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L-type calcium channel as a cardiac oxygen sensor

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Abstract

Acute oxygen sensing in the heart is thought to occur through redox regulation and phosphorylation of membrane channels. Here we report a novel O_2-sensing mechanism involving the C-terminus of the L-type Ca^{2+} channel and regulated by PKA phosphorylation. In patch-clamped myocytes, oxygen deprivation decreased I_{Ca} within 40 s. The suppressive effect of anoxia was relieved by PKA-mediated phosphorylation only when Ca^{2+} was the charge carrier, whereas phosphorylated I_{Ba} remained sensitive to O_2 withdrawal. Suppression of Ca^{2+} release by thapsigargin did not alter the response of I_{Ca} to anoxia, suggesting a mandatory role for Ca^{2+} influx and not Ca^{2+}-induced Ca^{2+} release (CICR) in O_2 regulation of the channel. Consistent with this idea, mutation of 80 amino acids in the Ca^{2+}/CaM-binding domain of the recombinant α_{1C} subunit that removes Ca^{2+} dependent inactivation (CDI) abolished O_2 sensitivity of the channel. Our findings suggest that the Ca^{2+}/CaM binding domain of the L-type Ca^{2+} may represent a molecular site for O_2 sensing of the heart.

Keywords

cardiac oxygen sensor; L-type calcium channel; C-terminus; PKA phosphorylation

Introduction

Voltage-gated calcium channels are widely expressed in the animal kingdom and in particular in the mammalian heart, brain, and muscle. All members of this family have a high selectivity for transport of calcium over other mono- or divalent cations. Although different types of voltage-gated Ca^{2+} channels have specific differences in their gating, voltage dependence, kinetics of activation and inactivation, pharmacology, and regulation by permeating Ca^{2+} and phosphorylation, they all transport Ca^{2+} efficiently, can be blocked by Ni^{2+}, Cd^{2+}, and La^{3+}, and share many overlapping properties. It is unclear as yet the evolutionary need for the development of this large family of voltage-gated Ca^{2+} channels, especially since Ca^{2+} serves mostly as a messenger signaling molecule. There are numerous reports that some members of this family, in addition to having a Ca^{2-}transporting function, may directly gate the release of Ca^{2+} from the ryanodine receptors of sarcoplasmic reticulum (SR) in skeletal muscle,[1] modulate CICR in cardiac muscle via specific domains of the C-terminus tail of the L-type Ca^{2+} channel,[2] or directly signal the nuclear transcription factors.[3]

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Conflicts of interest
The authors declare no conflicts of interest.

The energy required by the heart to carry out its enormous workload, for some 2 billion beats over a lifetime, requires critical regulation of the ATP and O_2 supply to the heart, and the rapid detection of their availability. Is there such an O_2 sensing mechanism in the heart? If so, what is the sensor and how does it detect O_2? In this report we probed the possible molecular site(s) for sensing of O_2 in the heart. Our experiments suggest that a motif closely associated with CaM-binding domains of the C-terminal tail of L-type Ca^{2+} channel may serve as the oxygen sensor of the heart.

Methods

All experiments were carried out on freshly isolated and enzymatically dispersed ventricular myocytes. Anoxic solutions (O_2 replaced by N_2) were applied rapidly (<50 ms) to an experimental whole-cell voltage-clamped myocyte, and I_{ca} was activated at 0–10 mV from a holding potential of −50 or −60 mV, chosen so as to fully inactivate I_{Na}. To prevent secondary effects arising from prolonged hypoxia that include mitochondrial dysfunction and generation of ROS, the experimental cell was exposed to anoxic solution for no longer than 2 min. Po_2 values of 5% were measured within 5–10 s of exposure of myocyte to the N_2 equilibrated anoxic solutions. All measurements were bracketed such that each cell served as its control and Student's *t*-test analysis was used to compare means.

Results

Figure 1 shows the direct effects of anoxic solutions on I_{Ca} measured either in ventricular myocytes or in HEK293 cells expressing the recombinant L-type Ca^{2+} channel. I_{Ca} started to decrease within the first 5 s of exposure to anoxic solutions (26 ± 3% in cardiomyocytes and 35 ± 6% in HEK cells within the first 50 s). I_{ca} was equally suppressed at all voltages tested. To exclude the effects of Ca^{2+} channel run-down, the suppression of I_{Ca} only in the first 50 s was quantified for the statistical analysis.

PKA phosphorylation prevents anoxic I_{Ca} suppression

PKA phosphorylation is well known to increase I_{Ca} amplitude and speed its inactivation rate.

Figure 2 compares the suppressive effects of anoxia on I_{Ca} in unphosphorylated and phosphorylated channels. Channel phosphorylation significantly relieved the suppressive effects of anoxia on I_{Ca} (average suppression decreases to 9 ± 2% from 27 ± 5% in unphosphorylated channels). In order to determine whether channel resistance to the suppressive effects of anoxia is specifically due to protein kinase A (PKA) phosphorylation, we included 10 μM of H-89, a specific PKA inhibitor in the patch pipette, and reexamined the degree of anoxic suppression in the presence of 200 μM cAMP. In a manner similar to that of unphosphorylated myocytes, H-89 exposure reversed the protective effects of PKA phosphorylation and decreased I_{ca} by 26 ± 5% ($N = 4$, $P = 0.002$) in response to anoxia. Since it has already been shown that phosphorylation of serine residues by PKA is enhanced under ischemic conditions,[4] it is likely that anoxia enhances PKA activity and in the presence of 200 μM cAMP leads to increased conductance of the channel, opposing the suppressive effects of oxygen removal.

Role of calcium svignaling in the anoxic response

Phosphorylation by PKA is known to affect Ca^{2+} channel gating directly by phosphorylating serine 1928 residue on the C-terminus and indirectly by enhancing Ca^{2+} release pools by phosphorylating phospholamban (PLB) associated with the Ca-ATPase of the SR.[5,6] To examine whether PKA-conferred resistance to anoxia was related to the increased Ca^{2+}

release, cAMP-dialyzed cells were incubated with 1 μM thapsigargin to deplete the SR calcium stores. Thapsigargin incubation did not modify the response of phosphorylated I_{Ca} to anoxic solutions, but did increase the current amplitude and delayed the fast component of its inactivation by an average of 10 ms (Fig. 3).

Thus, it appears that direct effects on the channel and not CICR are the critical steps in relieving the suppressive effect of anoxia on the phosphorylated channel. Furthermore, the protective effect of PKA phosphorylation against anoxia appears to be strongly mediated by Ca^{2+} influx through the channel. This assertion is supported by the finding that I_{Ba} is not only more sensitive to anoxic suppression, but is also insensitive to PKA-mediated protection, suggesting that both phosphorylation and the nature of permeating cation through the channel are critical in the O_2-sensing mechanism. In phosphorylated channels, the anoxia-induced suppression of I_{Ba} occurred within the first 5 s of exposure to oxygen removal, reaching a maximum value of 38% within 40 s. Under the same conditions and time frame, phosphorylated Ca^{2+} currents were suppressed by only 6% (Fig. 3). Given that the influx of Ba^{2+} through the channel fails to gate Ca^{2+} release from the ryanodine receptors, these results suggest that the observed effects of PKA are directly linked to molecular interactions between the channel and the permeating Ca^{2+}.

Where on the channel is oxygen sensed?

The rapid (~5 s) suppressive effects of O_2 removal on I_{ca}, the enhancement of this inhibitory effect when Ba^{2+} is transported through the channel, and the relief of this suppressive effect only when the phosphorylated channel is transporting Ca^{2+} suggests that a domain of the channel closely associated with permeating Ca^{2+} may contain the O_2 sensor motif. To further identify the site of the sensor, we probed the role of the CaM-binding domain of the C-terminus in O_2 sensing. Taking into account that CaM is unable to fully associate with the channel in presence of Ba^{2+}, we hypothesized that sensitivity of I_{Ba} to anoxia may result from loss of channel regulation by CaM, which results in exposure of possible O_2-sensing residues within the CaM-binding region. Thus, mutation of this region should eliminate the anoxic response of the channel even with Ba^{2+} as the charge carrier. Because the exact C-terminal site involved in CaM-mediated regulation is a matter of debate and the proposed binding region ranges from 75 residues (1572–1647) down to the 11 residue of the putative IQ motif,[7–10] we examined the effect of anoxia on the α_{1c86} channel, which has an alternative splice variance in exon 40–41 (residues 1572–1651) within the C-tail. The 80 amino acid mutation of α_{1c86} channel incorporates all the Ca^{2+}/CaM-binding sites (LA, IQ, and K motifs) and lacks Ca^{2+}-dependent inactivation.[10,11] When we measured I_{Ba} under anoxia in control (α_{1c77}) and the mutant (α_{1c86}) channels, we found that I_{Ba} through the α_{1c86} channel was totally insensitive to deprivation of O_2 in sharp contrast to 38% suppression of I_{Ba} through the α_{1c77} channel (Fig. 4). This finding supports the idea that the differential anoxic responses seen between Ca^{2+} and Ba^{2+} are due to association of Ca^{2+}/CaM with the C-terminus and that oxygen-sensing ability of the channel is masked by local Ca^{2+}/CaM-dependent regulation.

Discussion

In this report we have demonstrated a novel oxygen-sensing property of the cardiac Ca^{2+} channel that is regulated by PKA phosphorylation and involves a sequence of amino acids within the Ca^{2+}-binding region of the C-terminus. Similar to previous reports[12,13] our findings also indicate that cardiac I_{Ca} is sensitive to extracellular O_2 levels. The sensitivity of I_{Ca} to oxygen deprivation has been attributed to various mechanisms involving redox modification of specific channel residues,[14] mitochondrial ROS-mediated regulation,[15] regulation by specific isoforms of PKC,[13,17] and involvement of 36 amino acids in the C-terminus region of the $alpha_{1c}$ subunit of the channel.[16] Aside from the latter study, most

proposed mechanisms involve indirect modulation of the channel by intermediary molecules. In this report, however, we have made several new observations that further support direct-channel O_2-sensing mechanisms. To directly study channel effects, we studied I_{ca} modulation within 5–50 s of O_2 deprivation to avoid activation of secondary pathways. Using this paradigm, we observed that the anoxic response of I_{ca} occurs as early as 5 s of O_2 deprivation and maximizes within 40 s. Furthermore, the channel sensitivity to anoxia was highly regulated by PKA phosphorylation such that intracellular addition of 200 μM cAMP completely masked O_2 sensing of the channel, while addition of PKA inhibitor H-89 to 200 μM cAMP-dialyzed cells restored the channel sensitivity to anoxia. The observed PKA-induced anoxic resistance was strongly dependent on Ca^{2+} entry through the channel, such that anoxic suppression of I_{Ba} was insensitive to PKA-mediated protection. Treatment of myocytes with thapsigargin further revealed that Ca^{2+} entry–dependent inactivation and not calcium release from the SR, CICR, mediates the anoxia-produced resistance with PKA activation. This observation revealed a direct link between Ca^{2+}-dependent inactivation and the channel's O_2-sensing ability. Ca^{2+}-dependent inactivation involves 75 amino acids (residues 1572–1647) spanning the CaM association region of the C-terminus, and indeed we have shown that mutation of these residues abolishes I_{Ba} sensitivity to anoxia.

This observation along with that of Fearon and colleagues,[16] involving different residues 1787–1822, of calcium channel, indicates that the C-tail of the cardiac Ca^{2+} channel is directly involved in oxygen sensing of the myocardium during the first few seconds of oxygen deprivation. In addition, the criticality of Ca^{2+}-dependent regulation and phosphorylation by PKA in mediating Ca^{2+} channel O_2-sensing capability under acute anoxia may provide some insight into the pathology of ischemic insult. Considering the complexity of the oxidative process and the involvement of numerous signaling pathways, identification of a direct O_2-sensing site on the cardiac Ca^{2+} channel is an important step toward understanding the pathobiology of oxygen deprivation.

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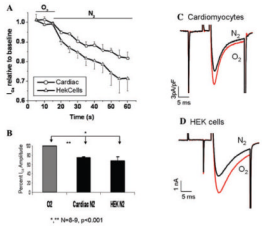

Figure 1.

Acute anoxia suppresses I_{Ca} in cardiomyocytes and HEK293 cells expressing recombinant L-type Ca^{2+} channel. (A) Time course of I_{ca} suppression in response to extracellular O_2 deprivation in cardiac and HEK cells. (B) Quantitative representation of I_{ca} suppression in response to acute extracellular anoxia. (C–D) Representative traces of I_{Ca} in cardiac (C) and HEK cells (D) before and within 40 s of extracellular O_2 deprivation.

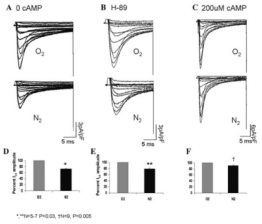

Figure 2.

PKA phosphorylation protects against anoxic suppression of I_{Ca}. (A–C) Representative *I–V* traces (−60 to 80 mV) of cardiac Ca^{2+} current before and after removal of extracellular O_2 in myocytes dialyzed with 0 cAMP (A), 10 μM H-89 + 200 μM cAMP (B) and 200 μM cAMP internal solutions (C). (D–F) Cumulative representation of anoxic I_{ca} suppression under conditions shown in A–C.

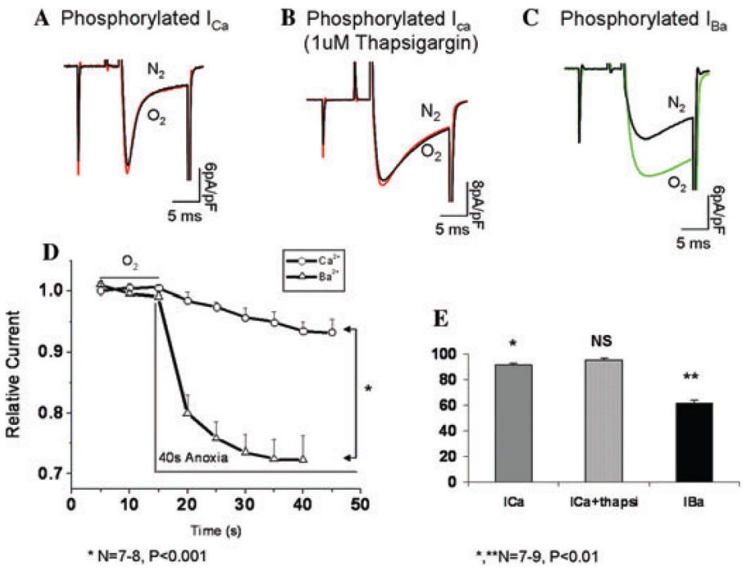

Figure 3.

Channel phosphorylation does not protect against the effects of O_2 deprivation when Ba^{2+} is the charge carrier through the channel. (A–C) Representative traces of phosphorylated I_{Ca} (A), phosphorylated I_{Ca} in presence of 1 uM thapsigargin, and phosphorylated I_{Ba} before and after oxygen deprivation in left ventricular myocytes. (D) Time course of current suppression in response to anoxia with Ca^{2+} and Ba^{2+} as charge carriers. (E) Quantitative representation of A–C (N = 7–9).

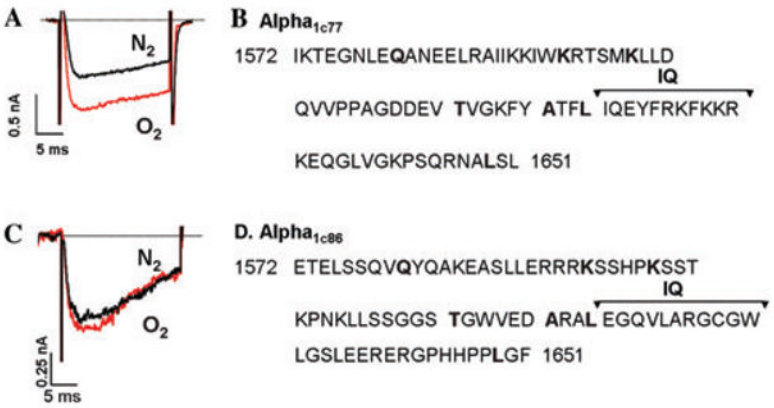

Figure 4.

Mutation of 80 amino acids in the Ca^{2+}/CaM binding domain of the C-terminus inhibits the effect of anoxia on Ba^{2+}-carried currents through the channel. (A) Representative I_{Ba} trace in a HEK cell expressing the $alpha_{1C77}$ subunit of the L-type channel before and after removal of extracellular oxygen. (B) Amino acid sequence 1572–1651 of native L-type channel C-terminus. (C) Representative trace showing I_{Ba} response to anoxia in a HEK cell expressing the mutant $alpha_{1c86}$ subunit. (D) Amino acid sequence 1572–1651 in the mutant 86 channel C-tail.