 mM) and Mg^{2+} (0.5 mM), and a minimum of 10,000 viable cells were acquired for each sample. GFP expressing cells were analyzed and sorted in the FL1 channel on the basis of the forward-scattered and side-scattered light in FACS Aria™ Cell Sorter (BD Bioscience) as the cells traversed the beam of an argon ion laser (488 nm). Data were analyzed by using Cell Quest software (BD Bioscience). The sorted cells were collected in corresponding differentiation medium and allowed to reattach to culture dishes. Gene expression studies were performed after 24–36 h.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**—Total RNA from D3 EB outgrowths or GFP-positive ANP-EGFP or MLC-2V-GFP expressing ES cells at different developmental stages was extracted by TRIzol reagent (Invitrogen). RT-PCR of genes of V1aR, V1bR, V2R, Gata-4, Mlc-2V, ANP, cardiac α-actin, myogenin, MyoD, and endothelial nitric-oxide synthase (eNOS) was performed with 2 μg of DNase-treated (Turbo DNase-free, Ambion Inc.) total RNA. For all PCR studies, dose-response curves were established for different amounts of RNA, and the samples were quantified in the curvilinear phase of PCR amplification (12).

The primers and PCR conditions are described in Table 1. The PCR products were size fractionated by 2% agarose gel electrophoresis and analyzed with the Storm 840 Imaging Systems and ImageQuant software (version 4.2, GE Healthcare). The concentrations of mRNA were normalized with respect to 18 S gene.

**Immunoblotting**—For Western blot analysis, enzymatically dispersed cells were lysed with radioimmunoprecipitation assay buffer. Protein concentration was quantified spectrophotometrically by Bradford's assay (Bio-Rad). 20 μg of total protein were loaded and fractioned by SDS-PAGE under reducing conditions. Separated proteins were transferred electrophoretically to polyvinylidene difluoride membrane (Amersham Biosciences). Blots were incubated with the primary antibody against mouse eNOS (Santa Cruz Biotechnology) overnight at +4 °C, followed by incubation with peroxidase-conjugated secondary antibody (Amersham Biosciences) the next day for 1 h at room temperature. The 140-kDa eNOS protein fragment was visualized with an ECL kit (Amersham Biosciences).
ImageJ software (National Institutes of Health, Bethesda, MD) was applied for densitometric analysis of selected band intensities. β-Actin was used as internal control.

Small Interfering RNA (siRNA)-mediated eNOS Gene Silencing—eNOS siRNA duplexes (sense, 5-CGUGGAUGGCCAGCUCAATT-3; antisense, 5-UUGAGCUCGCAUCCACGTG-3) targeted to the 21-nucleotide mouse eNOS mRNA sequence (CACGTGGATGAGCCAGCTCAA) and validated siRNA negative control (scrambled siRNA) were designed commercially (Qiagen, ON, Canada) and prepared as recommended by the manufacturer.

The methodology of siRNA transfection in ES cells was adopted and modified from previous reports (13, 14). Differentiating D3 EBs in suspension were transferred to 24-well plates, followed by transfection with eNOS siRNA (Qiagen) according to the instructions provided. After 24 h, the cells were retransfected as before and incubated in differentiation media in the presence or absence of AVP or AVP with the additional antagonists. EB outgrowths were examined for beating activity and the protein levels of eNOS and β-actin were detected by Western blotting.

Electrophysiology—Single cells were obtained by dissection of spontaneously beating EB areas and collagenase dispersion. AP and I_{Ca,L} recordings were carried out in the whole cell patch clamp configuration with an Axopatch 200B amplifier (Axon Instruments, CA) while sampling at 10 kHz and filtering at 2 kHz. The recording bath solution contained (in mmol/liter): NaCl 135, KCl 5, CaCl₂ 2, MgCl₂ 1, glucose 10, HEPES 10; pH was adjusted to 7.4 with NaOH, and temperature was kept constant at 35–36 °C. Recordings and analysis of I_{Ca,L} were performed as indicated in Ref. 15. For I_{Ca,L} recordings, NaCl was replaced with tetraethylammonium chloride (TEA-Cl) (135 mmol/liter) and 4-aminopyridine (4 mmol/liter) was added to block I_{to}.

The pipette solution contained (in mmol/liter): Cs-aspartate 120, CsCl 15, NaCl 5, MgCl₂ 1, Mg₂ATP 4, EGTA 5, HEPES 5; pH was adjusted to 7.4 with CsOH. Borosilicate microelectrodes were pulled with tip resistances of 2–4 MΩ (Narishige Inc., Japan).

Statistical Analysis—Results are presented as mean ± S.E. Comparisons between groups were evaluated by one-way analysis of variance. p values of <0.05 were considered as significant.

RESULTS

AVP Enhances Cardiomyogenesis in ES Cells—First, our study addressed the role of AVP in ES cell-derived cardiomyogenesis. Spontaneous cardiomyocyte differentiation of D3 cells was assessed in the presence of AVP or AVP with additional V1aR, V2R, or OTR antagonist. The incidence of spontaneously beating activity was analyzed by microscopy of 120 EB outgrowths in each subgroup. As shown in Fig. 1A, 10^{-7} M AVP exposure significantly increased the percentage of EBs with beating foci in D3 cells compared with the untreated (control) group (39.2 ± 4.9 versus 23.3 ± 3.4%; p < 0.05). The effect of AVP on the number of contracting D3 EBs tended to be inhibited by both V1aR and V2R antagonists.

To determine the impact of AVP on specialized cardiac phenotypes,
further experiments were performed on lineage-specific ANP-EGFP and MLC-2V-GFP expressing ES cell lines. Treatment with AVP significantly increased the percentage of beating EBs expressing ANP-EGFP (47.5 ± 3.9 versus 25.9 ± 4.0%; \( p < 0.05 \)) (Fig. 1B) and MLC-2V-GFP (45.0 ± 5.8 versus 32.5 ± 3.6%; \( p < 0.05 \)) (Fig. 1C). In the ANP-EGFP cell line, AVP-induced effects were strongly inhibited when V2R was specifically blocked (30.8 ± 4.7%; \( p < 0.05 \) versus AVP), whereas only a minor reduction in the number of contracting EBs was detected for V1aR antagonist (43.3% ± 6.2). An opposite but nonsignificant effect was observed in MLC-2V-GFP expressing ES cells, where the inhibitory effect of the V1aR blocker (42.4 ± 6.9%) prevailed over that of the V2R blocker (42.4 ± 6.9%). No inhibitory action of OTR antagonist was apparent in the investigated ES cell lines (Fig. 1, A–C).

The role of NO in AVP-mediated ES cell differentiation was evaluated in EB aggregates of all 3 cell lines subjected to additional treatment with L-NAME, a nonspecific NOS inhibitor. As illustrated in Fig. 1, A–C, AVP effects were largely abolished if EBs were exposed to L-NAME.

Next, we quantified the GFP expression pattern in AVP-treated transgenic ES cells by flow cytometry. Generally, MLC-2V-GFP-positive EBs displayed lower fluorescent intensities (as evidenced by fluorescence intensity ranging from \( 10^3 \) to \( 10^4 \) counts), but a greater number of green fluorescent cells than ANP-EGFP expressing myocytes (Fig. 2, A–D). However, in both cell lines, AVP induced a significant increase in the number of cells emitting green fluorescence compared with the untreated population (Fig. 2, E and F), without affecting the fluorescence intensities. This was particularly evident in ANP-EGFP expressing cardiac cells (7.6 ± 0.9 versus 4.4 ± 0.3%, \( n = 12; p < 0.05 \)), whereas the effect on the MLC-2V-GFP-positive cell population was less pronounced (11.9 ± 1.3 versus 8.2 ± 0.7%, \( n = 13; p < 0.05 \)). Application of L-NAME significantly reduced the percentage of GFP-positive cells in both cell lines (ANP-EGFP, 2.9 ± 0.7%; \( p < 0.05 \) versus AVP; MLC-2V-GFP, 6.3 ± 0.4%; \( p < 0.05 \) versus AVP).

GFP expressing cells, isolated from EBs at various differentiation stages, were sorted by FACS, and the cardiogenic potency of AVP was further investigated by RT-PCR analysis of cardiac marker genes. In both cell lines, consistent enhancement of Gata-4 expression by AVP was found throughout cardiac differentiation (Fig. 3, A and B). Terminally differentiated, AVP-treated cardiomyocytes exhibited higher Anp, Mlc-2V, and cardiac \( \alpha \)-actin mRNA levels (Fig. 3, B and E). Interestingly, V1aR was involved predominantly in the AVP-induced increase of Gata-4 and Mlc-2V expression, whereas the considerably V2R-mediated inhibition was observed for the Anp gene transcript in differentiated cardiac cells.

The specificity of AVP actions was further examined by the investigation of myogenin and MyoD expression in GFP-positive ES cells. However, no specific expression for both genes was found by RT-PCR (data not shown).

**Developmental Profiles of AVP Receptor Expression**—Detailed developmentally regulated expression profiles of AVP receptors were established by RT-PCR in untreated D3 EBs at days 1, 3, 6, 10, 15, 20, and 25 after plating. The results demonstrated that V1aR, V1bR, and V2R were abundantly expressed in the early developmental stage (days 1–6) (Fig. 4, A and B). However, as development progressed, all 3 investigated receptors exhibited major differences with respect to their relative...
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**FIGURE 4. Developmentally regulated mRNA expression of V1aR, V1bR, and V2R genes in D3 ES cells.** Representative ethidium bromide-stained agarose gels of RT-PCR products (A) and densitometric analysis of band intensities (B) from three independent experiments. EBs were subjected to cardiac differentiation for 1, 3, 6, 10, 15, 20, and 25 days after plating. V1aR, V1bR, and V2R were abundantly expressed at days 1–6. V1aR expression markedly decreased with further development. In contrast, V2R expression remained high followed by a minor decrease at days 20–25. Additionally, V1bR mRNA was detected throughout cardiac differentiation.

Abbreviations in text:
- AVP: Vasopressin
- eNOS: Endothelial nitric oxide synthase
- V1aR: Vasopressin receptor type 1a
- V1bR: Vasopressin receptor type 1b
- V2R: Vasopressin receptor type 2
- OTR: Oxytocin receptor
- L-NAME: N’-nitro-L-arginine methyl ester

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**AVP Increases eNOS Expression**—To evidence the possible link between AVP and NO signaling pathways in cardiogenesis, we investigated eNOS expression, a NO isoform abundantly synthesized during ES cell-derived differentiation (16), using RT-PCR and Western blotting. In these experiments, ES cells of the D3 line were treated with AVP in the presence or absence of V1aR, V2R, OTR antagonists, or L-NAME.

RT-PCR analysis (Fig. 5A) showed up-regulation of eNOS mRNA under AVP exposure. This effect was reversed by treatment with V2R antagonist or L-NAME. Incubation of EBs with the V1aR antagonist did not influence eNOS mRNA level.

Western blotting confirmed the involvement of eNOS in AVP-mediated cardiomyocyte differentiation (Fig. 5B), because the exposure of EBs to AVP increased eNOS protein. The AVP-induced rise in eNOS expression was reduced by V2R antagonist, and an even stronger decline was detected in L-NAME-treated ES cells. In contrast, application of the V1R antagonist only slightly diminished eNOS protein expression in AVP-treated EB aggregates. The AVP-mediated increase in eNOS expression seems to be partly regulated also by OTR, as lower eNOS mRNA and protein levels were observed in ES cells treated with OTR antagonist.

**AVP Effects in eNOS siRNA-transfected Cells**—To further validate the functional relevance of the above described findings, we used specific siRNA-mediated targeting of the eNOS gene and evaluated whether a decrease in endogenous eNOS expression would affect the AVP-mediated effects on contracting EBs. The siRNA-induced knockdown of gene expression was specific to the targeted gene, as eNOS protein was unaffected by scrambled siRNA transfection at days 2 and 5, and down-regulated in ES cells transfected with eNOS siRNA (Fig. 6, A and B). Because the highest decrease (by 75%) in relative eNOS protein expression was detected at day 5 after the second transfection (0.3 ± 0.09 versus 1.2 ± 0.16%, p < 0.05) (Fig. 6C), the spontaneously beating activity of transfected EB outgrowths (n = 192 in each subgroup) was analyzed at day 5. Indeed, beating incidence in transfected ES cells was lower (13.0 ± 1.8%; p < 0.05) compared with the non-transfected (24.0 ± 2.7%) or scrambled siRNA group (22.4 ± 2.6%) (Fig. 6D). An increased number of contracting EBs was found in AVP-treated transfected cells (21.4 ± 1.7%; p < 0.05). AVP failed to raise spontaneously beating in EB outgrowths with additional exposure to the V1aR antagonist (14.1 ± 1.7%). In contrast, the V2R antagonist showed no impact in AVP-induced cardiomyogenic effects with eNOS depletion (20.3 ± 3.1%).

**Electrophysiological Profile of AVP-treated Cardiomyocytes**—The precise phenotypic characteristics were established by examining AP recordings of cardiomyocytes isolated from spontaneously beating, AVP-treated (n = 38) and control EBs (n = 27) of the D3 line. Based on the typical AP configuration and electrophysiological parameters described in Table 2, all cardiac cell subtypes (pacemaker-, ventricular-, and atrial-like) could be identified among differentiated ES cells (Fig. 7, A and B). AVP tended to increase the number of atrial-like cells (37 ± 1.0, n = 14, versus 30 ± 2.6%, n = 8) while decreasing the pacemaker-like phenotypes (13 ± 0.8, n = 5, versus 22 ± 1.1%, n = 6). Interestingly, AVP-

**FIGURE 4. Developmentally regulated mRNA expression of V1aR, V1bR, and V2R genes in D3 ES cells.** Representative ethidium bromide-stained agarose gels of RT-PCR products (A) and densitometric analysis of band intensities (B) from three independent experiments. EBs were subjected to cardiac differentiation for 1, 3, 6, 10, 15, 20, and 25 days after plating. V1aR, V1bR, and V2R were abundantly expressed at days 1–6. V1aR expression markedly decreased with further development. In contrast, V2R expression remained high followed by a minor decrease at days 20–25. Additionally, V1bR mRNA was detected throughout cardiac differentiation.
treated ventricular-like myocytes displayed a more prominent plateau phase compared with the same control phenotype (Fig. 7B). This finding was reflected in the significantly increased action potential duration at 90% of repolarization (APD<sub>90</sub>) values measured in ventricular-like cells isolated from AVP-stimulated EBs (312 ± 13 ms, n = 19, versus 240 ± 11 ms, n = 13; p < 0.05). Unlike the ventricular phenotype, no significant changes of AP pattern and basic electrophysiological properties in response to AVP were found for pacemaker- and atrial-like cardiomyocytes (Table 2).

Guided by the AVP-induced specific changes in AP profile in ventricular cells, we investigated whether AVP influences the expression of <i>I<sub>Ca,L</sub></i>, a key determinant of the AP plateau phase in ventricular cells, in terminally differentiated MLC-2V-GFP expressing cardiomyocytes. As shown in Fig. 8, AVP markedly increased the <i>I<sub>Ca,L</sub></i> current density (18.7 ± 2.0 pA/pF at 0 mV, n = 17) compared with controls (11.4 ± 1.2 pA/pF, n = 14; p < 0.05) without affecting the voltage dependence.

**DISCUSSION**

The results presented in this work suggest a new role for AVP and its receptors in cardiac development. We demonstrate that:

1) AVP induces differentiation of atrial and ventricular precursor cells through V2R and V1aR, respectively; 2) both effects are mediated at least in part by NO as they can be inhibited by L-NAME; 3) V2R mediates the AVP action predominantly via eNOS up-regulation, whereas, in addition, AVP promotes cardiac differentiation in an eNOS-independent manner (as shown in siRNA-transfected cells) mainly via V1aR.

The hypothesis that AVP is involved in the regulation of cardiomyogenesis emerged from previous reports showing an essential role of AVP in the differentiation of skeletal muscle (7, 8, 17). The additional support was provided by the finding that a functional AVP system exists in the adult heart (18) and high AVP concentrations were detected in the newborn rat heart followed by a significant decrease during maturation.3

Our initial results revealed that AVP increased the number of beating EBs of the D3 cell line. To determine whether AVP promotes cardiac differentiation toward a specific cell phenotype, experiments were performed on ANP-EGFP and MLC-2V-GFP expressing ES cells. Both <i>Anp</i> and <i>Mlc-2V</i> promoters were shown previously to display lineage-specific cardiac expression, with <i>Anp</i> mainly found in atrial, and <i>Mlc-2V</i> in ventricular cells (19, 20). Interestingly, AVP-induced increases in the percentage of contracting EBs and green fluorescent cells were more evident in ANP-EGFP expressing ES cells. Indeed, more higher AVP concentrations were observed in atria than in ventricle in 21- and 66-day-old rats. Moreover, our results provide the first evidence of a distinct role of AVP receptors in cardiomyogenic differentiation, as demonstrated by RT-PCR of cardiac genes and the observation of spontaneous cardiac differentiation in AVP-stimulated transgenic cell cultures.

We also assessed the implication of OTR in AVP-mediated effects on the cardiac differentiation. As previously shown, oxytocin induces the cardiomyogenesis of embryonic carcinoma cells, and the OT/OTR system is expressed at its highest levels in developing rather than in adult hearts (21). In addition, some physiological AVP effects are thought to be mediated by OTR, as AVP and OT receptors share high primary sequence homology (1, 22). However, our experiments suggest no OTR involve-

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ment in the AVP-mediated cardiac effects due, probably, to the lower affinity of OTR for AVP compared with V1aR and V2R (23).

To obtain further insights into the functional relevance of AVP receptors in cardiac differentiation, we examined the expression of V1aR, V1bR, and V2R in a developmentally regulated manner. All 3 receptor isoforms were abundantly expressed in ES cells at the very early stages of cardiac differentiation. Interestingly, V2R was strongly expressed throughout cardiac differentiation until day 15, and decreased only slightly at day 20–25. Because V2R expression remained high at the late developmental stage, where prevalently terminally differentiated cardiomyocytes were detected (24), we assumed that it is not exclusively linked to the initiation of cardiac differentiation. In contrast to V2R, striking differences in V1aR expression were detected depending on the differentiation stage. V1aR expression levels were high during the first days after EB plating, followed by sharp down-regulation, starting from day 6. The mRNA expression pattern of V1aR suggests the importance of this receptor, particularly during very early differentiation of cardiac precursor cells. Additionally, our results showed that ES cell-derived cardiac cells exhibited V1bR. So far, there are no reports about V1bR expression in heart tissue. However, a recent study by Oshikawa et al. (25) suggested that AVP stimulated insulin release from pancreatic islet cells through V1bR. Considering this and other newly published findings supporting the implication of insulin-mediated pathways in (cardio)myogenic differentiation (26–28), it is tempting to speculate that V1bR signaling relates to the growth effects induced by insulin during cardiac differentiation.

In this respect, a potent V1bR antagonist SSR149415 has been more recently described and extensively studied, mostly in human (41). However, there are no validated pharmacological or biochemical data with SSR149415 application in cultured mouse cells limiting its application in the present study. Moreover, new evidence suggest that SSR149415 is a mixed V1b/
OTR antagonist, as it exhibited a significant antagonism with nanomolar affinity at OTR (42), most likely due to the high amino acid sequence homology between both receptors (43).

Because the available data suggest an essential role of NO in cardiomyogenesis (10, 16), we examined NO signaling in AVP-mediated cardiac effects. In mammalian cells, NO is synthesized by at least 3 isoforms of NOS: neuronal NOS, inducible NOS, and eNOS. Each of the NOS isoforms is encoded by a different gene, and exhibits a distinct expression pattern in developing ES cells (29). Our results suggest an important modulatory role of L-NAME in eNOS expression. In line with these findings, L-NAME has been reported to inhibit NO synthesis by eNOS and, to a lesser degree, by inducible NOS (30, 31). The functional relevance of inhibition of the NO pathways in cardiomyogenesis was corroborated by the finding that application of NOS inhibitors to ES cell cultures markedly reduced the myofibrillogenesis within the developing EBs and resulted in a prominent diminution of terminally differentiated cardiomyocytes (16). Consistently, L-NAME decreased the incidence of beating foci in ES cell-derived cardiomyocytes (10).

Based on AVP-induced eNOS up-regulation, we provide a biochemical link between V2R and eNOS expression. Previous reports have suggested the involvement of either V1aR or V2R in NO production in the rat kidney (32, 33). In osteoblast-like cells, NO production was found to be mediated through V1aR (9). In contrast to V1aR and V1bR, which are linked to the phosphoinositol signaling pathway with intracellular Ca\(^{2+}\) acting as second messenger, V2R is involved in the adenylate cyclase signaling pathway with cAMP as second messenger. Our observation that AVP induces eNOS expression through V2R activation is in agreement with the previous report by Kaufmann et al. (6), showing cAMP-mediated eNOS activation by DDAVP and other cAMP-raising substances in endothelial cells. The cross-link between eNOS and V2R-mediated pathways is also substantiated by the finding that the V2R antagonist did not influence AVP-induced spontaneously beating activity in eNOS mRNA knockdown ES cells. In this context, the AVP-mediated cardiomyogenic effects in ES cells transfected with eNOS siRNA reveal an additional V2R- and eNOS-independent AVP action, at least in part being mediated by V1aR. Among the considerable molecular pathways of AVP effects, the specific mechanism that activates cardiogenesis remains yet to be determined. However, enhanced Gata-4 expression, consistently observed throughout cardiac differentiation in response to AVP exposure in ES cells, might significantly contribute to the cardiomyogenic actions of AVP and, thus, represent another major signaling target for AVP-induced differentiation pathways. On the other hand, the AVP-mediated increase in spontaneously beating activity might be the result of additional activation of other NOS isoforms, which for their part can induce the cardiomyogenic differentiation (10). The latter notion is bolstered by our finding...
that AVP actions are largely abolished in the presence of 1-NAME, a nonspecific NOS blocker.

Consistent with previous reports, cardiomyocytes isolated from AVP-treated EBs at the terminally differentiated stage exhibited diverse AP configurations, as described for pacemaker, atrial, and ventricular cells (34). Furthermore, patch clamp recordings revealed specific changes in the electrophysiological properties of ventricular-like cells in response to AVP, as evidenced by the prominent plateau phase and prolongation of AP duration. These findings may imply an altered Ca\(^{2+}\) homeostasis in response to AVP, given that Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels underlies the molecular basis of the AP plateau phase. The AVP-induced increase of \(I_{\text{Ca,L}}\) in MLC-2V-GFP positive cardiomyocytes is in line with several reports demonstrating elevation of intracellular Ca\(^{2+}\) in cardiac tissue in response to AVP (35–37). AVP was also found to potentiate the unitary \(I_{\text{Ca,L}}\) currents in guinea pig ventricular cells (38).

In addition, the calcium-dependent signaling pathway has been implicated in cardiac and skeletal muscle differentiation. Particularly, cardiac and skeletal subtype-specific profile and our results suggest that AVP is involved in the regulation of the essential role of AVP in cardiomyogenesis. Moreover, REFERENCES

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