A Novel Long N-terminal Isoform of Human L-type Ca$^{2+}$ Channel Is Up-regulated by Protein Kinase C*

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Human L-type voltage-dependent Ca$^{2+}$ channels ($\alpha_{1C}$, or Ca$_{1.2}$) are up-regulated by protein kinase C (PKC) in native tissues, but in heterologous systems this modulation is absent. In rat and rabbit, $\alpha_{1C}$ has two N-terminal (NT) isoforms, long and short, with variable initial segments of 46 and 16 amino acids, respectively. The initial 46 amino acids of the long-NT $\alpha_{1C}$ are crucial for PKC regulation. However, only a short-NT human $\alpha_{1C}$ is known. We assumed that a long-NT isoform of human $\alpha_{1C}$ may exist. By homology screening of human genomic DNA, we identified a stretch (termed exon 1a) highly homologous to rabbit long-NT, separated from the next known exon of $\alpha_{1C}$ (exon 1b, which encodes the alternative, short-NT) by an ~80 kb-long intron. The predicted 46-amino acid protein sequence is highly homologous to rabbit long-NT. Reverse transcriptase PCR showed the presence of exon 1a transcript in human cardiac RNA. Expression of human long-NT $\alpha_{1C}$ in Xenopus oocytes produced Ca$^{2+}$ channel enhanced by a PKC activator, whereas the short-NT $\alpha_{1C}$ was inhibited. The long-NT isoform may be the Ca$^{2+}$ channel enhanced by PKC-activating transmitters in human tissues.

Voltage-dependent L-type Ca$^{2+}$ channels are crucial for cardiac and smooth muscle contraction and hormone secretion, and they regulate gene expression in the brain (1–3). Their function is highly regulated by hormones and neurotransmitters, largely via activation of protein kinases (3, 4). Regulation by PKC$^1$ is believed to be of substantial physiological importance, mediating all or part of the effects of several hormones and intracellular messengers (4). PKC enhances L-type Ca$^{2+}$ currents in diverse human tissues and cell lines: heart, neuroblastoma, T-cells, and endocrine cells (5–10). Dual modulation by PKC is often observed with activation followed by, or concomitant with, inhibition (5, 10). Similar enhancement by PKC, sometimes followed by inhibition, has been described in other mammals (11, 12) and was reproduced in Xenopus oocytes expressing the cloned rabbit cardiac L-type Ca$^{2+}$ channels (13, 14). However, expression of human L-type channels, encoded by all cDNA cloned to date, yielded Ca$^{2+}$ channels that were only inhibited by PKC; the enhancement could not be reconstituted (15). The reason for the inability to reproduce the PKC modulation of human L-type channels remained unknown.

The main, pore-forming subunit of cardiac/smooth muscle L-type channel ($\alpha_{1C}$ or Ca$_{1.2}$), also present in the brain, is the product of the $\alpha_{1C}$ gene, CACNA1C (16). Several splice variants of CACNA1C are known (17, 18). The resulting isoforms of human $\alpha_{1C}$ protein show differential distribution in human tissues, and in failing versus normal myocardium. They play important roles in Ca$^{2+}$-dependent inactivation, oxygen sensing, and drug sensitivity (18–22). However, the genomic structure of the beginning of N-terminal region of human $\alpha_{1C}$ is not entirely clear. In the two best studied mammalian species, rat and rabbit, two N-terminal isoforms of $\alpha_{1C}$ cDNA are known, which most probably represent variable splicing products of the same gene (23). These splice variants encode long- and short-NT $\alpha_{1C}$ proteins, with variable initial segments of 46 and 16 aa, respectively (23–26) (the total length of the cytosolic part of the NT region of $\alpha_{1C}$ is ~154 aa in the long-NT $\alpha_{1C}$). Traditionally, the short-NT isoform is called “neuronal” in rat and “smooth muscle” in rabbit, whereas the long-NT isoform is considered “cardiac” in rabbit. However, in rat, $\alpha_{1C}$ protein containing the long-NT is found in both heart and brain (27). The known human $\alpha_{1C}$ is highly homologous to short-NT isoforms of rat and rabbit; the long-NT isoforms of rat and rabbit are also highly homologous to each other. Because the human L-type channel is up-regulated by PKC, and because the initial 20 aa of the long-NT isoform are crucial for this regulation in rabbit $\alpha_{1C}$ (27, 28), we reasoned that a long-NT isoform of human $\alpha_{1C}$ should also exist.

Here we demonstrate the presence of an exon encoding the initial 46-aa long-NT segment in the human genomic DNA. The existence of $\alpha_{1C}$ RNA containing this segment was confirmed by RT-PCR gel analysis and DNA sequencing of the RT-PCR product. Using the RT-PCR products, we have demonstrated that cDNA coding for a full-length long-NT $\alpha_{1C}$ isoform expressed in Xenopus oocytes produces a Ca$^{2+}$ channel that is enhanced by a PKC activator. The identification of the long-NT isoform of human $\alpha_{1C}$ will enable the study of the molecular mechanisms of PKC modulation, which has previously been hampered by the inability to reconstitute this modulation in expression systems.

**EXPERIMENTAL PROCEDURES**

**Tissues and Oocyte Culture**—All experiments with human and animal tissues were approved by the Tel Aviv University Helsinki Committee and the Sackler School of Medicine Animal Use Committee, respectively. Rat atria and ventricles were obtained from 19-day-old Wistar rats after decapitation performed under ether anesthesia. Xenopus frogs were maintained and operated on, and oocytes were prepared as described (29). Each oocyte was injected with 2.5 ng of RNAs of $\alpha_{1C}$, $\alpha_{1C}/\delta$, and $\beta_{2A}$ subunits and incubated for 3–4 days at
20–22 °C in NDE96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 2.5 mM sodium pyruvate, 50 μM/ml gentamycin, 5 mM HEPES, pH 7.5).

**RT-PCR Analysis**—9 μg of total human cardiac RNA purchased from Ambion, Inc (cat. no. 7266; lot 110P43E) was reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen) with primer 3 (see text below and Fig. 2A). Each PCR reaction (50 μl) contained 2.4 μl of the product of RT reaction, 1 μl of 10 mM dNTPs, 20–50 pmol of primers, 5 μl of 10× PCR buffer, 2 μl of MgCl₂ (2 mM), and 0.7 μl of Taq DNA polymerase (Promega). PCR was performed under the following conditions: 95 °C for 1 min, 49 °C for 1 min, and 72 °C for 2 min, repeated 35 times. The final elongation was performed at 72 °C for 5 min. The PCR products were analyzed on a 1% agarose gel.

The primers used for RT and PCR were: No. 1, CTTCGACCCCTTGGTTCAGAT (nt 4–21 from A in ATG of exon 1a); No. 2, TCAATGGAATACCCAGAGGA (nt 5–22 in exon 1b; numbering from ATG of the short-NT isoform α₁C,77; 17); No. 3, ATTTGAGCTTGGTGAATC (within the proposed intron between exon 1a and exon 1b; nt 9529–95546 in contig 3810573); +149 nt from A in ATG of exon 1a; No. 5, ATACCATCTGAATAA (within the same intron, nt 95596–95913 in contig 3810573; +516 nt from A in ATG in exon 1a); No. 6, AACTGAGAAGTGGCTTT (2369 nt upstream from A in ATG of exon 1a; nt 90301–90327 in contig 3810573); No. 7, GCAGGTTAGTGTAGGAAT (1339 nt upstream from A in ATG of exon 1a; nt 94040–94067 in contig 3810573); No. 9, CCATTGCAGAATGGTGAATC (nt 369–382 in exon 2; numbering from ATG of exon 1b of α₁C,77; No. 10, GATACGAGATCGCATGT (within the proposed 5’-UTR of exon 1a, 147 nt upstream from A in ATG of exon 1a; nt 95232–95239 in contig 3810573). The DNA Constructs—DNAs of α₂β and αβ2 were as described (30). cDNA of human short-NT isoform α₁C,77, (Ref. 31; GenBankTM accession No. Z34815) was obtained from Dr. R. Zühlke and subcloned into pGEM-HJ vector (which contains 5′- and 3′-UTRs of Xenopus a-globin flanking the polylinker). In the resulting construct, termed α₁CTTTC, the 5′-UTR of α-globin is followed by a BamHI restriction site and then immediately by the initial ATG. The coding sequence of α₁CTTTC is followed by the 3′-UTR of α-globin and then by a SalI site. The DNA of the long-NT, termed α₁C,77, was constructed as follows. The human heart cDNA obtained in the RT reaction described above was subjected to PCR with the reverse primer No. 3 (see above) and a forward primer ATTTGAGCTTGGTGAATC which overlaps the first 18 nt of the coding part of exon 1a (starting with ATG) and also creates a BamHI restriction site preceding ATG. The PCR product was digested with BamHI and MfeI (a unique MfeI site is present in exon 3 upstream of protein-coding region, 2 and inserted between these restriction sites into BamHI/MfeI-digested α₁CTTTC, in place of the original DNA segment flanked by these sites. The 5′ portion of the resulting DNA was sequenced. The sequence of the BamHI-MfeI segment obtained by this RT-PCR subcloning procedure was 100% identical to that predicted for the long-NT splice variant (based on the DNA sequence of chromosome 12), in which exon 5a is followed by exons 5c and 3 (see Fig. 1C). RNA was synthesized as described (29).

**Electrophysiology**—Whole cell currents were recorded using the Gene Clamp 500 amplifier (Axon Instruments, Foster City, CA) using the two-electrode voltage clamp technique, as described (30), in a solution containing 40 mM Ba(OH)₂, 50 mM NaOH, 2 mM KOH, and 5 mM HEPES, titrated to pH 7.5 with methanesulfonic acid. PMA and bisindolylmaleimide (BIS) were purchased from Sigma. Oocytes were treated with BIS essentially as described (32). In brief, the oocyte was injected with 30 nl of a water solution of BIS at 150 μM and, in addition, incubated in 5 μM BIS 2–4 h before recording. Stimulation, data acquisition, and analysis were performed using pCLAMP software (Axon Instruments).

**RESULTS AND DISCUSSION**

The initial exon of the previously described (17) human α₁C clone, which we now designate exon 1b, contains a 5′-UTR and encodes the first 16 aa of the short-NT α₁C protein. To find out whether DNA sequences that encode an alternative, long-NT isoform of human α₁C exist, we performed a standard BLAST search of human genomic DNA, as our query the cDNA sequence encoding the first 46 aa of the rabbit cardiac long-NT clone (24). The initial search showed the presence of a highly homologous sequence in the contig 3810573 from human chromosome 12, locus p13.3 (Fig. 1A; the region of the initial search is highlighted by bold letters). This is within the region where the gene of α₁C, CACNA1C, is located. Significantly, the sequence upstream from this segment showed significant homology to the 5′-UTR of the rabbit α₁C (Fig. 1A), supporting the possibility that it may be part of an initial exon of a human L-type channel. We have designated this tentative exon as 1a. Within the presumably protein-coding part of exon 1a, a 45-base-long DNA segment, starting at base 16 (from the initial ATG) also shares 40% homology with the protein-coding part of exon 1b (Fig. 1A).

The availability of the draft sequence of the human chromosome 12 on the NCBi Human Genome site allowed us to map the location of the putative exon 1a relative to the known exons of the human α₁C gene (Fig. 1B). Exon 1a precedes exon 1b and is separated from the latter by ~80 kb. We assume that this is a large intron. Two other large introns are found, according to the draft map of the chromosome, between exon 1b and exon 2 (~60 kb) and between exons 3 and 4 (~330 kb). These large introns have not been fully sequenced previously; introns of >5 and 2.4 kb have been reported (17). The location of exon 1a supports the possibility that it constitutes the first alternative exon of the CACNA1C gene. The screening procedure also confirmed the presence and the correct spatial location on chromosome 12 of all of the constant and alternative exons described by Soldatov in 1994 (17) (Fig. 1B show 10 of the 50 exons, excluding exon 1a).

Our working hypothesis was that exons 1a and 2a are alternative initial exons of the human CACNA1C gene (Fig. 1C). Exons 2 and 3 are probably constant (17), as supported by the conservation of the corresponding protein sequences in known long- and short-NT α₁C isoforms in human, rat, rabbit, and mouse (Fig. 1D and Refs. 23, 27, and 28). The protein sequences of N termini of the proposed human α₁C short- and long-NT isoforms are shown in Fig. 1D and are compared with the rabbit long-NT α₁C. The segment corresponding to exon 2 starts after aa 46 in the long-NT isoform and after aa 16 in the short-NT isoform. The protein segment encoded by exon 3 starts at aa 155, exactly at the proposed junction between the cytosolic NT and the first transmembrane segment of the channel, S1C (24).

To further substantiate our working hypothesis, we performed RT-PCR on total human cardiac RNA. cDNA was obtained by reverse transcription using primer No. 3 (Fig. 2A), which corresponds to the 3′ end of exon 3. The analytical PCR was done with primers corresponding to segments of chromosome 12 DNA, as published at the NCBI Human Genome site, within the putative exons and introns of the 5′ region of CACNA1C. The location of primers is shown in Fig. 2A. Clear bands were detected for all DNAs that contained exon 1a (Fig. 2B): protein-coding sequences of exon 1a + exon 2 + exon 3 (lane 1) and [exon 1a + exon 2] (lane 3); [exon 1a including 5′UTR + exon 2] (lane 5). The sizes of the DNAs corresponded to those expected for an RNA transcript that contains exons 1a, 2, and 3 without 1b. Control reactions, with primers within the presumed intron regions (lanes 6 and 7) and ~1.4–2.4 kb upstream from the beginning of exon 1a (lane 8) did not yield signals. Another cDNA segment that included protein-coding sequences of exon 1a, 2, and (part of) 3 was obtained for subcloning purposes (see “Experimental Procedures”) and sequenced. The size and the DNA sequence of this RT-PCR product were exactly as expected for [exon 1a + exon 2 + exon 3 up to MfeI site] without exon 1b and encoded the first 46 aa of the.

2 Found on the Web at ncbi.nlm.nih.gov/BLAST.

3 NCBI accession number AC005342.
Fig. 1. The newly identified exon 1a and the proposed DNA and protein structure of two NT isoforms of human \( \alpha_{1C} \). A, nucleotide homology between rabbit heart (RH) long-NT cDNA, the homologous part of human chromosome 12 corresponding to the proposed exon 1a (ex1a), and the coding sequence of the known exon 1b (ex1b). Full homology is indicated by asterisks. Bold letters show the protein-coding sequence of long-NT isoforms. B, the location of exons and introns according to the draft map of chromosome 12 available at the NCBI site. C, the DNA sequences corresponding to proposed alternative splice variants encoding long- and short-NT isoforms of \( \alpha_{1C} \). The middle section shows the 5’ part of the CACNA1C gene, with boxes representing exons and angled lines representing introns. D, comparison of the protein sequences of rabbit cardiac \( \alpha_{1C} \) and the two proposed isoforms of the human \( \alpha_{1C} \). Asterisks show fully conserved aa; bold letters highlight the PKC phosphorylation sites responsible for the inhibitory effect of PMA in rabbit \( \alpha_{1C} \); the underlined residues are those that differ in rabbit and human long-NT \( \alpha_{1C} \).

Fig. 2. RT-PCR supports the existence of human long-NT isoform. A, the location of primers used for RT-PCR. B, RT-PCR products obtained with the primer pairs shown below the lanes. Primers were added to the PCR reactions at 50 pm. The sizes of DNAs expected from the hypothesis of Fig. 1 A were, by lanes (in nt): 1, 547; 2, 463; 3, 449; 4, 365; 5, 591; 6–8, no PCR products were expected. C, RT-PCR with 20 pm primers, showing lanes 3 and 4 as in panel B.

A band corresponding to the DNA of exons [1b + 2 + 3] was also obtained; the size was exactly as expected for a short-NT isoform, without 1a (Fig. 2B, lane 2). Under standard conditions the band corresponding to [exon 1b + exon 2] was not detected (Fig. 2B, lane 4), but varying the conditions of PCR revealed this band, although it still remained relatively weak (Fig. 2C). Thus, RNA of the previously described short-NT isoform is also present in human cardiac tissue.

Previously, the existence of a long-NT \( \alpha_{1C} \) protein in rat has been demonstrated by Western blot with a polyclonal antibody directed against the unique initial 46 aa of the rabbit long-NT isoform (27). The same antibody detected a \( \sim 220 \) kDa protein (presumably the L-type \( \mathrm{Ca}^{2+} \) channel) in a human colon cancer cell line (33). These results corroborate the presence of the long-NT \( \alpha_{1C} \) protein in human tissues. Taken together, our data and those of Ref. 33 support the notion that human cardiac (and probably other) tissues contain two N-terminal isoforms of \( \alpha_{1C} \) protein, a long-NT and a short-NT one, which are products of alternatively spliced RNA transcripts of the 5’-terminal region of the CACNA1C gene (as shown in Fig. 1, C and D).

According to our initial hypothesis, the long-NT \( \alpha_{1C} \) should be up-regulated by the activation of PKC. To test this prediction, we constructed a cDNA encoding a long-NT \( \alpha_{1C} \) on the basis of a human short-NT \( \alpha_{1C} \) DNA, \( \alpha_{1C,77} \) (19). Both cDNAs were subcloned into the pGEM-HJ vector and verified by DNA sequencing (see “Experimental Procedures”). The long-NT clone was designated \( \alpha_{1C,77L} \) and the short-NT clone \( \alpha_{1C,77S} \). The corresponding RNAs were synthesized in vitro and injected into Xenopus oocytes with RNA of the short-NT clone \( \alpha_{1C,77S} \). The same antibody detected a \( \sim 220 \) kDa protein (presumably the L-type \( \mathrm{Ca}^{2+} \) channel) in a human colon cancer cell line (33). These results corroborate the presence of the long-NT \( \alpha_{1C} \) protein in human tissues. Taken together, our data and those of Ref. 33 support the notion that human cardiac (and probably other) tissues contain two N-terminal isoforms of \( \alpha_{1C} \) protein, a long-NT and a short-NT one, which are products of alternatively spliced RNA transcripts of the 5’-terminal region of the CACNA1C gene (as shown in Fig. 1, C and D).
channels ranged from 100 to 800 nA without β2A and from 1500 to 7000 nA with β2A. The kinetics of $I_{Ba}$ of α1C,77L and α1C,77S (e.g. Fig. 3C) were very similar to each other and to those directed by rabbit cardiac α1C. The other parameters also resembled those previously reported for L-type Ca$^{2+}$ channels of various species. For instance, as described previously for the rabbit α1C (30, 34), coexpression of the β2A subunit increased the current amplitude 9.5 ± 0.4-fold in α1C,77L and 9.3 ± 0.6-fold in α1C,77S, and shifted the voltage dependence of activation to more negative potentials, as shown by normalized current-voltage curves (Fig. 3A). Peak $I_{Ba}$ was similar in the long-NT and short-NT isoforms; the small difference was statistically significant (Fig. 3B), but at present we do not know whether this reflects differences in protein expression or in the gating properties of the two isoforms.

The prediction that the long-NT human α1C should be enhanced by PKC has been fully confirmed. The main effect of the phorbol ester β-PMA (a PKC activator) was to increase the Ba$^{2+}$ current of α1C,77L coexpressed with αβ2 with or without β2A (Fig. 3, C–E). As in the rabbit α1C channel (13), the increase reached a maximum within 5–8 min and was followed by a decrease, sometimes below the basal current level (Fig. 3D). In contrast, the short-NT α1C,77S channels were inhibited by PMA (Fig. 3D), in line with the previous report (15). On average, PMA increased the current via the long-NT channels by 22.7 ± 4.4% in the absence of β2A subunit and by 21.2 ± 4.2% in its presence (Fig. 3E). The PMA-induced increase in $I_{Ba}$ was fully blocked by the specific PKC inhibitor, bisindolylmaleimide (Fig. 3E; bar marked “BIS”), supporting the notion that the enhancement of $I_{Ba}$ by PMA was indeed mediated by PKC. Thus, in general, the modulation of the human long-NT isoform by PKC is similar to that of the rabbit cardiac channel. With rabbit cardiac L-type channel, coexpression of β2A substantially weakens the enhancement of the current by PMA (13), whereas in the human channel this action of β2A is less pronounced. It would be of interest to see whether this is due to one of the few differences in the primary amino acid composition of the 46-aa-long initial NT segment in human and rabbit (Fig. 1D, underlined amino acids).

In a different expression system (human embryonic kidney cell line tsA-201), only inhibition of the rabbit long-NT α1C by PMA has been observed (35). The inhibition crucially depended on the presence of each of the two threonines, Thr$^{27}$ and Thr$^{31}$ (shown in bold in Fig. 1D), in which phosphorylation by PKC was proposed to underlie this modulation. It has been proposed that the lack of PKC-induced enhancement in tsA-201 cells may result from the absence of a specific PKC isozyme (35). At present, we do not know whether the phosphorylation of Thr$^{31}$ (Thr$^{27}$ is absent in the long-NT human α1C; Fig. 1D) plays a role in any of the PKC effects observed in Xenopus oocytes. We suspect that the mechanism of PKC-induced inhibition of α1C in oocytes is different from that observed in tsA-201 cells, because a short-NT isoform of rat α1C was not sensitive to PMA in this system (35), whereas the homologous short-NT human α1C is inhibited by PKC in Xenopus oocytes (Ref. 15 and Fig. 3). Furthermore, the decrease of $I_{Ba}$ is still observed in the presence of BIS (Fig. 3E), whereas in tsA-201 cells PKC inhibitors blocked the effect of PMA (35). The PMA-induced inhibition of human L-type Ca$^{2+}$ channels observed in Xenopus oocytes requires further study.

Because the most widely observed effect of PKC activators on human L-type Ca$^{2+}$ channels is an enhancement of the current (sometimes accompanied by an inhibition (5–10)), and because this regulation is reproduced with the long-NT α1C in Xenopus oocytes, we propose that the long-NT α1C is the isoform that underlies the PKC-induced enhancement. The homologous long-NT rabbit α1C isoform behaves in the same way when expressed in oocytes (13, 28), probably by means of an identical molecular mechanism as in human α1C. The four aa following the initial methionine in rabbit long-NT α1C, LRAL, are necessary for PKC-induced enhancement. Because this protein segment contains no putative phosphorylation sites, we have proposed that PKC phosphorylation occurs elsewhere in α1C or in an auxiliary protein, whereas the first five aa play a role in PKC anchoring or in channel gating (27). The short-NT isoform lacks these four amino acids; its initial 15-aa segment (following the first methionine) carries a partial homology to amino acids 6–20 of the long-NT isoform (Fig. 1D). In rabbit long-NT α1C, the first 20 aa play the role of the inhibitory gating element, which reduces the open probability of the L-type channel4 (27, 28). It would be of great interest to see whether this is also the case in human long-NT α1C. We hope that the discovery of the long-NT isoform of human α1C and the demonstration of its modulation by PKC, as described in this report, will provide the basis and the incentive for future studies of PKC targets (α1C or an auxiliary protein?) and of the interaction between the phosphorylated and gating parts of α1C in PKC modulation.

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A Novel Long N-terminal Isoform of Human L-type Ca\(^{2+}\) Channel Is Up-regulated by Protein Kinase C

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