Splice Variants Reveal the Region Involved in Oxygen Sensing by Recombinant Human L-Type Ca\(^{2+}\) Channels

Ian M. Fearon, Gyula Varadi, Sheryl Koch, Idit Isaacsohn, Stephen G. Ball, Chris Peers

Regulation of vascular smooth muscle Ca\(^{2+}\) channels by oxygen tension contributes importantly to hypoxic vasodilatation. We previously described the inhibitory effects of hypoxia on the recombinant human cardiac L-type Ca\(^{2+}\) channel \(\alpha_{1C}\) subunit (hHT isoform) expressed in HEK 293 cells. We now demonstrate that hypoxia inhibits only one of the three naturally occurring splice variants of this channel that differ only in the C-terminal domain, permitting identification of a 71-amino acid insert in the C-terminal region of the channel that confers oxygen sensitivity. Selective restriction of the spliced insert allowed determination of a 39-amino acid region essential for oxygen sensing. This represents the first identification of the structural region of an ion channel required for sensing changes in oxygen tension.

Regulation of ion channels by oxygen tension was first observed in chemoreceptive type I cells of the carotid body, where K\(^+\) channels were inhibited by hypoxia.\(^1\) Similar inhibitory effects of hypoxia on K\(^+\) channels have since been demonstrated in a variety of cells.\(^2\) Native L-type Ca\(^{2+}\) channels in carotid body type I and vascular smooth muscle cells are also regulated by hypoxia.\(^3,4,6\) We have previously shown that hypoxia reversibly inhibits the recombinant human cardiac L-type Ca\(^{2+}\) channel \(\alpha_{1C}\) subunit when expressed in human embryonic kidney (HEK 293) cells,\(^7\) indicating that auxiliary subunits are not required for oxygen sensing.

Several studies have examined the mechanism(s) involved in oxygen sensing by ion channels,\(^2\) and candidate mechanisms include redox modulation or sensing by membrane-bound, heme-containing structures. However, no studies have examined the structural requirements for oxygen sensing by ion channels. In the present study, we studied the oxygen sensitivity of the three splice variants of the human L-type (\(\alpha_{1C}\)) Ca\(^{2+}\) channel.\(^8\) Only one, possessing a 71-amino acid C-terminal insert, was oxygen-sensitive. Mutation of the splice insert allowed further determination of the region of the L-type Ca\(^{2+}\) channel responsible for oxygen sensing.

Materials and Methods
Experiments were conducted in HEK 293 cells, cultured as previously described\(^3\) and transiently expressing the required L-type Ca\(^{2+}\) channel construct. Production of constructs and transient transfection methods are detailed in the online-only data supplement (available at http://www.circresaha.org).

Coverslip fragments with attached cells were continually perfused (4 mL/min, bath volume 80 \(\mu\)L), and whole-cell patch-clamp recordings\(^9\) were made using pipettes of resistance 4 to 7 M\(\Omega\). Per fusate composition was as follows (in mmol/L): NaCl 95, CsCl 5, MgCl\(_2\) 1.2, BaCl\(_2\) 20, HEPES 5, d-glucose 10, and TEA-Cl 20 (pH 7.4). Pipette solution composition was as follows (in mmol/L): CsCl 120, TEA-Cl 20, MgCl\(_2\) 2, EGTA 10, HEPES 10, and ATP 2 (pH 7.2).

Cells were clamped at \(-80\) mV, and whole-cell currents were evoked by 100-ms step depolarizations to various test potentials (0.1 Hz). Series resistance compensation of 70% to 90% was applied. Currents were filtered at 5 kHz and digitized at 10 kHz. Capacitative transients were minimized by analogue means, and corrections for leak current were made by the scaling and subtraction of the average leak current evoked by small hyperpolarizing and depolarizing steps (\(\leq \pm 20\) mV). Analysis and voltage protocols were performed with the use of an Axopatch 200A amplifier/Digidata 1200 interface (Clampex software, pCLAMP 6.0.3, Axon Instruments Inc). Results are expressed as mean±SEM, and statistical comparisons were made using paired or unpaired Student’s \(t\) tests, as appropriate.

Bath hypoxia was achieved by bubbling the reservoir leading to the bath with 100% \(N_2\). The level of hypoxia was measured as previously described.\(^10\) The time course of the fall in \(P_{O_2}\) in the recording chamber was highly reproducible and was always stable within 30 to 60 seconds of switching solution.

Results
We first compared the effects of hypoxia on the three splice variants (hHT, rHT, and fHT) of the human L-type Ca\(^{2+}\) channel.\(^8\) In cells expressing the hHT splice variant, hypoxia (\(P_{O_2}\) 20 mm Hg) caused rapid and reversible reductions in Ba\(^{2+}\) current amplitude (Figure 1A; mean degree of inhibition 22.6±1.5%, \(n=6\)). By contrast, in cells expressing the rHT splice variant (\(n=22\); eg, Figure 1B) or the fHT splice variant (\(n=8\); Figure 1C), the same degree of hypoxia caused no reduction in Ca\(^{2+}\) channel activity in any cell examined.

The hHT splice variant contains a 71-amino acid insert in the C-terminal domain of the channel that is absent in the rHT clone,\(^8\) and the above data suggest that this region is responsible for oxygen sensing by this channel. Furthermore, when this insert was removed from the hHT clone (creating an hHT(–) clone), the channel was rendered oxygen-insensitive (\(n=10\); Figure 2A). When the insert cleaved from the hHT clone was inserted into the rHT clone (creating an rHT(+) clone), this clone became oxygen-sensitive (\(n=7\); Figure 2B). The mean degree of inhibition in cells expressing the rHT(+) clone was 23.3±2.4%, a value not significantly different from that seen in cells expressing the hHT splice variant (22.6±1.5%, \(n=6\); \(P=0.82\), unpaired Student’s \(t\) test). Thus, the 71-amino acid insert is responsible for oxygen sensing by this channel.

Given the differential oxygen sensitivity of the hHT and rHT splice variants, studies were carried out to further elucidate the region of the channel involved in oxygen

Received August 2, 2000; revision received August 22, 2000; accepted August 22, 2000.
From the Institute for Cardiovascular Research (I.M.F., S.G.B., C.P.), The University of Leeds, Leeds, UK; Institute of Molecular Pharmacology and Biophysics (I.L., S.K., G.V.), University of Cincinnati College of Medicine, Cincinnati, Ohio.
Correspondence to Dr Ian Fearon, Institute for Cardiovascular Research, The University of Leeds, Leeds LS2 9JT, UK. E-mail cvsinf@leeds.ac.uk.
(Circ Res. 2000;87:537–539.)
© 2000 American Heart Association, Inc.

Circulation Research is available at http://www.circresaha.org
sensing. As demonstrated in Figure 2C, when the distal 24 amino acids of the spliced insert were removed (creating an hHTΔ24 clone), the channel retained its oxygen sensitivity. The mean degree of inhibition at a PO₂ of 20 mm Hg in cells expressing this clone was 28.0±4.9% (n=9), a value not significantly different to that seen in cells expressing the hHT α₁C subunit present in the isoform examined.

**Discussion**

In humans, the L-type (α₁C) Ca²⁺ channel exists as several different isoforms.⁸¹¹ Of these, the hHT variant contains a 71-amino acid insert at position 1786. This is not present in the rHT isoform, although the proteins are otherwise identical. Our data demonstrate that Ba²⁺ currents through the hHT isoform were oxygen-sensitive, whereas those through the rHT variant were oxygen-insensitive. The fHT variant contains a different splice region starting at position 1786, and we found that this also generated oxygen-insensitive currents. Therefore, the spliced insert of hHT is essential for oxygen sensing. To confirm this finding, the spliced insert was removed from the hHT clone, eliminating the channel’s sensitivity to hypoxia. Moreover, when this insert was inserted into the rHT clone, the resulting channel became oxygen-sensitive.

To further elucidate the region involved in oxygen sensing by the Ca²⁺ channel, two mutants in which either the proximal or distal portion of the hHT spliced insert was removed were created. These studies showed that removal of 24 amino acids at the distal end of the insert (amino acids 1832 to 1856) had no effect on hypoxic sensitivity. In contrast, removal of the proximal 39 amino acids of the insert (amino acids 1784 to 1823) resulted in the channel becoming oxygen-insensitive. These data identify a 39-amino acid region in the C-terminal critical to oxygen sensing. The mechanism of this property remains to be elucidated and warrants further investigation into the mechanism whereby this small region of the Ca²⁺ channel plays a role in the physiological response to hypoxia.

**Acknowledgment**

This work was supported by the British Heart Foundation and National Institutes of Health Grant HL 22619-22.
References


Key Words: L-type Ca²⁺ channel ■ α₁C subunit ■ hypoxia ■ inhibition
Splice Variants Reveal the Region Involved in Oxygen Sensing by Recombinant Human L-Type Ca²⁺ Channels

Ian M. Fearon, Gyula Varadi, Sheryl Koch, Idit Isaacsohn, Stephen G. Ball and Chris Peers

_Circ Res._ 2000;87:537-539
doi: 10.1161/01.RES.87.7.537

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/87/7/537

Data Supplement (unedited) at:
http://circres.ahajournals.org/content-suppl/2000/09/22/87.7.537.DC1

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the _Permissions and Rights Question and Answer_ document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/
Splice variants reveal the region involved in oxygen sensing
by recombinant human L-Type Ca\(^{2+}\) channels

Online Data Supplement

Ian M. Fearon\(^1\), Idit Isaacsohn\(^2\), Sheryl Koch\(^2\), Gyula Varadi\(^2\),
Stephen G. Ball\(^1\) & Chris Peers\(^1\)

\(^1\)Institute for Cardiovascular Research, The University of Leeds, Leeds LS2 9JT, U.K. &
\(^2\)Institute of Molecular Pharmacology and Biophysics, University of Cincinnati College of
Medicine, Cincinnati, Ohio 45267-0828, U.S.A.

Correspondence: Dr. Ian Fearon
Institute for Cardiovascular Research
The University of Leeds
Leeds LS2 9JT, U.K.
Tel: (0113) 233 4821 Fax: (0113) 233 4803 E-mail: cvsimf@leeds.ac.uk

Short title: Oxygen sensing region of recombinant Ca\(^{2+}\) channels
Subject codes: 84, 94, 152
Acknowledgment: Supported by the British Heart Foundation and by an NIH Grant (HL 22619-22)
MATERIALS AND METHODS

Transient transfection of HEK 293 cells

The day before transfection, wild-type HEK 293 cells were split by trypsinisation and plated out in a 75 cm$^2$ flask such that they were 75% confluent on the day of transfection. In experiments using either the pAGS3-hHT, pAGS3-rHT or pAGS3-fHT constructs, cells were co-transfected with the pHOOK-1 vector (using a 3:1 ratio) to allow cells to be selected following transfection (see below). All other constructs were sub-cloned into the pHOOK-2 expression vector and transfected alone. 10 µg of the required plasmid DNA (concentration 0.8 – 1.7 µg/µl) was diluted in 300 µl of serum-free Minimal Essential Medium (Gibco BRL, Paisley, Scotland). To this solution, 50 ml of Superfect (Qiagen) lipid reagent was added. After allowing 10 min for the formation of DNA:lipid complexes, the volume of the transfection solution was brought up to 6 ml with serum-containing medium and placed onto the cells which had been previously washed with PBS. Cells were incubated for 3 h at 37°C in a humidified environment of 95% O$_2$/5% CO$_2$, after which time the transfection solution was removed and replaced with fresh medium following wash with PBS. The day after transfection, successfully transfected cells were selected by magnetic affinity cell sorting$^1$ and plated onto glass coverslips for use in electrophysiological studies 48-72 h following transfection.

Construction of mutant Ca$^{2+}$ channels

Swapping C-tail segments between hHT and rHT cDNA clones

Human heart α$_{IC}$ full-length cDNA clones have been described and deposited in Genbank (accession numbers are L04569, L29534, and L29536 for hHT, fHT and rHT,
respectively). The genomic sequences for the C-tail alternatively spliced region are also deposited under accession numbers L29530-L29539. The nucleotide numbering in this study is based on the hHT sequence (accession number: L04569), however, the 1422 base pair 5'-untranslated region was removed for clarity. Thus, the A of the ATG initiator codon received the number +1 and the subsequent nucleotides of the open reading frame are numbered accordingly. The hHT clone has a 213 base pair (71 amino acid) insertion at amino acid position 1786, otherwise the cDNA is a human homolog of the rabbit heart clone. The rHT clone is a perfect human homolog of that cloned by Mikami et al from rabbit heart. The fHT clone contains a structurally dissimilar 105 base pair (35 amino acid) cDNA insertion starting at amino acid position 1786, coded for by a separate alternative exon to the hHT insertion. Gene expression studies, monitored by quantitative PCR, have shown that the rHT variant is more prevalent in human heart while in aortic tissue, the calcium channel population is composed of 40% of the rHT and 60% of the hHT mRNA species, and only a very small amount of fHT is expressed. The fHT isoform is predominantly expressed in fibroblasts, though only provides around 10% of total fibroblast Ca²⁺ channel mRNA, with the rest of the fibroblast mRNA species composed of equal amounts of hHT and rHT.

HHT and rHT cDNAs were truncated both at 5'- and 3'-ends in order to remove most of the non-translated sequences but the Kozak's consensus sequence was completely saved in both clones. The truncated versions were inserted into pBluescript KS(-) between the HindIII and XbaI sites. Plasmid DNAs from both cDNAs were grown in SCS110 cells (dam-, dcm-) and cleaved with BclI and SalI, and the appropriate 1.8 and 2.0 kb fragments were isolated from rHT and hHT, respectively. Then the C-tail cassettes were exchanged between the two types of constructs, thus creating the hHT(-) clone in which the 213 base pair C-tail
insert is removed, and the rHT(+) into which the 213 base pair segment is added. The presence and absence of insert was verified in both clones by sequencing. Finally, the full-length cDNAs were liberated by cleavage with HindIII and XbaI and ligated into the corresponding sites of pHOOK-2 (Invitrogen).

Deletion of the Distal Segment (between amino acids 1832 to 1856) of the 71 aa Insertion at the C-tail

This deletion was performed by inserting an NdeI site at nucleotide 5558, cleaving with MscI (nucleotide 5496) and NdeI, followed by Mung Bean nuclease treatment and religation. In the first step, the NdeI site was introduced by the MegaPrimer PCR strategy. A forward primer: 5'- GGC TGC ACA GCC CCC CCA TAT GCC AGG TGC CAC TCC-3' (carried the NdeI site underlined) was designed between nucleotides 5545-5580, and a reverse primer: 5'- GGA CTC GAC CCC CTC AGG-3' (nucleotide 6063-6046) was utilized in the first amplification step. The resulting product (518 base pairs) was isolated and utilized in the second PCR reaction as a reverse primer. The forward primer was: 5'- GTC TTC TAC TTC ATC AGC-3' located between nucleotides 4270-4287. The resulting product (1.8 base pairs) was gel-isolated and cloned into the Eco RV site of pBluescript KS(-). This plasmid DNA (30 µg) was digested with NdeI and treated with 300 units of Mung Bean nuclease (Stratagene) for 15-30 min in a buffer supplied by the manufacturer. The reaction mixture was then phenol/chloroform extracted and an additional cleavage was performed with MscI. A 4.8 kb DNA band was gel-isolated and religated. Clones resulted from this ligation step were sequenced across the junction region to identify clones that contain the desired deletion and an in-frame religation. Finally, clones that fulfilled the above criteria were digested with BclI and AvrII, the 1.7 kb fragment isolated and used to replace the homologous segment in
hHT-1. The full-length cDNA was liberated by cleavage with HindIII and XbaI and ligated into the corresponding sites of pHOOK-2.

*Deletion of the Proximal Segment (between amino acids 1784 to 1823) of the 71 aa Insertion at the C-tail*

The deletion of this segment was performed by introducing two *NruI* sites flanking the desired stretch, then cutting with *NruI* and religating the DNA. Introducing *NruI* sites was achieved by the MegaPrimer PCR strategy. First, a PCR amplification was done by using a forward primer: 5'GCG TGG AAG CTC AGC **TCG CGA** AGG ATG CAC TGC TGT G-3' (between nucleotides 5335-5370, the mutated region that carries the *NruI* site is underlined) and a reverse primer: 5'-GGA CTC GAC CCC CTC AAG-3' (nucleotides 6063-6046). The 728 base pair PCR product was gel-isolated and utilized as reverse primer in the second PCR reaction together with a forward primer: 5'-GTC TTC TAC TTC ATC AGC-3' (nucleotides 4270-4287). The resulting 1.8 kb amplification product was subcloned and the presence of mutation was verified by sequencing. The latter clone was used as a template in the second set of PCR reactions to introduce an additional *NruI* site. The forward primer was: 5'-GGT TCC CTG GCC GGG **TCG CGA** GAG GAC ACA CCC TGC-3' (between nucleotides 5448-5484, the mutated region that introduces an *NruI* site at nucleotide 5466 is underlined), the reverse primer was: 5'-GGC CTC GAC CCC CTC AAG-3' (nucleotides 6063-6046). Similarly to the procedure manipulating in the first *NruI* site, the 614 base pair PCR product was isolated and used as a reverse primer in combination with the 4270-4287 forward primer. The 1.8 kb PCR product was subcloned and the presence of both *NruI* sites was confirmed by sequencing. Then the DNA fragment isolated and religated. DNAs from positive clones that carried the deletion between the two *NruI* sites was cleaved with BclII and AvrII, the 1.7 kb segment isolated and used for replacing the analogous stretch in hHT-1. The full-length
cDNA was liberated by cleavage with *Hind*III and *Xba*I and ligated into the corresponding sites of pHOOK-2.

**RESULTS AND DISCUSSION**

Previous studies of native L-type Ca$^{2+}$ channels in cells isolated from both the systemic and pulmonary vasculature have demonstrated that the inhibitory effect of hypoxia is strongly voltage-dependent.$^5$ Moreover, our own studies on the recombinant $\alpha_{1C}$ subunit (hHT isoform)$^6$ also showed that the effects of hypoxia were voltage-dependent. An example of this effect is shown in Figure 2A of this Online Data Supplement; inhibition was greatest at test potentials up to and including the peak of the I-V relationship, and was reduced or even absent at the higher activating test potentials. It is possible, therefore, that when examining the effects of hypoxia in time-series experiments where cells are step depolarised to a single test potential, any effect of hypoxia could be masked by altered voltage dependence. To examine this possibility, current-voltage (I-V) relationships were constructed in cells expressing either the rHT or fHT isoforms of the $\alpha_{1C}$ subunit. Representative I-V relationships are shown in Figure 2A, and these demonstrate that hypoxic inhibition could not be seen at any test potential examined. Therefore, the lack of observation of an effect of hypoxia in the time-series experiments cannot be explained by an altered voltage-dependence.

I-V relationships were also constructed in cells expressing either the hHT(-), RHT(+), hHTΔ24 or hHTΔ39 clones (Figure 2B). Our time-series experiments had demonstrated that hypoxia was without effect in cells expressing either the hHT(-) or the hHTΔ39 construct. The I-V relationships taken from cells expressing either of these clones demonstrate that hypoxia was without effect on Ba$^{2+}$ currents at any test potential examined. Therefore, the lack of observation of a hypoxic response was not due to any altered voltage-dependency of
the effects of hypoxia. The I-V relationships in Figure 2B also demonstrate that the effects of hypoxia in two mutant clones created for these studies, namely the rHT(+) and hHTΔ24 clones, also showed strong voltage-dependency, with the effects of hypoxia greatest at the lower activating test potentials. Therefore as well as retaining oxygen sensitivity, the Ca^{2+} channels resulting from expression of these mutant clones also retain other biophysical properties of the response to a hypoxic stimulus.

The rHT isoform is the predominant isoform in human heart, although the hHT isoform is also expressed in cardiac cells to a high degree. No studies to date have examined the effects of hypoxia directly on cardiac Ca^{2+} channels, and it would therefore be of interest to examine the effects of hypoxia on native cardiac Ca^{2+} channels. It is also possible that the differing expression of human Ca^{2+} channel isoforms in different areas of the heart may also lead to a variable effect of hypoxia on native cardiac Ca^{2+} channels. Similarly, differing effects of hypoxia have been demonstrated on native Ca^{2+} channels in smooth muscle cells isolated from various regions of both the pulmonary and the systemic circulation. It would also be of interest therefore to examine both the relative abundance of Ca^{2+} channel α_{1C} subunit isoforms in vascular myocytes from different vessels, and also whether differing levels of expression of these isoforms plays a role in the differing effects of hypoxia on Ca^{2+} currents in cells isolated from different regions of the cardiovascular system.
REFERENCES


FIGURE LEGENDS

Figure 1. Proposed structure of the human L-type Ca$^{2+}$ channel $\alpha_{1C}$ subunit. Cylinders represent putative $\alpha$-helical segments. Bold lines represent the polypeptide chains of each subunit, with length approximately proportional to the number of amino acid residues. The location and relative length of the C-terminal spliced inserts for the three naturally-occurring splice variants (hHT, rHT and fHT) is shown in the box. Numbers represent amino acid numbers.

Figure 2. Examining the voltage-dependency of the effects of hypoxia on naturally-occurring and mutant $\alpha_{1C}$ subunits. (A) Representative current-voltage (I-V) relationships constructed in HEK 293 cells transiently expressing either the hHT (left), rHT (centre) or fHT (right) naturally-occurring isoform of the human L-type Ca$^{2+}$ channel $\alpha_{1C}$ subunit. Relationships were constructed under normoxic (•) and hypoxic (○; Po$_2$, 20 mmHg) conditions. Currents were evoked by step depolarising cells (100 ms, 0.1 Hz) to the appropriate test potential from a holding potential of -80 mV. (B) as in (A) except I-V relationships were constructed in cells expressing mutant $\alpha_{1C}$ subunits, as indicated above each relationship. All I-V relationships are representative of those obtained from between 5 and 7 cells for each construct.