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COMMENTARY

Negative inotropic effects of endothelin-1 in mouse cardiomyocytes: evidence of a role for Na^+-Ca^{2+} exchange

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Endothelin-1 (ET-1) is a peptide hormone produced within the myocardium which may modulate myocardial contractility in a paracrine-autocrine fashion. In the majority of species, ET-1 has a direct positive inotropic effect on the myocardium that involves both increased myofilament Ca^{2+} sensitivity and increased Ca^{2+} transients. Ca^{2+} entry through reverse-mode Na^+-Ca^{2+} exchange, involving both indirect effects via elevation of intracellular [Na^+] and direct activation of the Na^+-Ca^{2+} exchanger, have been suggested to contribute to the increase in Ca^{2+} transients. Conversely, mouse cardiomyocytes show an exclusively negative inotropic response to ET-1. Here, Nishimaru and colleagues present novel evidence that the negative inotropic effect of ET-1 in mouse cardiomyocytes involves both a reduction in myofilament Ca^{2+} sensitivity and increased Ca^{2+} extrusion, via Na^+-Ca^{2+} exchange. Data obtained using the selective Na^+-Ca^{2+} exchange blocker, SEA0400, suggest that a re-assessment of the role of the exchanger in Ca^{2+}-handling by mouse cardiomyocytes may be necessary.
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Abbreviations: $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; ET, endothelin; ET_A, ET_B, A- and B-subtypes of endothelin receptor; $I_{Ca,L}$, L-type Ca^{2+} current; KB-R7943, an NCX blocker, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate; $[Na^+]_i$, intracellular Na^+ concentration; NCX, Na^+–Ca^{2+} exchanger; NHE, Na^+–H^+ exchanger; NIE, negative inotropic effect; PIE, positive inotropic effect; PKC, protein kinase C; SEA0400, a selective NCX blocker, 2-[4-[(2,5-difluorophenyl) methoxy]phenoxy]-5-ethoxyaniline

It is very nearly two decades since the discovery of endothelin (ET) as a potent vasoconstrictor peptide hormone released by endothelial cells (Yanagisawa *et al*., 1988). The demonstration of a direct positive inotropic effect (PIE) on the myocardium followed very soon thereafter (Ishikawa *et al*., 1988). The peptide is thought to be released within the heart, to modulate myocardial function in a paracrine–autocrine fashion (Mebazaa *et al*., 1993; MacCarthy *et al*., 2000) and may be involved in the contractile and hypertrophic responses to stretch (Yamazaki *et al*., 1996; Alvarez *et al*., 1999). The finding of elevated plasma concentrations of ET in various cardiac disorders and the beneficial effect of ET receptor antagonism in animal models of heart failure have focused attention on ET as a potential therapeutic target (Russell and Molenaar, 2000). Nevertheless, the actions of ET on the heart remain incompletely understood, in part due to the complexity of action of the hormone, involving direct and indirect effects on the myocardium and a multiplicity of signalling pathways, and partly due to species differences in the action of the peptide.

Although ET was originally proposed to act, like certain peptide toxins, by direct interaction with voltage-gated Ca^{2+} channels (Ishikawa *et al*., 1988; Yanagisawa *et al*., 1988), this peptide (now renamed ET-1) was demonstrated to act through two subtypes of G-protein-coupled receptor, ET_A and ET_B, ET_A being predominant in cardiomyocytes (Russell and Molenaar, 2000). Although both receptors couple to $G\alpha_q$ signalling pathways, including phosphatidylinositol hydrolysis and protein kinase C (PKC) mobilization, ET receptors are relatively promiscuous in their coupling with G proteins, and signalling varies between cell types. In cardiomyocytes, not only does ET-1 signal via $G\alpha_q$, but ET_A receptors couple to $G\alpha_i$ pathways, including adenylyl cyclase inhibition (Hilal-Dandan *et al*., 1992). Thus, there are many reports of the inhibition by ET-1 of currents regulated by β-adrenoceptors,

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such as the L-type Ca^{2+} current ($I_{Ca,L}$) (for example, Tohse *et al*., 1990; James *et al*., 1994; Xie *et al*., 1996). Nevertheless, although transient negative inotropic effects (NIE) are often observed on administration of the peptide, which may involve $I_{Ca,L}$ inhibition (Woo and Lee, 1999), in the majority of species a more slowly developing, sustained PIE is also observed. The PIE has been associated with an increase in the Ca^{2+} sensitivity of the myofilaments and/or (depending on species) an increase in the Ca^{2+} transients (for example, Krämer *et al*., 1991; Takanashi and Endoh, 1991; Kelso *et al*., 1996a). The mechanisms of the increase in myofilament Ca^{2+} sensitivity are controversial but may involve Na^+–H^+ exchanger (NHE) activation and intracellular alkalinization (Krämer *et al*., 1991; Kang and Walker, 2006). Similarly, the basis of the increase in the Ca^{2+} transient is yet to be fully elucidated: With limited cell dialysis, ET-1-mediated increases in $I_{Ca,L}$ can be observed (see Kelso *et al*., 1996b; Woo and Lee, 1999; He *et al*., 2000), demonstrating the sensitivity of ET-1 responses to experimental conditions. NHE may also have a role in increasing Ca^{2+} transients, as stretch activation of NHE via ET-1 has been suggested to increase intracellular Na^+ concentration ($[Na^+]_i$), thereby favouring Ca^{2+} entry via 'reverse-mode' Na^+–Ca^{2+} exchange (Alvarez *et al*., 1999). The Na^+–Ca^{2+} exchanger (NCX) is an electrogenic transporter ($3Na^+$:Ca^{2+}) that represents the major route for Ca^{2+} extrusion from cardiomyocytes; the driving force for Ca^{2+} extrusion being the difference between the equilibrium potential for NCX and the membrane potential (Bers, 2002). Thus, NCX can also contribute to Ca^{2+} entry when the driving force for NCX transport reverses (for example, early in the action potential), an event facilitated by elevation of $[Na^+]_i$. Results obtained using the NCX blocker, KB-R7943, are consistent with the contribution of reverse-mode NCX in ET-1 responses (Yang *et al*., 1999; Perez *et al*., 2001). In addition, ET-1 increases NCX activity directly via PKC-dependent phosphorylation (Zhang *et al*., 2001).

It is against this background that Nishimaru *et al*. (in press) have investigated the actions of ET-1 in mouse cardiomyocytes, which appears on pages XX–YY of this volume of the journal. In contrast to most other species, the adult mouse shows exclusively a NIE to ET-1 (Sekine *et al*., 1999; Sakurai *et al*., 2002). Using epifluorescence and videometric methods, the authors investigated the effects of ET-1 on Ca^{2+} transients and cell shortening in isolated mouse cardiomyocytes loaded with the ratiometric Ca^{2+} fluorophore, Indo-1. Their conclusions contrast beautifully with the extant literature on ET-1-mediated PIE; the NIE of mouse cardiomyocytes involves reduction of myofilament Ca^{2+} sensitivity and activation of NCX. ET-1 concentration-dependently reduced both Ca^{2+} transients and cell shortening, although the percentage reductions in cell shortening were greater than those in the Ca^{2+} transient. Elevation of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) in the presence of ET-1 restored the amplitude of cell shortening and of the Ca^{2+} transient. Plotting cell shortening against Indo-1 fluorescence ratio during a single twitch in control and in the presence of ET-1 plus elevated $[Ca^{2+}]_o$ provided key evidence for the reduction in Ca^{2+} sensitivity; the slope of this relationship was less steep in the presence of ET-1, despite similar amplitudes of the Ca^{2+} transient and contraction. The mechanisms for this reduction in myofilament sensitivity remain unclear, although phosphorylation of troponin I by ET-1 has been suggested to reduce myofilament Ca^{2+} sensitivity (Cuello *et al*., 2007). As ET-1-induced phosphorylation of troponin I also occurs in species other than mouse (Damron *et al*., 1995), presumably additional mechanisms account for the increased myofilament Ca^{2+} sensitivity in these species.

The selective NCX blocker, 2-[4-[(2,5-difluorophenyl) methoxy]phenoxy]-5-ethoxyaniline (SEA0400) provided evidence for the involvement of NCX in the NIE through reduction in Ca^{2+} transients: blockade of NCX completely abolished the ET-1-induced reduction in Ca^{2+} transient, suggesting a role for increased Ca^{2+} extrusion via NCX. On the other hand, inhibition of $I_{Ca,L}$ was not required for the NIE as the action of ET-1 on Ca^{2+} transients was unaffected by L-type blockade. At first sight, this result may seem surprising since evidence from transgenic models suggests that NCX plays only a minor role in the recovery of Ca^{2+} transients in mouse (reviewed in Bers, 2002). Although the evidence in the present study is largely pharmacological, the authors were wise in their selection of NCX blocker: in contrast to KB-R7943, which blocks $I_{Ca,L}$, SEA0400 is relatively selective for NCX (Birinyi *et al*., 2005). Indeed, the increase in Ca^{2+} transients produced by SEA0400 is consistent with a significant role for NCX in Ca^{2+} extrusion. So how can increased NCX activity be involved in both the NIE of ET-1 in mouse and the PIE in other species? The answer may lie, at least partially, in species differences in the driving force for NCX-mediated Ca^{2+} transport during the cardiac cycle (Bers, 2002). In mice, the action potential is relatively short, with no clear plateau phase, and $[Na^+]_i$ is comparatively high (Bers, 2002). As a consequence, although the driving force for Ca^{2+} extrusion via NCX at the resting membrane potential during diastole is small, it is markedly increased by the Ca^{2+} transient during the repolarization phase of the action potential. In contrast, the longer action potential, with a clear plateau, and lower $[Na^+]_i$ of other species (for example, rabbit, human) lead to a greater Ca^{2+} extrusion during diastole and less during repolarization (Bers, 2002). Thus, ET-1-induced $[Na^+]_i$ increase reduces diastolic Ca^{2+} extrusion and favours reverse-mode NCX in these species (Aiello *et al*., 2005). By extension, one might speculate that in mouse cardiomyocytes perhaps ET-1 does not increase $[Na^+]_i$ and thus NCX operates predominantly in the forward mode, extruding Ca^{2+} during repolarization of the action potential.

In summary, this elegant study not only demonstrates the existence of two parallel mechanisms for the ET-1-induced NIE in mouse, but also provides important insights into species differences in hormone action. Further studies are warranted to elucidate the underlying mechanisms of ET-1 action in the mouse. Importantly, although the evidence is limited, the study suggests that a re-assessment of the role of NCX in Ca^{2+} handling by mouse cardiomyocytes may be necessary.

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