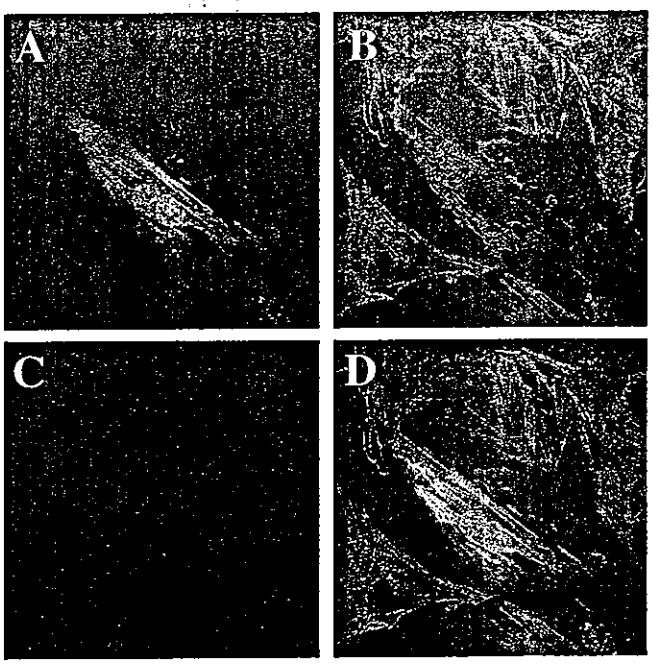

Figure 1. Immunostaining of skeletal muscle-derived cells after cocultured with cardiomyocytes. Skeletal muscle-derived cells that were prepared from GFP-transgenic mice were cultured with contracting cardimyocytes for 5 days. Skeletal muscle-derived cells expressed GFP (A, *D*, green). Cells were double-stained by anti-cardiac TnT antibody visualized with Cy3-conjugated secondary antibody (*B, D*, red), and anti-ANP antibody visualized by Cy5-conjugated secondary antibody (*C, D*, blue). A GFP-positive skeletal muscle-derived, cell-expressed cardiac TnT and ANP. The skeletal muscle-derived cells that expressed cardiac TnT and ANP showed fine striated pattern (*D*).

4. Skeletal muscle-derived cells showed cardiomyocyte-like action potential in coculture with cardiomyocytes

An electrophysiological study was performed on the GFP-positive skeletal muscle-derived cells that contract synchronously with neighboring cardiomyocytes. The contracting GFP-positive cell demonstrated cardiomyocyte-like action potentials. This action potential was characterized by *1)* a relatively long action potential duration and *2)* a relatively shallow resting membrane potential. These properties are consistent with the action potential observed in cardiomyocytes of early developmental stage.

5. Direct contact was necessary for transdifferentiation of skeletal muscle-derived cells

All these transdifferentiated cells were adjacent to cardiomyocytes, suggesting that cell–cell contact is necessary for transdifferentiation of skeletal muscle-derived cells into cardiomyocytes. To prove this hypothesis, skeletal muscle-derived cells were cultured in three different ways: monoculture, coculture, and double chamber system, which has cell culture inserts. In the double chamber system, cardiomyocytes and skeletal muscle cells were cultured separately but in the same culture media. Skeletal muscle-derived cells expressed cardiac TnT and ANP in the coculture condition but not in monoculture condition or double chamber condition. These results suggest that humoral factors are not sufficient but direct cell–cell contact is necessary for transdifferentiation of skeletal muscle-derived cells into cardiomyocytes.

6. Nifedipine treatment inhibited the transdifferentiation of skeletal muscle-derived cells and cyclic stretch restored the inhibitory effect of nifedipine

The cultured cardiomyocytes of neonatal rats were rhythmically beating. To access whether contraction of cardiomyocytes is necessary for transdifferentiation of skeletal muscle-derived cells, cells were cultured in the presence or absence of nifedipine. Nifedipine (5 μM) inhibited contraction of ~half of cardiomyocytes and 20 μM nifedipine abolished the beating. Nifedipine treatment suppressed transdifferentiation of skeletal muscle-derived cells in a dose-dependent manner (**Fig. 2**). To further examine the effect of mechanical stretch on the transdifferentiation of skeletal muscle-derived

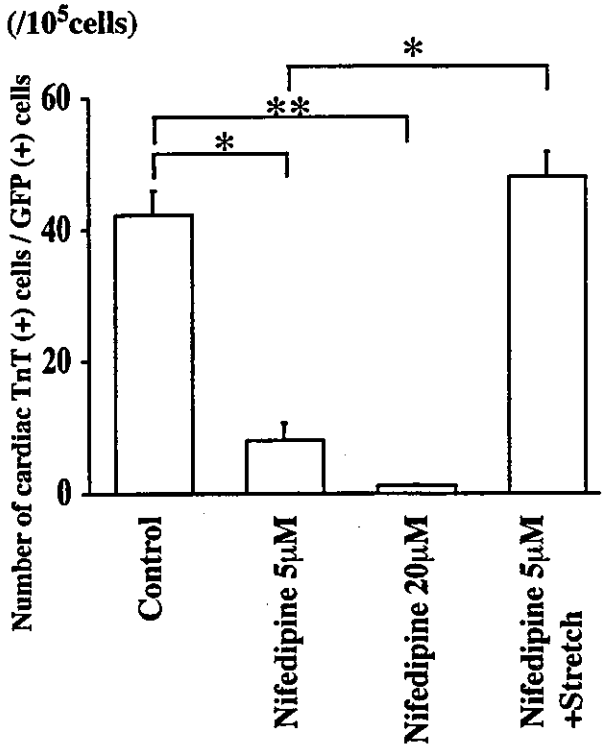

Figure 2. Role of contraction. Skeletal muscle cells and cardiomyocytes were cocultured in silicone dishes in the absence (Control) or presence of nifedipine (nifedipine 5 μM, nifedipine 20 μM). Cyclic stretch was applied in the presence of 5 μM nifedipine (nifedipine 5 μM +stretch). (*P<0.05 **P<0.01) The number of cells that are double positive for cardiac TnT and GFP was counted and normalized by total number of GFP-positive cells in each silicone dish.

cells, cells were cultured on the silicone dish and passive cyclic stretch (60 cycles/min) was applied to the skeletal muscle-derived cells cocultured with cardiomyocytes whose spontaneous beating was inhibited with 5 μM nifedipine. After 48 h treatment, cells were stained by anti-cardiac TnT antibody and anti-ANP antibody. Treatment of 5 μM nifedipine markedly reduced the number of cardiac TnT-positive cells compared with control; cyclic stretch completely restored this inhibition (Fig. 2). These results suggest that mechanical load on the skeletal muscle-derived cells is important for the transdifferentiation of skeletal muscle-derived cells.

CONCLUSIONS AND SIGNIFICANCE

In the present study, we demonstrate that skeletal muscle-derived cells can transdifferentiate into cardiomyocytes when cocultured with contracting cardiomyocytes. This is demonstrated by the expression of *1)* cardiac-specific proteins (cardiac TnT and ANP), *2)* cardiac transcription factors (Nkx2E and GATA4), and *3)* adhesion and gap junction proteins (cadherin and connexin43) in the skeletal muscle-derived cells. The anti-cardiac TnT antibody does not react with adult skeletal muscle and stained specifically cardiomyocytes. ANP expression is known to be restricted to the heart but not to skeletal muscle. Expression of these two cardiac-specific proteins suggests that skeletal muscle-derived cells transdifferentiated into cardiomyocytes. We also demonstrated the expression of two cardiac transcription factors (Nkx2E and GATA4) in skeletal muscle-derived cells. ANP gene expression is activated by Nkx2E and GATA4 synergistically. Cardiac TnT also contains potential Nkx2E binding site and GATA binding site in its promoter region. Expression of Nkx2E and GATA4 was recognized 1 day earlier than cardiac TnT and ANP in skeletal muscle-derived cells, suggesting that the skeletal muscle-derived cells acquire phenotype of cardiomyocytes by the transcriptional regulation of cardiac-specific genes.

N-Cadherin is a major adhesion molecule of the adherence junction and connexin 43 is a gap junction protein. They are located at the intercalated disc of myocardium. Gap junction forms low resistance pathway of cardiac action potential. In our study, these two proteins were clearly expressed at the border of the transdifferentiated skeletal muscle-derived cells and cardiomyocytes. Action potentials recorded from contracting GFP-positive skeletal muscle cells had cardiomyocyte-like properties clearly different from action potentials of the skeletal muscle. These results suggest that skeletal muscle-derived cells not only express cardiac-specific proteins but also show cardiac electrical properties.

To investigate the mechanisms of transdifferentiation, we examined whether direct cell–cell contact and beating of cardiomyocytes are needed to transdifferentiation. The double chamber experiment revealed that humoral factors are not sufficient but direct cell–cell contact is needed for transdifferentiation of skeletal muscle-derived cells into cardiomyocytes. Treatment of nifedipine, an L-type calcium channel antagonist, and Ca^{2+}-free culture media inhibit spontaneous beating of cardiomyocytes. Both treatments clearly reduced the number of transdifferentiated cells. Moreover, the cyclic stretching restored this inhibition by 12%, suggesting that mechanical stress is important for the transdifferentiation. Mechanical load has been reported to activate various signaling pathways through autocrine/paracrine secretable factors, Ca^{2+}-dependent signaling, and adhesion molecules, including integrins. Further investigation is necessary to clarify how mechanical load is connected to transdifferentiation of skeletal muscle-derived cells.

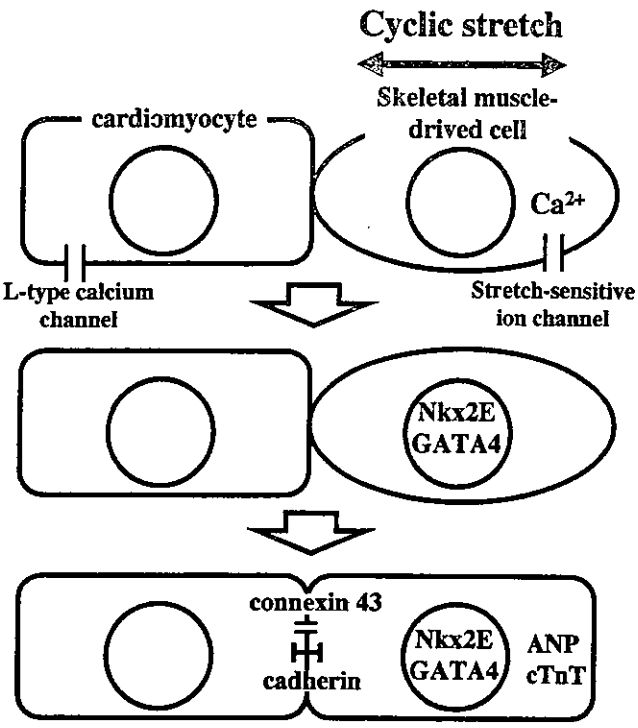

Figure 3. Schematic diagram

Skeletal muscle has been reported to contain stem cell populations besides satellite cells. We enriched the stem cell population of skeletal muscle cells by collecting the SP fraction. Cells of the SP fraction showed much higher rate (~10-fold) of transdifferentiation than unfractionated cells when cocultured with cardiomyocytes (unpublished data). Although this is not direct evidence, it suggests that multipotent muscle-derived stem cells differentiate into cardiomyocytes.

In conclusion, we demonstrated that skeletal muscle-derived cells could transdifferentiate into cardiomyocytes and that direct cell–cell contact and mechanical force of beating cardiomyocytes were important for transdifferentiation. FJ

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Review

Stretch-modulation of second messengers: effects on cardiomyocyte ion transport

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Abstract

In cardiomyocytes, mechanical stress induces a variety of hypertrophic responses including an increase in protein synthesis and a reprogramming of gene expression. Recently, the calcium signaling has been reported to play an important role in the development of cardiac hypertrophy. In this article, we report on the role of the calcium signaling in stretch-induced gene expression in cardiomyocytes. Stretching of cultured cardiomyocytes up-regulates the expression of *brain natriuretic peptide (BNP)*. Intracellular calcium-elevating agents such as the calcium ionophore A23187, the calcium channel agonist BayK8644 and the sarcoplasmic reticulum calcium-ATPase inhibitor thapsigargin up-regulate *BNP* gene expression. Conversely, stretch-induced *BNP* gene expression is suppressed by EGTA, stretch-activated ion channel inhibitors, voltage-dependent calcium channel antagonists, and long-time exposure to thapsigargin. Furthermore, stretch increases the activity of calcium-dependent effectors such as calcineurin and calmodulin-dependent kinase II, and inhibitors of calcineurin and calmodulin-dependent kinase II significantly attenuated stretch-induced hypertrophy and *BNP* expression. These results suggest that calcineurin and calmodulin-dependent kinase II are activated by calcium influx and subsequent calcium-induced calcium release, and play an important role in stretch-induced gene expression during the development of cardiac hypertrophy.

Keywords: Mechanoreceptor; Ion channel; Calcium; Gene expression; Hypertrophy; Natriuretic peptide

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Contents

1. Introduction

Although cardiac hypertrophy can be regarded as an adaptation to increased workload (Hunter and Chien, 1999), this process eventually leads to congestive heart failure, arrhythmia and sudden death. Therefore, it is important to understand the molecular mechanisms underlying cardiac hypertrophy. Hemodynamic overload is clinically the most important stimulus for cardiac hypertrophy (Komuro et al., 1990, 1991b; Komuro and Yazaki, 1993; Sadoshima and Izumo, 1997), however, it remains to be determined how mechanical loading is sensed by cardiomyocytes and is converted into intracellular biochemical signals leading to cardiac hypertrophy.

It is well known that stretching of cardiomyocytes enhances calcium (Ca^{2+}) transient and modulates their contractility (Allen and Kurihara, 1982; Hongo et al., 1996). Recently, Ca^{2+} signaling has been reported to play a critical role in the generation of cardiac hypertrophy, as well (Frey et al., 2000). Increased intracellular Ca^{2+} binds to and modulates calcium-binding proteins including calmodulin. The calmodulin inhibitor, W7, completely blocks the hypertrophic responses that are induced by α-adrenergic activation (Sei et al., 1991). In addition, both an increase in Ca^{2+} levels in cultured cardiomyocytes (Sei et al., 1991), and cardiac overexpression of calmodulin in mice lead to cardiac hypertrophy (Gruver et al., 1993). Calmodulin undergoes conformational change upon binding to intracellular Ca^{2+}, and regulates several downstream effectors such as calcineurin and Ca^{2+}/calmodulin-dependent protein kinases (CaMKs). Activation of calcineurin (Molkentin et al., 1998) and CaMKs (Ramirez et al., 1997; Passier et al., 2000) induces cardiac hypertrophy both in vivo and in vitro. Inhibition of calcineurin activity attenuated load- (Sussman et al., 1998; Meguro et al., 1999; Shimoyama et al., 1999, 2000; De Windt, 2001; Zou et al., 2001a, b) and agonist- (Molkentin et al., 1998; Zhu et al., 2000; De Windt et al., 2001) induced cardiac hypertrophy. Stretching of cardiomyocytes induces Ca^{2+} influx through stretch-activated ion channels (Ruknudin et al., 1993), which evokes Ca^{2+}-induced calcium release (CICR) (Sigurdson et al., 1992). We have demonstrated that the Ca^{2+}/calmodulin pathway is involved in stretch-induced gene expression in cultured cardiomyocytes and that

mechanical stretch up-regulates *brain natriuretic peptide (BNP)* gene expression through calcineurin and CaMKII.

2. Stretch induces gene expression in cultured cardiomyocytes

Mechanical stress has been considered to be one of the major stimuli that evoke hypertrophic responses including reprogramming of gene expression in cardiac myocytes (Komuro et al., 1990, 1991b; Komuro and Yazaki, 1993). To investigate the signaling pathways that translate mechanical stress into cardiac hypertrophy, we developed an in vitro device by which mechanical stress can be imposed on cardiac myocytes cultured in the serum-free condition. Prolonged stretch of neonatal rat cardiomyocytes cultured on an elastic silicone dish induces several hypertrophic responses such as activation of protein kinases (Yamazaki et al., 1993, 1995; Komuro et al., 1996; Pan et al., 1999), gene expression of immediate early genes or fetal type genes (Komuro et al., 1990, 1991b; Komuro and Yazaki, 1993; Sadoshima and Izumo, 1997), and an increase in protein synthesis (Mann et al., 1989). *BNP* has been reported to be up-regulated in the hypertrophied heart (Dagnino et al., 1992; Hanford et al., 1994), and Northern blot analysis reveals that *BNP* gene expression is strongly up-regulated by stretch (Fig. 1a).

3. Ca^{2+} is involved in stretch-induced gene expression

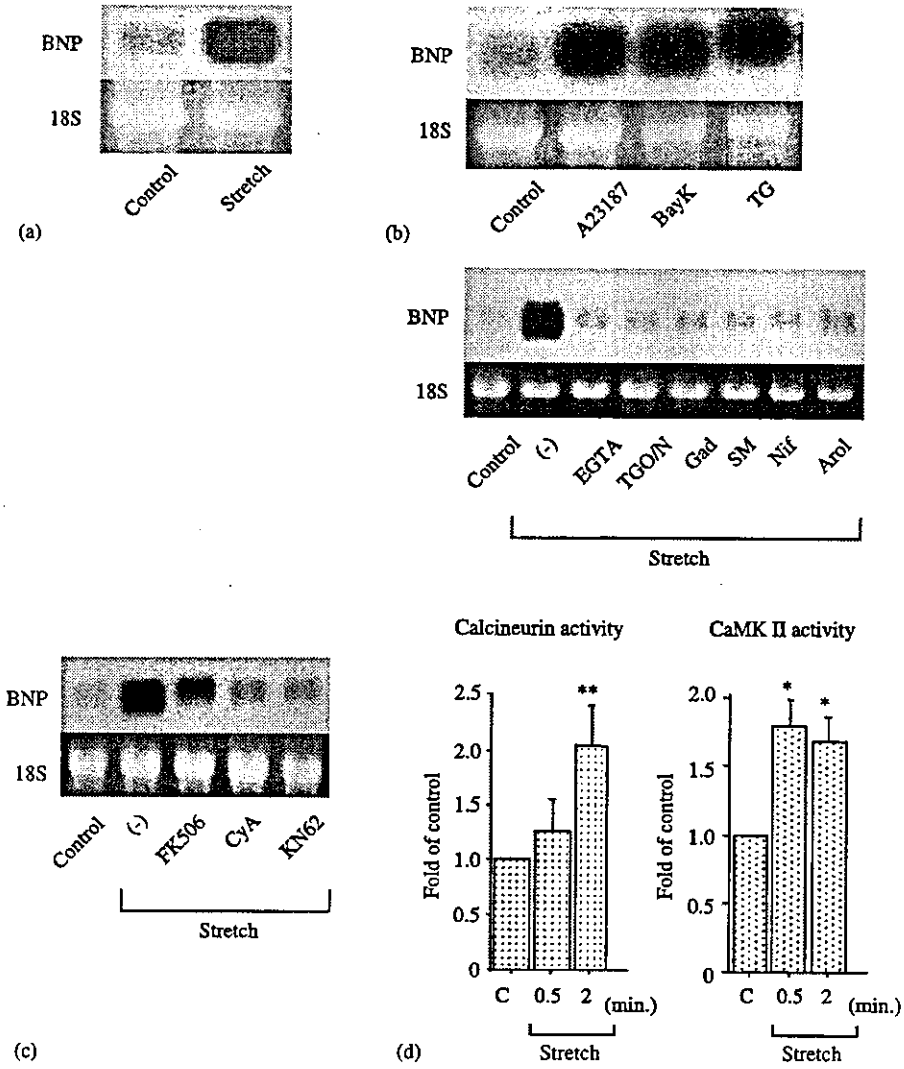
Since it has been recently demonstrated that Ca^{2+} acts as an important mediator for various hypertrophic stimuli (Frey et al., 2000), we examined whether Ca^{2+} is involved in stretch-induced *BNP* gene expression. Addition of a Ca^{2+} ionophore A23187 increased the expression levels of *BNP* in cardiac myocytes (Fig. 1b). Up-regulation of *BNP* gene expression was also induced by an increase in intracellular Ca^{2+} concentration following treatment with BayK8644, a calcium channel agonist, and thapsigargin (Thastrup et al., 1990), a sarcoplasmic reticulum (SR) calcium ATPase inhibitor (Fig. 1b). On the contrary, pretreatment with EGTA, an extracellular Ca^{2+} chelator, strongly inhibited stretch-induced *BNP* gene expression (Fig. 1b). It has been reported that long-term treatment of cardiac myocytes with thapsigargin causes depletion of Ca^{2+} in SR and decreases the intracellular Ca^{2+} transient (Kirby et al., 1992). Long exposure with thapsigargin also inhibits stretch-induced *BNP* gene expression (Fig. 1b).

Our results indicate that an increase in intracellular Ca^{2+} levels induced by Ca^{2+} ionophore, Ca^{2+} channel agonist, or the SR calcium ATPase inhibitor, was sufficient to activate *BNP* gene expression (Fig. 1b). Intracellular Ca^{2+} regulates the expression of other cardiac genes such as the *atrial natriuretic factor (ANF)* (Irons et al., 1992; McDonough and Glembotski, 1992) and the *β-type myosin heavy chain (β-MHC)* (Zhu et al., 2000). However, the mechanisms by which intracellular Ca^{2+} regulates gene expression are not completely understood. Chelation of extracellular Ca^{2+} with EGTA abolished stretch-induced *BNP* expression (Fig. 1b), suggesting that Ca^{2+} influx from extracellular space is necessary. Since depletion of Ca^{2+} in the SR suppressed the stretch-induced *BNP* gene up-regulation, a large increase in intracellular Ca^{2+} level accomplished by CICR may be necessary for gene induction.

4. Ion channels as mechanosensors

Stretch-induced *BNP* gene expression is also abolished by the treatment with stretch-activated ion channel inhibitors such as gadolinium and streptomycin (Ohmori, 1985; Yang and Sachs, 1989), or voltage-dependent Ca^{2+} channel antagonists such as nifedipine and amlodipine (Fig. 1b), suggesting that Ca^{2+} influx through voltage-dependent Ca^{2+} channels and possibly through stretch-activated ion channels may be involved in stretch-induced gene expression.

Ion channels and exchangers on the cell membrane have been postulated to be potential "mechano-receptors", because they may respond promptly to stretch and convert it into intracellular signals (Komuro and Yazaki, 1993). It was reported that mechanical stretch of cultured chick cardiac myocytes evokes calcium influx and subsequent CICR from the SR, which



was abrogated by gadolinium (Sigurdson et al., 1992). Previously, we and others have reported that inhibitors of stretch-activated ion channels, such as gadolinium and streptomycin, do not block stretch-induced expression of *c-fos* (Komuro et al., 1991a; Sadoshima et al., 1992) and activation of MAP kinases (Yamazaki et al., 1998). Pan et al. reported that gadolinium and EGTA did not attenuate stretch-induced phosphorylation of *s*ignal *t*ransducers and *a*ctivators of *t*ranscription (STAT)1 and STAT3 (Pan et al., 1999). In our study, however, pretreatment of gadolinium and streptomycin significantly inhibited *BNP* gene expression induced by stretch (Fig. 1b). Although gadolinium and streptomycin are not specific to stretch-activated ion channels (Hu and Sachs, 1997), these results suggest that Ca^{2+} entry through stretch-activated ion channels may be involved in *BNP* gene expression, but not in expression of *c-fos* gene or activation of MAP kinases and Janus kinase/STAT pathway.

Voltage-dependent Ca^{2+} channel antagonists, nifedipine and amlodipine, significantly suppress *BNP* gene expression by mechanical stretch. Furthermore, Ruwhof et al. have reported that the voltage-dependent Ca^{2+} channel antagonist diltiazem blocks the stretch-induced increase in intracellular calcium levels by ~50% in cardiomyocytes (Ruwhof et al., 2001), suggesting the following two possibilities; (1) activity of voltage-dependent Ca^{2+} channel is stretch-activated or (2) the inward current of cations through stretch-activated ion channels causes membrane depolarization which in turn opens the Ca^{2+} channels. Transgenic mice which overexpress voltage-dependent Ca^{2+} channel α1-subunit in the heart exhibit cardiac hypertrophy (Muth et al., 1999, 2001). Furthermore, we found that blocking of the voltage-dependent Ca^{2+} channels by continuous infusion of nifedipine was sufficient to suppress the activity of calcineurin and prevent the development of cardiac hypertrophy in spontaneously hypertensive rats (Zou et al., 2002). These results suggest that an increase in Ca^{2+} influx through the voltage-dependent Ca^{2+} channels are necessary for the generation of cardiac hypertrophy in vivo.

Na^+–H^+ exchanger (NHE), another potential mechanosensitive ion exchnager, is activated by mechanical stretch (Cingolani et al., 1998). We have reported that pretreatment with an NHE

Fig. 1. Involvement of Ca^{2+} signaling in stretch-induced gene expression. (a) Mechanical stretch induces *BNP* gene expression. Cultured cardiac myocytes of neonatal rats were stretched by 20% for 30 min (stretch). *BNP* gene expression was examined by Northern blot analysis. 18S ribosomal RNA was stained with ethidium bromide, and used as a loading control. (b) Roles of Ca^{2+} in stretch-induced *BNP* gene expression. Cardiac myocytes were incubated with a Ca^{2+} ionophore A23187 (3 mM), a Ca^{2+} channel agonist BayK8644 (10 mM), or an SR calcium ATPase inhibitor thapsigargin (2 mM) for 30 min. Ca^{2+} influx and subsequent CICR are critically involved in stretch-induced *BNP* gene expression. On the contrary, pretreatment with an extracellular Ca^{2+} chelator EGTA (5 mM) strongly inhibited stretch-induced *BNP* expression. Pretreatment with inhibitors for stretch-activated ion channels, gadolinium (10 mM) or streptomycin (500 mM), and specific inhibitors for voltage-dependent Ca^{2+} channels nifedipine (1 mM) or amlodipine (1 mM), also attenuated stretch-induced *BNP* expression. Depleting Ca^{2+} store in SR by treatment with thapsigargin overnight inhibited stretch-induced *BNP* expression. SM, streptomycin; Nif, nifedipine; Amlo, amlodipine; TG, thapsigargin; TG O/N, treatment with thapsigargin overnight. (c) Calcineurin and CaMK are critically involved in stretch-induced *BNP* gene expression. Pretreatment with specific inhibitors for calcineurin FK506 (10 mM) and cyclosporin A (0.4 mM), and a specific inhibitor for CaMK KN62 (0.5 mM) significantly inhibited stretch-induced *BNP* expression. CyA, cyclosporin A. (d) Stretch increases the activity of calcineurin and CaMK in cultured cardiac myocytes. Cultured cardiac myocytes were stretched, and the activities of calcineurin and CaMK were determined by measuring the radioactivity of liberated ^{32}P from phosphorylated GST-RII and by assaying the phosphorylation of synthetic peptide autocamtide-2, respectively. Data were shown as mean ±SE **, $p<0.01$ compared with control. *, $p<0.05$ compared with control. *C*, control.

inhibitor HOE694 inhibited stretch-induced activation of the protein kinase cascades and protein synthesis (Yamazaki et al., 1998). A rise in intracellular Na^+ may stimulates the reverse mode of the Na^+–Ca^{2+} exchanger (NCX) and causes increase in intracellular Ca^{2+}. In cat myocardium, stretch-induced increase in contractile force is attenuated by inhibition of either NHE or the reverse mode of NCX (Perez et al., 2001; Aiello et al., 2002). It remains unclear whether NHE and NCX are involved in stretch-induced activation of Ca^{2+} signaling and gene expression.

5. Role of calcineurin and CaMKII in stretch-induced gene expression

Increased intracellular Ca^{2+} binds to calmodulin and evokes a variety of events through Ca^{2+}/calmodulin-dependent enzymes such as calcineurin and CaMKs. Recently, the importance of these two pathways for the development of cardiac hypertrophy has been reported. Transgenic mice overexpressing constitutively activated mutant of calcineurin develop marked cardiac hypertrophy (Molkentin et al., 1998). Calcineurin inhibitors, FK506 (Shimoyama et al., 2000) and cyclosporin A (Meguro et al., 1999; Zhu et al., 2000; Zou et al., 2001a, b), inhibit AngII-, ET-1-, phenylephrine-, and isoproterenol-induced hypertrophic responses including *BNP* gene expression in cultured cardiac myocytes (Molkentin et al., 1998; Zhu et al., 2000). Furthermore, we and others have reported that calcineurin is critically involved in load- and agonist-induced cardiac hypertrophy in vivo (Sussman et al., 1998; Meguro et al., 1999; Shimoyama et al., 1999, 2000; Zou et al., 2001a, b). With regard to CaMK signaling, CaMKI and CaMKIV enhanced *ANF*- and *skeletal α-actin*-promoter activity, and transgenic mice overexpressing CaMKIV developed cardiac hypertrophy (Passier et al., 2000). Furthermore, the CaMKII inhibitor, KN62 (Zhu et al., 2000), prevented ET-1-induced hypertrophic responses such as expression of *β-MHC* and *ANF* genes, an increase in protein synthesis and induction of sarcomere organization (Irons et al., 1992; Zhu et al., 2000).

To elucidate the pathways downstream of Ca^{2+} involving stretch-induced gene expression, we examined the effects of specific inhibitors for these Ca^{2+}/calmodulin-dependent enzymes on stretch-induced *BNP* gene up-regulation. Pretreatment FK506 and cyclosporin A or a CaMKII inhibitor KN62 significantly inhibited stretch-induced *BNP* gene upregulation (Fig. 1c). We also found that calcineurin and CaMKII activities were increased by stretch (Fig. 1d), suggesting that both molecules may be involved in stretch-induced *BNP* gene expression.

6. Divergence of the signaling pathway in stretch-induced gene expression

Mechanical stretching of postnatal cardiomyocytes up-regulates expression of various kinds of genes, that are not typical fetal genes or natriuretic peptide genes. For example, myocardial stretch in an isolated rat heart model increases *vascular endothelial growth factor* (VEGF) expression (Li et al., 1997) by enhancing nuclear translocation of hypoxia-inducible factor-1 (HIF-1), which is a key transactivator of the *VEGF* gene under hypoxic conditions (Kim et al., 2002). Stretch-induced VEGF expression by HIF-1 is attenuated by gadolinium but not by diltiazem. In this model, phosphatidylinositol 3-kinase (PI3 K)-dependent Akt phosphorylation is activated by mechanical stretch, and a PI3 K inhibitor wortmannin suppressed up-regulation of

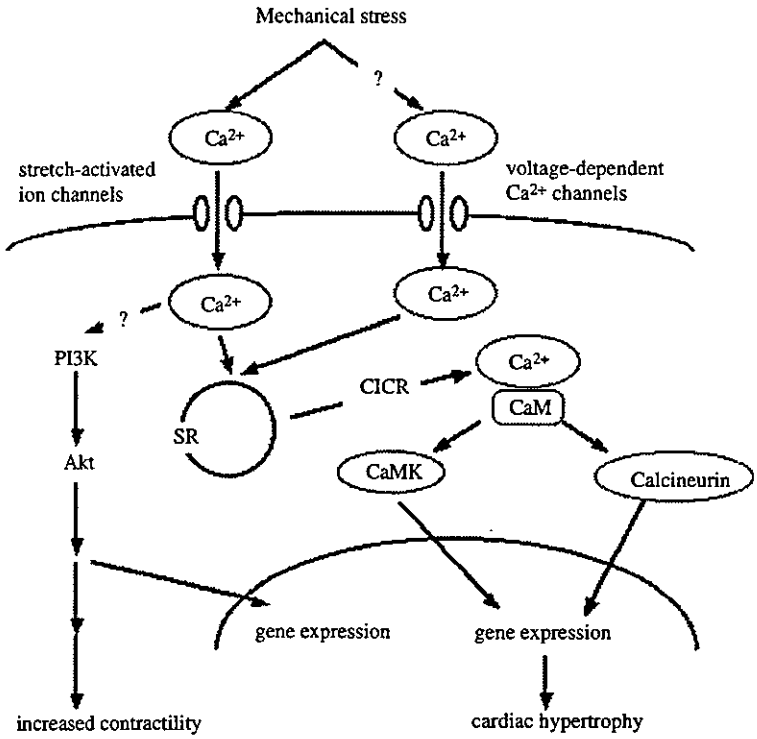


Fig. 2. Involvement of Ca^{2+} in mechanical stress-induced cardiac hypertrophy. Ca^{2+}/calmodulin-dependent enzymes are activated by calcium influx through ion channels and subsequent CICR, and play an important role in stretch-induced gene expression during the development of cardiac hypertrophy. Other pathways including the PI3K/Akt axis are involved in stretch-induced increase in myocardial contractility and gene expression.

the *VEGF* gene. These result suggest that stretch-activated ion channels and the PI3K/Akt pathway are critically involved in stretch-induced gene expression, although the link between stretch-activated ion channels and PI3K remains undetermined. Independence of the voltage-dependent Ca^{2+} channels, the mechanism of which is also observed in stretch-induced activation of heat-shock factor 1 (Chang et al., 2001), is a good contrast to their involvement in stretch-induced *BNP* expression. The PI3K/Akt pathway also contributes to stretch-induced activation of myocardial contractility (Petroff et al., 2001). Therefore, the Ca^{2+}/calmodulin pathway is involved in the hypertrophic responses, but distinct signaling pathways such as the PI3K/Akt pathways may be involved in other phenomena induced by mechanical stretch (Fig. 2).

7. Conclusion

Ca^{2+} plays a critical role in stretch-induced gene expression and ion channels may be one of "mechano-receptors" in cardiac myocytes. Further studies are necessary to clarify the role of voltage-dependent Ca^{2+} channels in mechanical stretch-induced gene expression.

8. Editor's note

Please see also related communications in this volume by Casadei and Sears (2003) and Calaghan et al. (2003).

Acknowledgements

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Editorial

Too much Csx/Nkx2-5 is as bad as too little?

Cardiac transcription factors rule over the intricate process of coordinated myocardial differentiation by regulating cardiac-specific gene expression [1]. *Csx/Nkx2-5* is a member of NK homeobox gene family that is conserved in evolution and acts as a DNA-binding transcriptional activator [2–4]. Extensive investigation for the last decade has established the notion that Csx/Nkx2-5 is essential in normal cardiac development. Although considerable data have elucidated the roles of Csx/Nkx2-5 in cardiogenesis, little is known about functional significance of Csx/Nkx2-5 in the adult heart. On pages 243–256 of this issue of the *Journal of Molecular and Cellular Cardiology*, Kasahara et al. [5] demonstrated the unexpected role of Csx/Nkx2-5 in terminally differentiated cardiomyocytes by overexpressing wild-type or mutated Csx/Nkx2-5 protein both in murine heart and cultured cardiomyocytes.

Targeted disruption of *Csx/Nkx2-5* resulted in embryonic lethality due to the arrested looping morphogenesis of the heart tube [6,7]. In the heart of *Csx/Nkx2-5*-deficient embryos, expression of several cardiac genes was down-regulated including *ventricular isoform of myosin light chain 2*, *atrial natriuretic peptide* (*ANP*), *brain natriuretic peptide*, *cardiac ankyrin-repeat protein* (*CARP*), *MEF2C*, *eHAND/HAND1*, *N-myc*, *Iroquois homeobox gene 4*, and *HOP* [6–12], indicating the crucial role of Csx/Nkx2-5 in transcriptional regulation of a repertoire of cardiac-specific genes. Furthermore, many reports have been accumulated with regard to the molecular framework of transcriptional regulation by Csx/Nkx2-5. More cardiac genes are identified as direct downstream targets, such as *ANP* [13,14], *cardiac α-actin* [15], *A1 adenosine receptor* [16], *calreticulin* [17], *cardiac sodium-calcium exchanger 1 gene* [18], and *connexin 40* [10].

Csx/Nkx2-5 has attracted more attention since mutations in human *Csx/Nkx2-5* were reported to be responsible for a spectrum of congenital cardiac malformations associated with progressive atrioventricular conduction disturbance [19,20]. The promoter of *connexin 40* gene as well as that of *ANP* [21] is directly transactivated by Csx/Nkx2-5 in combination with T-box transcription factor, Tbx-5 [10]. Connexin 40 is a major constituent protein of gap junctions in the specialized conduction systems, and loss of *connexin 40* in mice enhances predisposition to cardiac conduction abnormalities [22,23]. Therefore, transcriptional regulation of gap junction proteins by Csx/Nkx2-5 is supposed to be relevant in maintaining the appropriate propagation of electrical activities in the heart.

Kasahara et al. [5] generated three types of transgenic mice, which overexpressed wild-type (TG-wild), a mutant lacking the C-terminus (TG-ΔC), or a DNA-nonbinding mutant with a missense mutation in the homeodomain (TG-I183P) under the control of the *α-myosin heavy chain* promoter to investigate the role of Csx/Nkx2-5 in the postnatal heart. To our surprise, all the transgenics, including TG-wild, displayed heart failure with conduction abnormalities. Furthermore, overexpression of *Csx/Nkx2-5* in cultured postnatal cardiomyocytes induced down-regulation of its potential downstream targets, such as *connexins*, *ANP* and *CARP*. From these findings, the authors came to the conclusion that transcriptional regulation of the individual target genes by Csx/Nkx2-5 diverges according to the developmental stages.

A number of works on molecular analysis of transcriptional regulation by Csx/Nkx2-5 were largely based on the experiments using the *Csx/Nkx2-5* mutant embryos or the data obtained by transient transfection assays using heterologous cell lines. Differential transcriptional regulation is implicated in cardiomyocytes with different spatial and temporal origins. Tbx-2 functions as a transcriptional repressor for *ANP* gene expression by displacing Tbx-5 and forming a complex with Csx/Nkx2-5 in the region of atrioventricular canal, inner curvature, outflow tract and inflow tract, where *Tbx-2* is expressed and *ANP* expression is exclusively absent [24]. Transcriptional activity of Csx/Nkx2-5 could be modulated positively and negatively by its respective endogenous-binding partner in a cell-type-specific manner. Identification of putative-binding partners of Csx/Nkx2-5 that determine its transcriptional mode in postnatal cardiomyocytes will be of great help to understand the intricate molecular mechanisms, where transcriptional activities switch from "promotive" to "repressive".

Another highly suggestive finding in this article is that, in neonatal cultured cardiomyocytes, adenoviral infection of wild-type *Csx/Nkx2-5* actually transactivated the *ANP* promoter, although endogenous mRNA of *ANP* was conversely down-regulated. As the authors mentioned in the text, it is possible that the promoter of *ANP* used in the reporter assays does not fully cover the regions where the *cis*-elements essential for practical transcriptional regulation in the biological context are located. The discrepancy may come from the intrinsic flaws of transient transfection assays, where the reporter plasmids are transactivated episomally. It would be

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necessary to reconstitute comprehensively the molecular framework of transcriptional regulation by Csx/Nkx2-5 in postnatal cardiomyocytes. Serial analysis of the regulatory regions of each target gene using transgenic or knock-in reporter mice will provide mechanistic insights into the issue whether Csx/Nkx2-5 transactivates or repress the individual genes in the *in vivo* context.

We generated transgenic mice overexpressing human *Csx/Nkx2-5* under the control of cytomegalovirus enhancer/chicken β-*actin* promoter (WT-CSX TG) and reported that they appeared normal and did not show significant differences in cardiac performance evaluated by transthoracic echocardiography [25]. Expression levels of cardiac genes, such as *ANP, brain natriuretic peptide, CARP* and *ventricular isoform of myosin light chain 2* were up-regulated in adult hearts of WT-CSX TG mice. Electron microscopic analysis revealed that a number of secretory granules, which might contain ANP, were present in the ventricles of transgenics. Furthermore, we generated transgenic mice overexpressing dominant-negative mutant of CSX/NKX2-5, in which a highly conserved leucine in the homeodomain was substituted to a proline, under the control of *α-myosin heavy chain* promoter (DN-CSX TG) [26]. In DN-CSX TG mice, cardiac function was impaired, and degenerative changes of cardiomyocytes including loss of myofilaments and increased number of mitochondria were observed by electron microscopic analysis. DN-CSX TG mice showed severer cardiac dysfunction and larger number of apoptotic myocardial cells by injection of doxorubicin than wild types. On the contrary, doxorubicin-induced myocardial damages were mild in WT-CSX TG. These results indicate the essential role of CSX/NKX2-5 in protecting the cardiomyocytes from cytotoxic stress in the adulthood. In a feline pulmonary artery banding model, pressure overload induced right ventricular hypertrophy with increased expression levels of *Csx/Nkx2-5* and its downstream target genes, *ANP* and *cardiac α-actin* [27]. In this model, *Csx/Nkx2-5* transcript levels were increased significantly at 2 days after banding, and declined toward baseline levels after 2 weeks. Expression levels of *ANP* and *cardiac α-actin* changed in parallel with those of *Csx/Nkx2-5*, suggesting correlation of *Csx/Nkx2-5* expression with *ANP* and *cardiac α-actin* expression in adult heart.

How should these apparently contradictory phenotypes be interpreted? As Kasahara et al. speculated in the article, the functional gaps of human CSX/NKX2-5 vs. mouse Csx/Nkx2-5 might be the possible cause of the phenotypical differences. However, on the basis of the equivalent data obtained in the *in vitro* experiments, it is likely that Csx/Nkx2-5 proteins in both species should have the fundamental properties in common. Another possibility is that the phenotypes are dependent on the expression level of Csx/Nkx2-5 proteins. We previously reported that overexpression of a *Csx/Nkx2-5* mutant, with truncation of the C-terminus, induced apoptosis in cultured rat neonatal cardiomyocytes, although this mutant showed enhanced transactivation of the *ANP* promoter [28]. We examined at least two lines of WT-CSX TG and DN-CSX TG and found that there were good correlations between phenotypes and expression levels of the transgenes. We have not observed any mice with cardiac dysfunction, although they abundantly expressed wild type of Csx/Nkx2-5 protein. However, in the article by Kasahara et al., only one line in each type of transgenics was analyzed due to unsuccessful inheritance or scarce expression of the transgenes. Analysis of transgenic mice of different lines with divergent expression levels of wild-type Csx/Nkx2-5 would be informative to elucidate the postnatal function of Csx/Nkx2-5 in the heart.

The authors recently reported that transgenic mice overexpressing I183P mutant of *Csx/Nkx2-5* in the embryonic heart under the control of β-*myosin heavy chain* promoter were born normal, but exhibited cardiac conduction defect after birth with reduced expression levels of *connexin 43*, encoding a major component of gap junctions in working myocardium, as well as *connexin 40* [29]. The similar phenotypes of the previous transgenics and TG-I183P are accountable because expression of I183P mutant protein persisted after birth. However, our and their groups reported that Csx/Nkx2-5 mutants with missense mutations in the homeodomain, including I183P, operate as a dominant-negative transcriptional repressor *in vitro* [28,30]. If endogenous Csx/Nkx2-5 is a transcriptional repressor in the adult heart as demonstrated by the authors, this dominant-negative mutant is supposed to function as a transactivator! The authors noted that endogenous *Csx/Nkx2-5* expression was up-regulated in these transgenics and proposed that this mutant may operate as a hypomorphic gain-of-function mutant. Biochemical analysis is necessary to elucidate how I183P mutant modulate the function of Csx/Nkx2-5 in the adult cardiomyocytes.

Csx/Nkx2-5 may be lost in deep contemplation. "To be a transcriptional activator or a repressor? To be cardioprotective or cardiotoxic?" Further investigation will unravel the tangled web of unsolved questions with regard to transcriptional regulation by Csx/Nkx2-5 in the terminally differentiated cardiomyocytes.

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Attenuated defense response and low basal blood pressure in orexin knockout mice

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Kayaba, Yuji, Akira Nakamura, Yoshitoshi Kasuya, Takashi Ohuchi, Masashi Yanagisawa, Issei Komuro, Yasuichiro Fukuda, and Tomoyuki Kuwaki. Attenuated defense response and low basal blood pressure in orexin knockout mice. *Am J Physiol Regul Integr Comp Physiol* 285: R581–R593, 2003. First published May 15, 2003; 10.1152/ajpregu.00671.2002.—The perifornical area of the hypothalamus has been known as the center for the defense response, or "fight or flight" response, which is characterized by a concomitant rise in arterial blood pressure (AP), heart rate (HR), and respiratory frequency (Rf). We examined whether orexin, a recently identified hypothalamic neuropeptide, contributes to the defense response and basal cardiovascular regulation using orexin knockout mice. Microinjection of a GABA-A receptor antagonist, bicuculline methiodide (0.1–1 mM in 20 nl), to the perifornical area in urethane-anesthetized wild-type mice elicited dose-dependent increases in AP, HR, and Rf. Although similar changes were observed in orexin knockout mice, intensities were smaller and duration was shorter than those in wild-type mice. Moreover, in an awake and freely moving condition, telemeter-indwelling orexin knockout mice showed diminished cardiovascular and behavioral responses to emotional stress in the resident-intruder test. We also found that basal AP in orexin knockout mice was significantly lower in both anesthetized (117 ± 8 mmHg in wild type and 92 ± 3 in knockout) and conscious (125 ± 6 mmHg in wild type and 109 ± 2 in knockout) conditions. α-Adrenergic blockade with prazosin or ganglion blockade with hexamethonium canceled the difference in basal AP. HR and cardiac contractile parameters by echocardiography did not differ between the two strains of mice. These results indicate lower sympathetic vasoconstrictor tone in knockout mice. The present study suggests that orexin-containing neurons in the perifornical area play a role as one of the efferent pathways of defense response and also operate as a regulator of AP at basal condition by activating sympathetic outflow.

hypothalamus; stress; respiration; sympathetic nervous system; circadian rhythm

OREXIN A and B, also known as hypocretin 1 and 2, are recently identified neuropeptides that consist of 33 and 28 amino acids, respectively (10, 46). They are proteolytically derived from the same precursor peptide (prepro-orexin) and exert a variety of functions by acting on orexin receptor type 1 and/or type 2. Orexin-containing neuron cell bodies are located exclusively in the lateral and dorsal hypothalamic areas and their axons diffusely innervate almost the entire central nervous system (6, 9, 10, 38, 39, 44, 46). This anatomic feature establishes the bases that orexin contributes to multiple physiological functions, including feeding behavior (46), energy homeostasis (46, 54), sleep-wake cycle (5), and regulation of the autonomic and neuroendocrine systems (9, 22, 44, 54).

Several laboratories have proposed a possible contribution of orexin in cardiovascular regulation by observing the effects of exogenously administered orexins. Orexins on intracerebroventricular injection increased arterial blood pressure (AP), heart rate (HR) (47), renal sympathetic nerve activity, and plasma catecholamines (50) in conscious, unrestrained rats. Intrathecal administration of orexins increased AP and HR (3). Microinjection of orexin A to the rostral ventrolateral medulla (RVLM) increased AP and HR in anesthetized rats (7) and awake rats (36). However, there is no report to date on whether the same is true for intrinsic orexin.

On the other hand, the perifornical area of the hypothalamus or dorsomedial hypothalamus, a region of the brain with the highest density of orexin-containing neurons (9, 10, 44, 46), has been known as the center for defense response and is sometimes called the defense area (29). Defense response, which is also known as "fight-or-flight" response, is characterized by a coordinated rise in AP, HR, respiratory frequency (Rf), and resistance in most vascular beds along with a fall in resistances in airway and blood vessels in the skeletal muscles when an animal encounters stressors. A pioneer work by Hess (23) showed that electrical stimulation of the posterior hypothalamus in cats elicited behavioral rage, along with the specific autonomic responses, that was termed the "defense response." Although some reports using chemical stimulation with

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excitatory amino acids (e.g., glutamate) had shown no effect or even a depressor effect (19, 25), later it was shown that negative results might have been caused by the stimulation of specific subregions in the posterior hypothalamus and/or dosage of drugs (14). Actually, the perifornical area was the most reliable region to elicit the cardiovascular defense response (29), and an overdose of excitatory amino acids sometimes inhibited neuronal activity, an effect known as excitation block phenomenon (34). Disinhibition with a GABA-A receptor antagonist, bicuculline methiodide, of the perifornical area reliably and dose dependently induced the defense response (13). Moreover, injection of a GABA agonist to the defense area inhibited the stress-induced rise in AP and HR (35).

There is only limited information about neurotransmitter(s) that subserve efferent pathways of the defense responses. Namely, glutamate was proposed to mediate at least the cardiovascular component of the defense response (52). However, there is no report on the molecular basis of the defense response underlying its multifaceted nature such as concomitant and coordinated changes in cardiovascular, respiratory, and behavioral parameters. We hypothesized that intrinsic orexin, synthesized in the perifornical area, may participate in the efferent pathway of the defense response and thought that knockout mice may be useful to test the hypothesis.

Prepro-orexin knockout mice, which completely lack both orexin A and orexin B, have been recently established (5). They exhibited a phenotype strikingly similar to human narcolepsy patients, such as behavioral arrest resembling cataplectic attack, sleep-onset rapid eye movement sleep, and diminished awake time in the dark period. This murine model and the canine model of narcolepsy that has mutation in the gene for orexin receptor type-2 (33) suggested the importance of orexin-orexin receptor type-2 interaction in the pathogenesis of narcolepsy. In fact, human narcolepsy seems to be an autoimmune disease, resulting in loss of orexin-containing neurons in the hypothalamus (53). On the other hand, there are only a few reports examining autonomic regulation in human narcolepsy (see DISCUSSION). Cardiovascular and autonomic phenotypes in orexin knockout mice have not been examined.

The aim of this study was to test our hypothesis by *1*) identifying hypothalamic subregion(s) in mice suitable to elicit the defense response and by *2*) comparing the effect of hypothalamic stimulation between orexin null mutated knockout mice and wild-type mice. Possible involvement of orexin in the defense response was further clarified by *3*) telemetric measurement of AP and HR during socioemotional stress of the resident-intruder test. In the course of the experiments, we found lower basal AP in orexin knockout mice. Therefore, an additional aim of this study was to examine possible involvement of intrinsic orexin in basal cardiovascular regulation by *4*) comparing AP and HR between mutant and wild-type mice in unanesthetized freely moving condition with or without pharmacological perturbations.

MATERIALS AND METHODS

Animals. Prepro-orexin mutated mice of genetic background of a mixture of 129/Sv and C57BL/6 were generated as reported previously (5). They were maintained in heterozygotes and crossed to obtain null mutants and wild-type littermates. Genotype of orexin knockout mice was identified by PCR on DNA extracted form a tail biopsy in a similar manner as had been reported except for primer sequences. We used a 5′ primer, GAC CTA TCA GGA CAT AGC GTT GGC and a 3′ primer, TCA CCC CCT TGG GAT AGC CCT TCC for the mutant allele and a 5′ primer, GAC GAC GGC CTC AGA CTT CTT GGG with the same 3′ primer to identify the wild-type allele. Mice used in this study were 18- to 40-wk-old male orexin null mutated mice and wild-type mice. Heterozygotes were not used. All mice were housed in plastic cages in a room maintained at 23–25°C with lights on at 7:00 AM and off at 7:00 PM. Mice had food and water available ad libitum. All animal procedures conformed to the "Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences" recommended by the Physiological Society of Japan.

Measurement of cardiorespiratory parameters and electroencephalogram in anesthetized mice. Mice were anesthetized with intraperitoneal injection of urethane (1.1 g/kg); supplementary doses of 0.1–0.5 g/kg were given when required to maintain a level of anesthesia (average total dose including all additional doses was 1.22 ± 0.05 g/kg, n = 20). We found this dose was adequate in both orexin knockout mice and wild-type mice as judged by baseline stability of AP, HR, respiration, and electroencephalography (EEG) throughout the experiment. Cardiorespiratory parameters were recorded as described in our previous reports (31, 42). In brief, AP was measured by a pressure sensor catheter (SPR-671 microtip pressure catheter for mouse/rat, Millar, Houston, TX) placed in the abdominal aorta through an incision of the right femoral artery. Mean AP (MAP) was calculated by damping (0.2 Hz low pass) the phasic AP signal. HR was recorded using an HR counter (AT-601G, Nihon Kohden, Tokyo, Japan) triggered by the AP pulse. A tracheal cannula (polyethylene tube, OD = 1.20 mm) was inserted through a midline incision. Respiratory flow signal was obtained through a Lilley type pneumotachograph (TV-241T and TP-602T, Nihon Kohden) connected to the tracheal tubing. Rf was counted by a pulse counter (ET-612J, Nihon Kohden). In *experiment 2* (see below), a pair of electrodes to record EEG was implanted through holes in the skull over the left and right frontal cortex. Cortical EEG arousal has been implicated as one of the functions of the perifornical lateral hypothalamus (11) and may be a part of defense response. EEG signals were amplified with band-pass filters of 0.25–100 Hz (AB-651J, Nihon Kohden). In *experiment 1*, we did not try to measure EEG because electrical stimulation in the brain makes serious noise in EEG.

In both *experiments 1* and *2*, the animal was placed in the prone position in a stereotaxic frame (ST-7, Narishige, Tokyo, Japan) so that bregma and lambda would be horizontal. A small hole was drilled on the skull for insertion of a metal electrode or a glass micropipette into the hypothalamus. After completion of surgery, at least 1 h was allowed to stabilize all the parameters. Throughout the experiment, rectal temperature was kept constant at 37.0 ± 1.0°C by a heating pad connected to a thermo controller (ATB-1100, Nihon Kohden).

Experiment 1: mapping of hypothalamic areas to induce defense response. We used 18- to 25-wk-old male wild-type mice (n = 10, body wt 32 ± 3 g). Electrical stimulations were

made with stainless steel electrodes. Explored brain regions were the perifornical area and surrounding hypothalamus (from 1.5 to 2.5 mm caudal to the bregma, from 0.3 to 1.5 mm lateral to the midline, and from 4.0 to 5.8 mm ventral to the bregma). An enamel-coated sharp stainless-steel electrode (0.1 mm of the tip exposed) was inserted into the targeted region, and the different lead was placed under the skin of the neck. The electrode was carried in a stereotaxic micromanipulator (SM-11, Narishige) and lowered along the track in steps of 0.3 mm. Three to seven tracks were completed in each animal, and each track was 0.5 mm apart from the other tracks. At each point, the brain was stimulated with a train of rectangular pulses of 0.5-ms duration at 100 Hz for 20 s. The stimulation currents were 0.3 mA with the negative electrode. Stimulations were separated by at least 5 min after all the parameters returned to baseline. We used electrical stimulation in *experiment 1* because repeated drug injection to such a tiny brain as in mice cannot be devoid of volume effect (34). However, electrical stimulation excites not only cell bodies but also fibers of passage. Because of such a disadvantage in electrical stimulation, we used the microinjection technique in *experiment 2* (see below) to stimulate only cell bodies but not fibers of passage.

Experiment 2: chemical stimulation of the perifornical area. Experiments were done in 18- to 25-wk-old male wild-type mice (n = 5, body wt 35 ± 1 g) and prepro-orexin knockout mice (n = 5, 36 ± 2 g). Microinjection of drugs was made with glass micropipettes (Drummond, 1–5 μl) shaped by a micropipette puller (PE-2, Narishige) and cut to fit the tip diameter of 20–30 μm. The pipettes were carried in a stereotaxic micromanipulator (SM-11, Narishige) and connected by silicon tubing to a pressure injector (IM-200J, Narishige). The micropipette was filled with a GABA-A receptor antagonist, bicuculline methiodide (0, 0.1, 0.3, 1.0 mM), dissolved in artificial cerebrospinal fluid (ACSF). The tip of the pipette was positioned in the perifornical area, where maximum response was observed in *experiment 1* (2.0 mm caudal to the bregma, 0.65 mm lateral to the midline, 5.0 mm ventral to the bregma, see also Fig. 1). While observing the fluid meniscus in the micropipette through a dissection microscope (OME, Olympus) equipped with an ocular micrometer, a volume of 20 nl was injected by adjusting pressure and time of injection. Bicuculline was administered sequentially from the lowest dose to the highest after recorded parameters returned to the baseline in each animal.

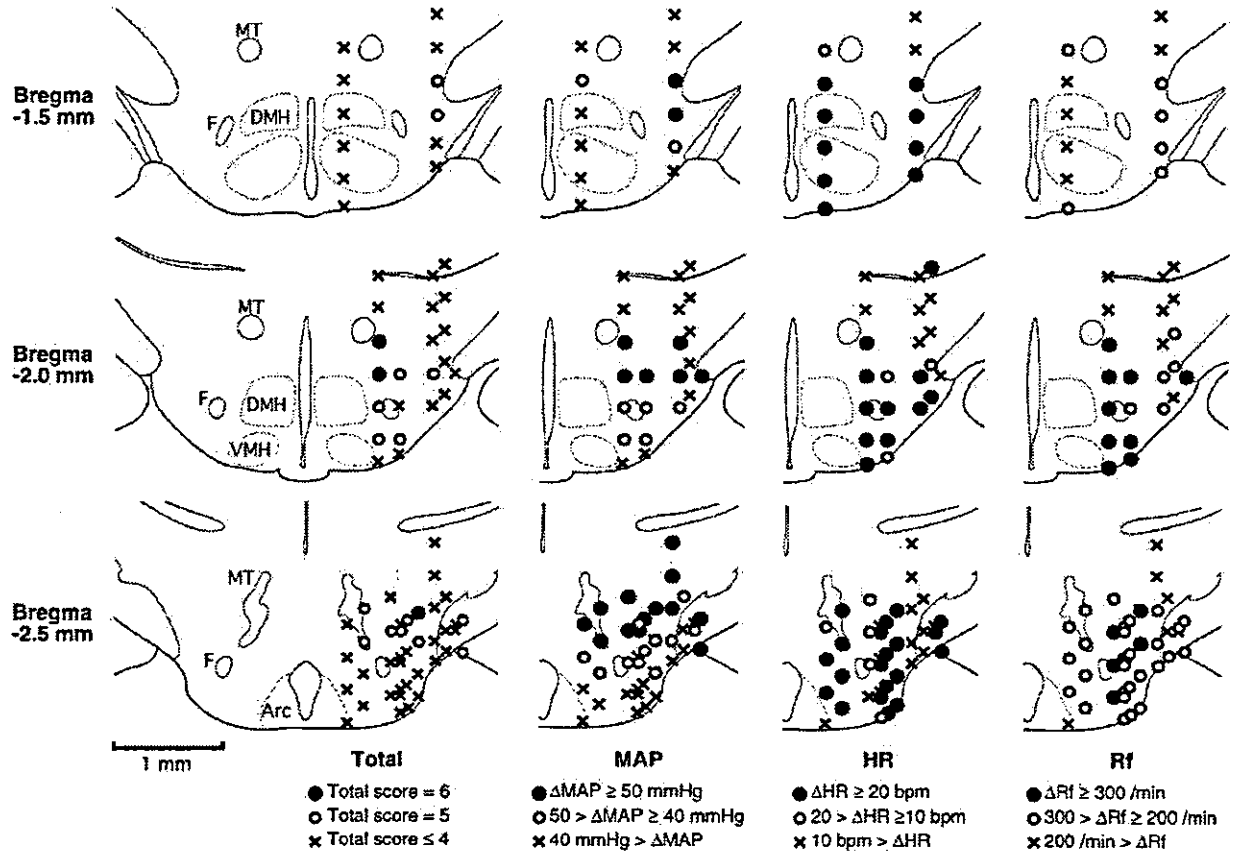

Fig. 1. Schematic coronal sections of the mouse hypothalamus from 1.5 to 2.5 mm caudal to the bregma showing effects of electrical stimulation on (*left* to *right*) consolidated defense response (Total), mean arterial blood pressure (MAP), heart rate (HR), and respiratory frequency (Rf). Data were obtained from 10 wild-type (WT) mice. Arc, arcuate hypothalamic nucleus; DM, dorsomedial hypothalamic nucleus; F, fornix; MT, mammillothalamic tract; VMH, ventromedial hypothalamic nucleus.

Experiment 3: measurement of cardiovascular parameters and activity by radiotelemetry. Experiments were done in 30- to 40-wk-old male wild-type mice (n = 6, body wt 46 ± 3 g) and prepro-orexin knockout mice (n = 6, 52 ± 2 g). A telemetry system (Data Sciences International) was used for measuring AP, HR, and locomotor activity. This system consisted of a radio-frequency transducer (TA11PA-C20) and a receiver (RLA1020). For implantation of the transducer, mice were anesthetized with 2–3% isoflurane and given an antibiotic (cephalosporin, 50 mg/kg sc). A midline incision was made on the neck, and the left common carotid artery was isolated. A small cut was made in the vessel wall, and the catheter-transducer was implanted in the thoracic aorta through the hole. The sensor was tunneled subcutaneously and fixed to the abdominal wall. Finally, the incision was closed with sutures. Care was taken to maintain body temperature during and after the surgery. Mice were returned to their home cages and housed individually during the recovery and recording period. All the mice were allowed at least 5 days of recovery from surgery until circadian rhythm was evident in both AP and HR (4).

After the recovery period, AP, HR, and activity were recorded for successive 2–3 days using Dataquest LabPRO software (Data Sciences) in unrestrained, freely moving, and unanesthetized conditions.

Two types of stressor were applied in the afternoon on the following day after the basal measurement was completed. The first one was a socioemotional stressor in the resident-intruder test (31). A group-housed, age-matched wild-type mouse (intruder) was placed in the cage for 5 min. When AP, HR, and activity returned to the baseline, a second trial of the resident-intruder test was performed, but the telemeter-indwelling animal served as the intruder this time. Cardiovascular and behavioral responses in the first and second trials in the resident-intruder test are called "resident response" and "intruder response," respectively, in this study. The second stressor was noxious stimuli. Vascular clamps of two strengths (60 and 125 g, Fine Science Tools, nos. 18055–01 and -04) were attached to the tail for 10 s.

Finally, on the following day in the light phase, urethane (1.1 g/kg) was intraperitoneally administered to see whether the difference in AP observed in *experiment 2* (see RESULTS) could be reproduced and to confirm that attenuation of defense response observed in *experiment 2* did not result from generally increased susceptibility of the knockout mice to anesthesia.

Experiment 4: measurement of cardiovascular parameters with indwelling catheter and pharmacological interventions. Experiments were done in 18- to 25-wk-old male wild-type mice (n = 21, body wt 33 ± 1 g) and prepro-orexin knockout mice (n = 14, 33 ± 1 g). For simultaneous AP measurement and intravascular drug delivery, we used an indwelling catheter to minimize surgical damage because telemetric AP measurement needs additional operation for this purpose. Under isoflurane anesthesia, polyethylene tubing was inserted into the femoral artery for both AP measurement and drug delivery as reported (41). On the following day during the light phase, AP and HR were measured continuously for 2 h under conscious and unrestrained conditions in a quiet environment after at least 1 h of acclimatization. Thereafter, the animals were divided into three groups. The first group was given intra-arterially a ganglion blocker, hexamethonium (20 mg/kg, n = 13 in wild-type mice and n = 6 in knockout mice), to examine possible contribution of the autonomic nervous system in the difference in basal AP between knockout and wild-type mice. The second group was given an angiotensin-converting enzyme inhibitor, captopril (30 mg/kg, n = 3 in both wild-type and knockout mice), to examine possible interaction of ANG II and orexin, since orexin has been shown to stimulate drinking behavior as potently as ANG II (30). The third group (n = 5 in both wild-type and knockout mice) was sequentially given two drugs: a vasopressin V1a receptor antagonist, [β-mercapto-β,β-cyclopentamethylenepropionyl[1], O-me-Tyr[2], Arg[8]]vasopressin (10 μg/kg), and an α-adrenergic blocker, prazosin (1 mg/kg). Effect of the V1 receptor antagonist was tested because orexin-containing neurons innervate neurons in the paraventricular nucleus containing vasopressin (51). Resulting AP and HR were calculated as the peak value within 1 h after the administration.

Experiment 5: measurement of echocardiography. Experiments were done in 14- to 18-wk-old male wild-type mice (n = 6) and prepro-orexin knockout mice (n = 6). Anesthetized (pentobarbital sodium, 50 mg/kg ip) animals were observed with a 2D-guided M-mode echocardiographic system equipped with a 12-MHz imaging transducer (Sonos 4500; Agilent Technologies, Osaka, Japan). Left ventricular (LV) diastolic diameter (LVDD) and LV systolic diameter (LVSD) were measured. LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV) were calculated by the cubed method as $(LVDD)^3\pi/3$ and $(LVSD)^3\pi/3$, respectively. We also calculated percent fractional shortening [%FS = 100 × (LVDD − LVSD)/LVDD], stroke volume (SV = LVEDV − LVESV), and ejection fraction (%EF = 100 × SV/LVEDV). Three beats were averaged for each measurement.

Data analysis and statistical procedure. All the signals were fed into a personal computer (Macintosh, Apple Computer) after analog-to-digital conversion (model 1401, CED, UK or MacLab, AD Instrument, Australia) together with event signals.

In *experiment 1*, the baseline value was the mean value before stimulation for 20–30 s, and the peak value was calculated as a mean value for 5–10 s around the maximum response.

In *experiment 2*, data were averaged for every 1 min. The baseline value was the mean value during 5 min before injection of bicuculline. Recovery time was defined as the time from injection until MAP, HR, or Rf returned to the value 5% above the baseline, because spontaneous fluctuation of these parameters during control period was within ±5% range of the mean value. We also calculated the area under the curve (AUC) above the baseline value during periods of 10, 15, 20, and 30 min from the injection for 0, 0.1, 0.3, and 1.0 mM of bicuculline, respectively. The length of the periods was determined from the result of recovery time in wild-type mice (see Table 1). To estimate the cortical arousal level, we calculated the power spectra of EEG using fast Fourier transformation (FFT; length of sections 5 s, FFT number 512). Arousal level was determined as relative β-band (13–50 Hz) power to the total band powers. For relative β-band power, recovery was defined as the time point when the value returned to a value 20% above the baseline, since spontaneous fluctuation of this parameter during control period was within ±20% range of the mean value.

For circadian measurement in *experiment 3*, AP, HR, and activity counting were averaged for every 30 min. Values in each animal were analyzed by the cosinor method (40) to calculate midline estimating statistic of rhythm (MESOR) and amplitude. The mean light and dark phase values were also calculated. In the resident-intruder test, AP, HR, and activity counting were averaged for every 1 min. AUC was calculated for 5 min when resident and intruder were in the same cage. In the tail-clip test, resulting AP and HR were calculated as the peak value within 30 s after the start of

Table 1. *Duration of responses by bicuculline*

Parameter	Dose of Bicuculline, mM in 20 nl	Wild-Type Mice	Knockout Mice
Blood pressure	0.1	10.8±2.6	2.6±1.7*
	0.3	15.8±2.9	5.0±1.6*
	1	26.0±2.7	14.0±2.2*
Heart rate	0.1	12.4±1.6	1.8±1.1*
	0.3	22.8±2.4	9.0±2.8*
	1	31.8±2.3	25.2±5.5
Respiratory frequency	0.1	12.4±1.6	1.6±1.0*
	0.3	19.2±2.8	9.4±1.4*
	1	21.2±2.6	19.6±3.7
EEG β-band power	0.1	11.0±1.0	2.2±0.9*
	0.3	18.8±2.2	8.6±2.6*
	1	28.2±4.7	11.0±1.4*

Values are means ± SE of response duration in min. *$P < 0.05$ vs. wild-type mice (repeated-measure ANOVA followed by post hoc test of Student-Newman-Keuls).

clipping. In urethane treatment, pretreatment value was calculated as the mean during the 0.5 h immediately before injection. Posttreatment value was the average during the period from 1 to 6 h after injection, since *experiment 2* was usually completed within 6 h.

All data are expressed as means ± SE. Effects of microinjection of bicuculline on cardiovascular, respiratory, and arousal responses were assessed by ANOVA with repeated measures design. Post hoc comparisons of Student-Newman-Keuls procedure or unpaired *t*-test were used to compare between genotypes. A value of $P < 0.05$ was considered significant.

Histological verification of stimulation sites. At the end of the experiment, the injection site was marked by injecting 20 nl of a 2% Evans blue solution in *experiment 2*. In *experiment 1*, the same Evans blue solution was injected to the site where electrical stimulation was finally performed. The animal was deeply anesthetized with additional urethane and transcardially perfused with 20 ml of heparin-added saline followed by 20 ml of 4% formalin. The brains were removed and stored in the formalin solution for at least 2 days before sectioning. Coronal or sagittal sections of 50-μm thickness were cut serially with a micro slicer (DTK-1000, Dosaka EM, Kyoto, Japan), mounted on poly-L-lysine-coated slides, and stained with 1% neutral red or 0.5% cresyl violet. The locations of the injection sites were determined according to the atlas of Paxinos and Franklin (43).

RESULTS

Experiment 1: mapping of hypothalamic areas to induce defense response. To examine whether hypothalamic stimulation induces defense response in mice as in other experimental animals, the effect of electrical stimulation on cardiorespiratory parameters was systematically explored. Ninety-three histologically verified sites within the targeted area were examined in 10 mice. Average MAP, HR, and Rf before stimulation were 115 ± 4 mmHg, 570 ± 15 beats/min, and 194 ± 5/min, respectively. Electrical stimulation in most sites resulted in increases in MAP, HR, and Rf except for 11 sites to which stimulation resulted in decreases in HR. Response magnitude of each parameter was ranked into three levels and is shown in Fig. 1. Stimulation at caudal hypothalamic sites induced a pressor response. The most effective sites were located in the dorsolateral region in the caudal hypothalamus, where electrical stimulation resulted in an increase in MAP by >50 mmHg (Fig. 1). In regard to HR, effective sites were roughly overlapped with but extended more widely compared with the effective sites for MAP. Electrical stimulation at most of the explored sites in the caudal hypothalamus produced an increase in HR by >20 beats/min. An increase in Rf was elicited in the middle to lateral part of the caudal hypothalamus, in which responses in Rf were >300/min.

To map the most effective sites for eliciting defense response, in other words coordinated increases in MAP, HR, and Rf, the rank of response magnitude in each parameter was scored as 2, 1, or 0 points from the highest to the lowest in this order, and summation of scores was calculated for each stimulating site (Fig. 1, *left*). The total score was highest at three sites in the dorsal part of the perifornical area. The most effective sites were located at 0.65 mm lateral to the midline, 4.7–5.0 mm ventral to the bregma in the plane of 2.0 mm caudal to the bregma, and 1.0 mm lateral, 4.7 mm ventral in the plane of 2.5 mm caudal to the bregma.

These results clearly show that defense response can be elicited by electrical stimulation of dorsal hypothalamus, especially the dorsal part of the perifornical area in mice. From these results, we decided to stimulate a point 2.0 mm caudal to the bregma, 0.65 mm lateral to the midline, and 5.0 mm ventral to the bregma in the following experiment.

Experiment 2: chemical stimulation of the perifornical area in orexin knockout mice. To test our hypothesis that orexin may contribute to expression of the defense response, we compared the effects of chemical stimulation with bicuculline to the perifornix on cardiorespiratory and EEG parameters between orexin null mutated knockout mice and wild-type littermates. Before stimulation, the average values of MAP, HR, Rf, and relative β-power in five wild-type mice were 117 ± 8 mmHg, 558 ± 12 beats/min, 178 ± 7/min, and 14 ± 2%, respectively. In wild-type mice, microinjection of 0.1–1 mM of bicuculline to the perifornical area elicited dose-dependent increases in MAP, HR, Rf, and relative β-power (Fig. 2), but ACSF (vehicle) did not cause any significant changes. By 0.1, 0.3, and 1.0 mM of bicuculline, peak increases in MAP (11 ± 2%, 13 ± 1, and 28 ± 2%), HR (13 ± 3, 17 ± 2, and 19 ± 4%), Rf (54 ± 6, 103 ± 7, and 167 ± 15%), and relative β-power (111 ± 22, 122 ± 39, and 121 ± 17%) were all statistically significant. These changes began within 15 s from injection of bicuculline and returned to baseline within 10–30 min depending on the dosage (Table 1).

In orexin knockout mice, basal values of MAP, HR, Rf, and relative β-power were 92 ± 3 mmHg, 562 ± 22 beats/min, 218 ± 11/min, and 13 ± 3%, respectively. MAP was significantly ($P < 0.05$) lower and Rf was significantly higher than that in wild-type mice (Fig. 2). Basal HR and relative β-power were not significantly different between the genotypes. At a glance, microinjection of bicuculline to the perifornical area in the mutant mice appeared to result in similar cardio-