Modulation of Iron Uptake in Heart by L-Type Ca\(^{2+}\) Channel Modifiers

Possible Implications in Iron Overload

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Abstract—Heart failure is the leading cause of mortality in patients with transfusional iron (Fe) overload in which myocardial iron uptake ensues via a transferrin-independent process. We examined the ability of L-type Ca\(^{2+}\) channel modifiers to alter Fe\(^{2+}\) uptake by isolated rat hearts and ventricular myocytes. Perfusion of rat hearts with 100 nmol/L \(^{59}\)Fe\(^{2+}\) and 5 mmol/L ascorbate resulted in specific \(^{59}\)Fe\(^{2+}\) uptake of 20.4 ± 1.9 ng of Fe per gram dry wt. Abolishing myocardial electrical excitability with 20 mmol/L KCl reduced specific \(^{59}\)Fe\(^{2+}\) uptake by 60 ± 7% \((P<0.01)\), which suggested that a component of myocardial Fe\(^{2+}\) uptake depends on membrane voltage. Accordingly, \(^{59}\)Fe\(^{2+}\) uptake was inhibited by 10 \(\mu\)mol/L nifedipine \((45±12%, \ P<0.02)\) and 100 \(\mu\)mol/L Cd\(^{2+}\) \((86±3%; \ P<0.001)\) while being augmented by 100 nmol/L Bay K 8644 \((61±18%, \ P<0.01)\) or 100 nmol/L isoproterenol \((40±12%, \ P<0.05)\). By contrast, uptake of 100 nmol/L ferric iron \((^{59}\)Fe\(^{3+}\)) was significantly lower \((1.4±0.3 ng \text{ Fe per gram dry wt}; \ P<0.001)\) compared with divalent iron. These data suggest that a component of Fe\(^{2+}\) uptake into heart occurs via the L-type Ca\(^{2+}\) channel in myocytes. To investigate this further, the effects of Fe\(^{2+}\) on cardiac myocyte L-type Ca\(^{2+}\) currents were measured. In the absence of Ca\(^{2+}\), noninactivating nitrendipine-sensitive Fe\(^{2+}\) currents were recorded with 15 mmol/L \([\text{Fe}^{2+}]_{\text{o}}\). Low concentrations of Fe\(^{2+}\) enhanced Ca\(^{2+}\) current amplitude and slowed inactivation rates, which was consistent with Fe\(^{2+}\) entry into the cell, whereas higher Fe\(^{2+}\) levels caused dose-dependent decreases in peak current. Fe\(^{3+}\) had no effect on current amplitude or decay. Combined, our data suggest that myocardial Fe\(^{2+}\) uptake occurs via L-type Ca\(^{2+}\) channels and that blockade of these channels might be useful in the treatment of patients with excessive serum iron levels. (Circ Res. 1999;84:1302-1309.)

Key Words: iron overload ▪ channels ▪ heart failure ▪ permeability ▪ Ca\(^{2+}\)

Iron is essential for cellular metabolism and enzyme function. Normally, tissue levels of iron are precisely regulated and stored as a ferritin complex, thereby minimizing the potential toxic effects of catalytically active iron. However, elevations in serum iron occur commonly and are associated with a number of disease conditions. For example, acute iron poisoning is the most common cause of overdose mortality in young children and often results in myocardial dysfunction. On the other hand, chronic elevations of serum iron are associated with a number of disease conditions, including cardiomyopathy, diabetes mellitus, hypopituitarism, and liver cirrhosis. Iron overload cardiomyopathy is the most common cause of heart failure in young adults and is often associated with arrhythmias. It generally results from primary or secondary hemochromatosis. Primary (or hereditary) hemochromatosis is an autosomal recessive disorder that results from mutations in the histocompatibility antigen HLA-A\(^{B}B\) and afflicts ∼10% of people from European extraction. Secondary hemochromatosis is the most common single gene disorder in humans; it causes major thalassemic syndromes and sideroblastic anemias associated with ineffective erythropoietic activity and parenchymal iron overload. In addition, iron overload in these patients is often compounded by additional iron loads that result from chronic blood transfusions.

Currently, no satisfactory therapies exist for the treatment of iron overload disorders. Iron chelators have aided the long-term survival of iron-overload patients and reduce the incidence of cardiac dysfunction. However, patient compliance is poor with deferoxamine mesylate, and recent evidence suggests that the oral chelator deferiprone is ineffective in thalassemic patients and may promote hepatic fibrosis. Although chelation treatment improves survival, these patients are still at risk for developing late iron-induced cardiomyopathy. Therefore, understanding the mechanisms involved in iron accumulation in the heart and other tissues may prove useful for the development of new treatment strategies in iron-overload patients.
In most cells, iron uptake is mediated through internalization of the transferrin-iron complex bound to high-affinity membrane receptors.\textsuperscript{16} A second mechanism of iron uptake occurs through a transferrin-independent process. This non-transferrin-bound iron (NTBI) transport process is considered to have a minor role in iron uptake under normal physiological conditions but becomes the primary uptake mechanism when serum iron is severely elevated (eg, primary and secondary hemochromatosis). Under these conditions, iron saturation of transferrin and reductions in the number of transferrin receptors occur, which results in excessive transferrin-independent iron uptake via an unknown transporter pathway.\textsuperscript{16,17} NTBI uptake has been demonstrated in a number of mammalian cells, including cardiac myocytes.\textsuperscript{18,19} It is calcium-dependent and can be enhanced by prior iron loading of the cell.\textsuperscript{20,21} Of importance to our studies, it has been shown that a critical step in NTBI uptake is the reduction of ferric iron (Fe\textsuperscript{3+}) to the ferrous state (Fe\textsuperscript{2+}) by a membrane-associated ferrireductase.\textsuperscript{21}

In the present study, we demonstrate that a significant component of myocardial uptake of reduced iron (ie, Fe\textsuperscript{2+}) is dependent on the electrical excitability of the heart and can be modulated by agents and interventions that affect the L-type Ca\textsuperscript{2+} channel activity. In addition, we show that Fe\textsuperscript{2+} permeates Ca\textsuperscript{2+} channels at high concentrations and can alter channel kinetics at lower concentrations. Our results suggest that L-type Ca\textsuperscript{2+} channels might contribute significantly to iron uptake by the heart and has many of the properties associated with the unknown NTBI uptake pathway.

Materials and Methods

\textbf{\textsuperscript{59}Fe Uptake in Isolated Perfused Hearts} 

Hearts were rapidly excised from heparinized and anesthetized (sodium pentobarbital, 75 mg/kg) male Sprague-Dawley rats (Charles River, Montreal, Canada; 200–250 g) and mounted for perfusion using the Langendorff technique. Isolated hearts were perfused with Tyrode’s solution (8 mL/min) containing (in mM/L) 140 NaCl, 4 KCl, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 20 butanedione monoxime, 10 glucose, 10 HEPES (pH 7.4 with NaOH), and gassed with 100% O\textsubscript{2}. Temperature was maintained at 37°C. The addition of 5 mM/L ascorbic acid to the perfusion media maintained the iron in the reduced state (Fe\textsuperscript{2+}) and prevented the formation of Fe\textsuperscript{3+}. For ferric (Fe\textsuperscript{3+}) iron experiments, nitrocatechol (5 mM/L) was used to prevent Fe\textsuperscript{3+} precipitation\textsuperscript{20,21} and the concentration of CaCl\textsubscript{2} was adjusted to maintain a 1 mM/L free Ca\textsuperscript{2+} level. After a 10-minute equilibration period to remove blood from the vasculature and to ensure proper vascular perfusion, hearts were perfused for 15 minutes with 100 mM/L \textsuperscript{59}Fe\textsuperscript{2+} (\textsuperscript{59}FeSO\textsubscript{4}, 27 to 31 mCi/mg; NEN Life Science Products) or \textsuperscript{59}Fe\textsuperscript{3+} (\textsuperscript{59}FeCl\textsubscript{3}, 27 mCi/mg; NEN) then perfused with ice-cold Tyrode’s solution for 10 minutes to remove the bound iron. Ascorbic acid (5 mM/L) or NTA (5 mM/L) was included in the external solution for the ferrous and ferric iron experiments, respectively. With NTA in the external solution, the levels of CaCl\textsubscript{2} were adjusted to maintain a free concentration of 2 mM/L. The pipette solution consisted of (in mM/L) 140 N-methyl-D-glucamine, 2 CaCl\textsubscript{2}, 1 MgSO\textsubscript{4}, 3 4-aminopyridine, 10 glucose, and 10 HEPES (pH 7.4 with methanesulfonic acid). Ascorbic acid (5 mM/L) or NTA (5 mM/L) was included in the external solution for the ferrous and ferric iron experiments, respectively.

Electrophysiology

Whole-cell Ca\textsuperscript{2+} currents were recorded at room temperature from enzymatically isolated rat cardiac ventricular myocytes\textsuperscript{22} using the patch-clamp technique\textsuperscript{23} (Axopatch 200A, Axon Instruments). L-type Ca\textsuperscript{2+} currents were elicited by 200-millisecond step depolarizations between --40 and 70 mV from a holding potential of --80 mV. A 100-millisecond prepulse to --45 mV was used to inactivate Na\textsuperscript{+} channels. Current records were sampled at 150 μs and filtered at 2 kHz (4-pole Bessel filter, --3 dB). The external solution contained (in mM/L) 140 N-methyl-D-glucamine, 2 CaCl\textsubscript{2}, 1 MgSO\textsubscript{4}, 3 4-aminopyridine, 10 glucose, and 10 HEPES (pH 7.4 with methanesulfonic acid). Ascorbic acid (5 mM/L) or NTA (5 mM/L) was included in the external solution for the ferrous and ferric iron experiments, respectively. With NTA in the external solution, the levels of CaCl\textsubscript{2} were adjusted to maintain a free concentration of 2 mM/L. The pipette solution consisted of (in mM/L) 140 N-methyl-D-glucamine, 5 MgATP, 2 phosphocreatine, 0.2 GTP, 5 BAPTA, and 10 HEPES (pH 7.2 with methanesulfonic acid). At the end of the experiments, 20 mM/L nitrendipine was used to determine dihydropyridine-sensitive currents. Nitrendipine was used for these experiments instead of nifedipine to minimize photoinactivation.\textsuperscript{24}

Data Analysis

Data acquisition and analysis was performed using custom written and Origin software (MicroCal, Inc). The dose-response relationship was fit with a Hill equation: \(I_{p}/I_{0} = 1/(1 + (\text{IC}_{50}/n)^{h})\), where \(\text{IC}_{50}\) is the half-maximal inhibitory concentration of Fe\textsuperscript{2+} and \(n\) is the Hill coefficient. Data are presented as the mean±SEM. Statistical analysis was performed by use of a 1-way ANOVA followed by a multiple comparison testing (Student-Newman-Keuls; SPSS 7.5; SPSS). A P value <0.05 was used to denote statistical differences between groups.

Results

Iron Uptake in Isolated, Perfused Hearts

Iron uptake in most mammalian cells is mediated primarily through a transferrin-dependent process, but under conditions of iron overload, NTBI uptake becomes the predominant mode of iron uptake.\textsuperscript{18,20} In rat myocardium, NTBI uptake can exceed the transferrin-dependent pathways by 300-fold.\textsuperscript{18} Because the redox state of iron is critical in NTBI uptake and

Figure 1. Specific \textsuperscript{59}Fe\textsuperscript{2+} uptake of isolated perfused rat hearts is voltage-dependent. Hearts were perfused with \textsuperscript{59}Fe\textsuperscript{2+} (100 nmol/L) for 15 minutes followed by a 10-minute washout period in the absence and presence of KCl (20 mmol/L), nifedipine (1 or 10 μmol/L), Cd\textsuperscript{2+} (100 μmol/L), Bay K 8644 (100 nmol/L), or isoproterenol (100 nmol/L). \textsuperscript{59}Fe uptake was measured from ventricular tissue and normalized to dry wt. Data represent the mean±SEM of 6 to 8 hearts. *P< 0.05, **P< 0.01.
Fe\(^{2+}\) is the primary ionic species translocated across the cell membrane.\(^{21-25}\) We initially examined iron uptake into myocardium by perfusing isolated rat hearts (Figure 1) with 100 nmol/L \(^{59}\)Fe\(^{2+}\). This concentration is well below the plasma levels of NTBI measured in patients with hemochromatosis (ie, 1 to 20 \(\mu\)mol/L; References 26–28) or in some children after acute iron poisoning (ie, >1 mmol/L; Reference 29). Perfusion of rat hearts for 15 minutes resulted in specific \(^{59}\)Fe\(^{2+}\) uptake of 20.4±1.9 ng Fe per g of dry wt (n=7).

Electrically arresting the hearts with 20 mmol/L KCl reduced \(^{59}\)Fe\(^{2+}\) uptake to 8.1±1.3 ng Fe per g dry wt (n=8; \(P<0.01\); Figure 1), demonstrating that a major component of myocardial \(^{59}\)Fe\(^{2+}\) uptake is voltage-dependent.

Under normal physiological conditions, voltage-gated cardiac L-type Ca\(^{2+}\) channels are selectively permeable for Ca\(^{2+}\) versus Na\(^{+}\) and K\(^{+}\).\(^{30,31}\) However, these channels are permeable to other divalent cations, such as Ba\(^{2+}\), Sr\(^{2+}\), Mn\(^{2+}\), and Zn\(^{2+}\).\(^{32-34}\) Therefore, we hypothesized that L-type Ca\(^{2+}\) channels contribute to \(^{59}\)Fe\(^{2+}\) uptake. Consistent with our expectations, 10 \(\mu\)mol/L nifedipine, an L-type Ca\(^{2+}\) channel antagonist,\(^{35}\) suppressed myocardial contractility in the isolated perfused rat hearts and decreased \(^{59}\)Fe\(^{2+}\) uptake to 11.2±2.5 ng Fe per g dry wt (n=6; \(P<0.02\), Figure 1), a level not significantly different from KCl-arrested hearts (\(P>0.26\)). A lower concentration of nifedipine (1 \(\mu\)mol/L) produced a more modest reduction in \(^{59}\)Fe\(^{2+}\) uptake (17.9±0.7 ng Fe per g dry wt, n=6). Conversely, augmenting Ca\(^{2+}\) channel activity with the specific L-type Ca\(^{2+}\) channel agonist\(^{35,36}\) (−)Bay K 8644 significantly increased \(^{59}\)Fe\(^{2+}\) uptake to 32.9±3.7 ng Fe per g dry wt (n=7; \(P<0.01\)), which is 2.3-fold greater than the nifedipine-sensitive component. Because L-type Ca\(^{2+}\) channel activity is enhanced by \(\beta\)-adrenergic receptor activation,\(^{37,38}\) 100 nmol/L isoproterenol was added to the perfusion media. This agent caused a significant 40% enhancement of \(^{59}\)Fe\(^{2+}\) uptake (28.6±2.5 ng Fe per g of wt; n=6; \(P<0.05\)).

Next, we examined the effects of the inorganic divalent cation Cd\(^{2+}\), which blocks L-type Ca\(^{2+}\) channels.\(^{31,34}\) As shown in Figure 1, 100 \(\mu\)mol/L Cd\(^{2+}\) markedly inhibited \(^{59}\)Fe\(^{2+}\) uptake by 86% (2.9±0.6 ng of Fe per g dry wt, n=6; \(P<0.001\)). The inhibition of \(^{59}\)Fe\(^{2+}\) uptake by Cd\(^{2+}\) was significantly greater (\(P<0.01\)) than with KCl or nifedipine, suggesting that transporters other than L-type Ca\(^{2+}\) channels may be involved in myocardial iron uptake. This is not unexpected because Cd\(^{2+}\) interferes with numerous other membrane transporters including iron transporters.\(^{21,39}\)

In contrast to these findings, uptake of radioactive oxidized iron (\(^{59}\)Fe\(^{3+}\)) by the perfused rat hearts (1.4±0.3 ng Fe per g dry wt, n=4) was 15-fold less than \(^{59}\)Fe\(^{2+}\) uptake (\(P<0.001\)). These results are consistent with previous publications showing that ferrous iron (Fe\(^{2+}\)) is the primary species entering the heart via the NTBI mechanism.\(^{21,25}\)

**Fe\(^{2+}\) Permeation of L-Type Ca\(^{2+}\) Channels**

The dependence of \(^{59}\)Fe\(^{2+}\) uptake on cellular excitability and agents that affect L-type Ca\(^{2+}\) channel function led us to examine the interaction of Fe\(^{2+}\) with the cardiac L-type Ca\(^{2+}\) channel. With 2 mmol/L Ca\(^{2+}\) in the external solution, Ca\(^{2+}\) currents (\(I_{Ca}\)) peaked at 0 mV (−7.8±0.9 pA/pF, n=8; Figure 2A and 2E). Replacement of external Ca\(^{2+}\) with 15 mmol/L Fe\(^{2+}\) reduced, but did not eliminate, the amplitude of the inward current (−0.20±0.03 pA/pF at 20 mV; n=8; Figure 2B and 2E). Subsequent exposure of the cells to 20 \(\mu\)mol/L...
nitrendipine completely blocked all inward current (Figure 2C). The nitrendipine-sensitive Fe\(^{2+}\) currents showed little current inactivation (Figure 2D). In addition, activation of the nitrendipine-sensitive Fe\(^{2+}\) currents was shifted by 16±2 mV (n=8), resulting in a large rightward shift in the peak of the current-voltage relationship with no measurable change in the reversal potential (Figure 2E). The depolarizing shift in the voltage dependence of activation most probably results from the screening of negative surface charges caused by the higher concentration of divalent cation concentration in the external media (15 mmol/L Fe\(^{2+}\) versus 2 mmol/L Ca\(^{2+}\)) as observed at high external Ca\(^{2+}\) and Ba\(^{2+}\) concentrations. These results demonstrate that Fe\(^{2+}\), like other divalent cations, is capable of permeating the L-type Ca\(^{2+}\) channel and shifting gating properties of L-type Ca\(^{2+}\) channels.

Under more physiological conditions, Fe\(^{2+}\) might compete with Ca\(^{2+}\) within the permeation pathway for ion conduction, as has been observed with Ba\(^{2+}\), Na\(^{+}\), and other cations. Therefore, the interaction of Fe\(^{2+}\) on \(I_{Ca}\) with 2 mmol/L Ca\(^{2+}\) present in the extracellular solution was examined. There was no significant change in peak \(I_{Ca}\) with 250 μmol/L Fe\(^{2+}\) in the external solution (2±2%, n=5); however, 500 μmol/L Fe\(^{2+}\) potentiated \(I_{Ca}\) by 21±3% (n=5, P<0.01; Figure 3A and 3C). At higher concentrations, Fe\(^{2+}\) reduced the peak \(I_{Ca}\) in a dose-dependent manner, with an IC\(_{50}\) of 2.1 mmol/L (Figure 3A and 3C), which is remarkably similar to previous estimates of 2.7 mmol/L, determined in single-channel studies.

Concomitant with the reduction in peak \(I_{Ca}\), current decay was slowed in a dose-dependent manner (Figure 3B). To quantify the effects of Fe\(^{2+}\) on current inactivation, the nitrendipine-sensitive Fe\(^{2+}\) current in a dose-dependent manner, with an IC\(_{50}\) of 2.1 mmol/L (Figure 3A and 3C), which is remarkably similar to previous estimates of 2.7 mmol/L, determined in single-channel studies.

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inactivation were reversible when the Fe\(^{2+}\) concentration was changed from 500 \(\mu\)mol/L to 2 mmol/L and then back to 500 \(\mu\)mol/L (Figure 6). These results suggest that irreversible modification of the channel protein did not occur and therefore is not responsible for the observed effects on channel inactivation.

**Discussion**

The present study demonstrates that myocardium Fe\(^{2+}\) uptake is inhibited in electrically arrested nonbeating hearts and is...
modulated by agents that affect L-type Ca\(^{2+}\) channel activity. Iron uptake was inhibited, consistent with the hypothesis that Fe\(^{2+}\) uptake into heart can occur via L-type Ca\(^{2+}\) channels. However, in addition to blocking L-type Ca\(^{2+}\) channels, the high concentrations of nifedipine used in the studies (Figure 1) can also inhibit other ion channels\(^{47-49}\) that might contribute to \(^{59}\)Fe\(^{2+}\) uptake, although no previous studies have demonstrated that Fe\(^{2+}\) permeation occurs through these channels. On the other hand, the selective L-type Ca\(^{2+}\) channel agonist Bay K 8644 produced a 2.3-fold increase in the nifedipine-sensitive Fe\(^{2+}\) uptake by the heart. Assuming complete block of L-type Ca\(^{2+}\) current by nifedipine (see below), this Bay K 8644-dependent enhancement of Fe\(^{2+}\) uptake matches the 1.5- to 2-fold increase of current produced by these agents in previous voltage-clamp studies.\(^{35}\) By contrast, minimal uptake of Fe\(^{3+}\), which does not permeate L-type Ca\(^{2+}\) channels, occurred in these experiments. Overall, these studies suggest that NTBI (ie, Fe\(^{2+}\)) uptake into the heart can occur, although probably not exclusively (see below), via L-type Ca\(^{2+}\) channels.

Under our experimental conditions, the extent of Fe\(^{2+}\) blockade by Cd\(^{2+}\) was 2-fold greater than by 10 \(\mu\)mol/L nifedipine. The differences between these 2 blockers of L-type Ca\(^{2+}\) channels in our experiments may result at least in part from incomplete blockage of Ca\(^{2+}\) channels by nifedipine, because nifedipine binding to L-type channels is very voltage-dependent.\(^{55,50}\) On the other hand, Cd\(^{2+}\) can inhibit many membrane transporters in addition to Ca\(^{2+}\) channels; therefore, it seems likely that a second nifedipine-insensitive iron uptake process exists in the myocardium. One potential candidate transporter is the voltage-dependent divalent-cation transporter (DCT1), which was cloned from rat duodenum and appears to be expressed in heart.\(^{51}\) However, Cd\(^{2+}\) is actually transported by DCT1,\(^{51}\) which would not readily explain our observed effects of Cd\(^{2+}\) on Fe\(^{2+}\) uptake. Although both NTBI uptake observed in the present study and DCT1 Fe\(^{2+}\) uptake display similar voltage dependency, it is unknown whether high K\(^{+}\), nifedipine, Bay K 8644, or phosphorylation affect DCT1 transport properties. In contrast to DCT1, voltage-independent Fe\(^{2+}\) uptake has been measured in cultured neonatal rat cardiac myocytes.\(^{25}\) In these studies, 20 \(\mu\)mol/L nifedipine inhibited 20% of \(^{45}\)Ca\(^{2+}\) uptake in cultured myocytes.\(^{52}\) This modest effect of nifedipine is not entirely inconsistent with our results because previous studies have demonstrated that L-type Ca\(^{2+}\) channel densities and activity are much lower in cultured neonatal myocytes than adult heart cells.\(^{52}\) Clearly, additional studies are required to determine whether multiple pathways for NTBI (ie, Fe\(^{2+}\)) uptake exist in heart and to characterize their relative importance in iron loading.

Our ability to measure Fe\(^{2+}\) currents in the absence of external Ca\(^{2+}\) and the effects of Fe\(^{3+}\), on inactivation kinetics of L-type Ca\(^{2+}\) currents further support the hypothesis that Fe\(^{2+}\) permeates the L-type Ca\(^{2+}\) channel. This conclusion is consistent with previous studies demonstrating that Fe\(^{2+}\) and other transition metals (Zn\(^{2+}\), Co\(^{2+}\), and Mn\(^{2+}\)) can permeate L-type Ca\(^{2+}\) channels in addition to impeding Ca\(^{2+}\) flux.\(^{30,31,13}\) However, permeation of divalents like Fe\(^{3+}\) through L-type Ca\(^{2+}\) channels is too slow to be readily detected using electrical recordings\(^{30,34}\) under relevant pathophysiological conditions. Previously, it has been suggested that measurement of intracellular accumulation would provide better evidence for the flux of divalent cations, like Fe\(^{2+}\), through L-type Ca\(^{2+}\) channels.\(^{54}\) Initially, we attempted to use an optical fluorescence method in isolated cardiac trabeculae and single myocytes. However, these methods were also relatively insensitive partly because redox cycling of iron occurs after Fe\(^{2+}\) enters myocytes (see below), as demonstrated previously by the measurable Fe\(^{2+}\) labile pools in iron-loaded cardiac myocytes.\(^{19}\) Our radiosotope \(^{59}\)Fe\(^{2+}\) flux measurements in the whole hearts avoided these problems, thereby allowing estimation of the rate of iron accumulation in heart.

The nifedipine-sensitive component of iron uptake in our Langendorff experiments is remarkably similar to the 14 to 19 pmol \(\cdot\) min\(^{-1}\) \(\cdot\) g\(^{-1}\) dry wt of Fe\(^{2+}\) that is predicted from our electrophysiological studies and those of others\(^{20}\) to enter myocytes via L-type Ca\(^{2+}\) channels. This calculation, outlined below, requires that the relative flux of Fe\(^{3+}\) versus Ca\(^{2+}\) is proportional to the ratio of their maximum current densities (or binding rates) and that binding isotherms adequately describe the dependence of current on the permeant divalent ion concentrations as established previously.\(^{53}\) Since the Fe\(^{2+}\) current density in rat ventricular myocytes at 0 mV was 0.20 pA/pF with 15 mmol/L Fe\(^{2+}\) and the dissociation constant was 2.1 mmol/L (similar to the 2.7 mmol/L reported previously),\(^{53}\) the maximum current (\(I_{\text{MAX}}\)) for Fe\(^{2+}\) is estimated to be 0.23 pA/pF. The corresponding \(I_{\text{MAX}}\) for Ca\(^{2+}\) current density at 0 mV was 38 pA/pF using an estimated \(I_{\text{CM}}=14\) mmol/L.\(^{53}\) Thus, the ratio of maximum currents is estimated to be 6.7 \(\times\) 10\(^{-3}\), which matches closely the corresponding ratio of 7.6 \(\times\) 10\(^{-3}\) that is based on the second order binding rate constants (ie, 3.4 \(\times\) 10\(^8\) mol \(\cdot\) L\(^{-1}\) \(\cdot\) s\(^{-1}\) for Fe\(^{2+}\) versus 4.5 \(\times\) 10\(^8\) mol \(\cdot\) L\(^{-1}\) \(\cdot\) s\(^{-1}\) for Ca\(^{2+}\)).\(^{30}\) The net Ca\(^{2+}\) flux that enters a typical myocyte in each beat at 2 mmol/L [Ca\(^{2+}\)], is 16 pC.\(^{54}\) Therefore, the predicted maximum net Ca\(^{2+}\) flux is 66.4 \(\mu\)mol Ca\(^{2+}\) per minute per gram of dry weight, assuming there are 1 \(\times\) 10\(^5\) myocytes per heart, the dry wt–wet wt ratio is 0.2, and the heart rate is about 200 bpm. In contrast, the predicted flux of Fe\(^{2+}\) at a concentration of 100 mmol/L is 19.1 pmol \(\cdot\) min\(^{-1}\) \(\cdot\) g\(^{-1}\) dry wt. Corresponding estimates with the ratios of the second-order rate constants to estimate the Fe\(^{2+}\) flux predict an uptake rate through the L-type Ca\(^{2+}\) channels of 14.3 pmol \(\cdot\) min\(^{-1}\) \(\cdot\) g\(^{-1}\) dry wt.

The above estimates suggest that sufficient Fe\(^{2+}\) can enter through L-type Ca\(^{2+}\) channels to account for the nifedipine-sensitive Fe\(^{2+}\) accumulation observed in our whole heart experiments, provided that extrusion is relatively slow. Is this, in fact, likely to be the case? Previous studies have established that in conditions of iron overload, NTBI entry into cells bypasses the transferrin-based system and overwhelms the normal regulatory capacity of the cell for iron.\(^{7,16}\) Consequently, NTBI entering the cell is not bound to ferritin but becomes weakly bound as low-molecular-weight complexes and subsequently undergoes redox cycling of iron.\(^{55}\) This not only produces free radicals but also leads to the irreversible precipitation of iron in the form of hemosiderin.\(^{55}\) Indeed, it is known that very little, if any, of the NTBI that accumulates in cardiac myocytes under conditions similar to
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those in our experiments is transported back out of the cell unless the cell is treated with iron chelators, suggesting that Fe$^{2+}$ that enters the cell under these conditions is effectively trapped.

If L-type Ca$^{2+}$ channels contribute to iron uptake in heart, it is reasonable to ask whether this uptake pathway can account for the iron levels observed clinically. Patients with secondary hemochromatosis often have total serum iron levels of 20 to 61 μmol/L, with estimated NTBI of ≈1 to 20 μmol/L. Under these conditions, the amount of iron accumulation predicted to occur via the L-type Ca$^{2+}$ channels in 10 to 15 years, a relevant period for patients not receiving chelation therapy, was 3 to 5 mg of iron per gram of heart. This compares favorably with the 2 to 8 mg of iron per gram of heart typically observed in these patients.

The Fe$^{2+}$-mediated slowing of Ca$^{2+}$ current inactivation in our studies is analogous to the slowing of Ca$^{2+}$ current inactivation by Ba$^{2+}$ after Ca$^{2+}$ permeates the channel pore. Accordingly, this slowing could arise from competition between Fe$^{2+}$ and Ca$^{2+}$ for the C-terminal cytoplasmic Ca$^{2+}$ binding site involved in Ca$^{2+}$-mediated inactivation of L-type Ca$^{2+}$ channels. It is, nevertheless, also conceivable that these effects could result from Fe$^{2+}$ uptake via an independent yet unidentified transporter. But this seems unlikely because previous single-channel studies have established that the transporter would need to be localized in very close proximity to the Ca$^{2+}$ channel in order to explain our observations. Alternatively, slowed inactivation by Fe$^{2+}$ might also be due to irreversible oxidation of the channel as a result of free radical production or sulfhydryl oxidation as reported previously. However, the observed effects of Fe$^{2+}$ on current inactivation rapidly reversed following washout, which is inconsistent with an oxidation-based mechanism because reversal requires the application of free radical scavengers or sulfhydryl reducing agents.

The Fe$^{2+}$-induced slowing of Ca$^{2+}$ current inactivation could have a number of important consequences. For example, the slowing of current decay by 500 μmol/L Fe$^{2+}$ resulted in a 50% increase in the time integral of the Ca$^{2+}$ current and thus net Ca$^{2+}$ influx. This is expected to significantly increase intracellular Ca$^{2+}$ levels and possibly contribute to contractile dysfunction (Ca$^{2+}$ overload) or impaired diastolic function observed during the early stages of iron overload. On the other hand, slowed inactivation of L-type Ca$^{2+}$ currents would increase NTBI Fe$^{2+}$ entry into the myocytes, which may explain the upregulation of Fe$^{2+}$ uptake that was previously reported to occur in iron-loaded cardiac myocytes.

In summary, our data supports the hypothesis that NTBI uptake and iron accumulation by myocardium occurs via L-type Ca$^{2+}$ channels. Consistent with this assertion, we have recently observed a 2-fold reduction in myocardial iron content and mortality with L-type Ca$^{2+}$ channel blockers amlodipine and verapamil using an in vivo murine model of cardiac iron overload (G.Y.O. and P.H.B., unpublished data, 1998). Poor patient compliance with deferoxamine treatment and the lack of efficacy with the oral iron chelator, deferiprone, warrants the development of alternative strategies for the treatment of iron overload cardiomyopathies.

Our results suggest that inhibition of Fe$^{2+}$ uptake by Ca$^{2+}$ channel blockers might be useful for the clinical management of iron overload disorders. Finally, our observations could also be of significance in other tissues, such as pancreas and pituitary glands, which also possess high L-type Ca$^{2+}$ channel activity for hormone secretion, because iron overload is commonly associated with both diabetes and pituitary hormone dysfunction.

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Modulation of Iron Uptake in Heart by L-Type Ca\(^{2+}\) Channel Modifiers: Possible Implications in Iron Overload

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