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Smooth muscle uses another promoter to express primarily a form of human $Ca_V1.2$ L-type calcium channel different from the principal heart form

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Abstract

Several different first exons and amino termini have been reported for the cardiac Ca channel known as α_{1C} or $Ca_V1.2$. The aim of this study was to investigate whether the expression of this channel is regulated by different promoters in smooth muscle cells and in heart in humans. Ribonuclease protection assay (RPA) indicates that the longer first exon 1a is found in certain human smooth muscle-containing tissues, notably bladder and fetal aorta, but that it is not expressed to any significant degree in lung or intestine. On the other hand, all four smooth muscle-containing tissues examined strongly express transcripts containing exon 1b, first reported cloned from human fibroblast cells [1]. In addition, primary cultures of human colonic myocytes and coronary artery smooth muscle cells express predominantly transcripts containing exon 1b. The promoter immediately upstream of exon 1b was cloned, and it displays functional promoter activity when luciferase-expressing constructs were transfected into three different cultured smooth muscle cells: primary human coronary artery smooth muscles cells, primary human colonocytes, and the fetal rat aorta-derived A7r5 cell line. These results indicate that expression in smooth muscle is primarily driven by a promoter different from that which drives expression in cardiac myocytes.

Keywords: Promoter; L-type calcium channel; Human smooth muscle; Human heart

The cardiac L-type Ca channel known variously as α_{1C} or $Ca_V1.2$ is expressed in humans not only with splice variants [1–8], but also with three different lengths of amino termini, depending upon whether exon 1a [9,10], exon 1b [1], or neither exon [11] is expressed. It is not known which of these forms is preferentially expressed in any human smooth muscle.

The promoters for the rat and human heart forms of the channel have been independently cloned by two groups [10,12,13], and the rat promoter has been shown to be functional not only in heart cells but also in PAC1 and A7r5 smooth muscle cell lines [12,14; Saada et al., unpublished data], consistent with its cloning from rat aorta [15]. However, since transcripts expressing a shorter downstream first exon 1b have been reported not only in human fibroblasts [1], but also in smooth muscle-rich rabbit lung [16], certain smooth muscles may express a form of the channel that lacks exon 1a. The present study was initiated to distinguish which transcript predominated in smooth muscle and whether it was driven by a promoter just upstream of exon 1a, as for human heart [10], or one just upstream of exon 1b. Portions of this work have previously been communicated in abstract form [17,18].

Materials and methods

Total RNA from human heart, small intestine, lung, bladder, and colon was obtained from Ambion (Austin, TX), that from human fetal

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aorta from Stratagene (La Jolla, CA), and that from human uterus from Clontech (BD Biosciences Clontech, Palo Alto, CA). Total RNA was prepared from primary cultures of human coronary artery and human circular colonic smooth muscle with Tri-reagent (Sigma, St. Louis, MO). RT-PCRs were carried out using AMV reverse transcriptase at 38 °C (using random primers) or 42 °C (using oligo(dT) priming) for 60 min, followed by PCR using Taq with default cycling parameters of 35 cycles each of 55 s at 52–57 °C hybridization, 1 min 72 °C extension, and 30 s 94 °C denaturation in a MiniCycler thermal cycler from MJ Research (Waltham, MA). Primers were synthesized commercially by IDT (Coralville, IA). Sequencing was carried out on ABI 310 or 377 sequencers (Advanced Biotechnologies, Columbia, MD) at an institutional core facility.

Ribonuclease protection assays. Ribonuclease protection assays (RPAs) were carried out utilizing the RPA III and MAXIscript T7 kits from Ambion (Austin, TX) according to the manufacturer's instructions.

One probe utilized for RPA was generated by RT-PCR from human heart RNA using a sense primer gcgatacgatacggccatgtc in the 5′ untranslated (utr) region of exon 1a and antisense primer tggagctgactgtggagatg in the second exon. This probe was subcloned with a TA cloning kit into pCR 2.1 (Invitrogen, Carlsbad, CA), sequenced, and digested with *Dra*I (shortening it by 112 nucleotides), and a riboprobe was generated using the T7 promoter [10].

A second probe was generated by RT-PCR from human bladder RNA using a sense primer cgtggctgctcctcctatta in the 5′ utr of exon 1b and antisense primer tggagctgactgtggagatg in exon 2. This probe was also subcloned into pCR 2.1 and digested with *Hin*dIII for use of the T7 promoter. Because it was susceptible to cleavage near one end, it was shortened by digestion with *Sma*I near the 3′ end of the insert and *Eco*RV on the vector. The fragment which included the rest of the insert and the vector was gel purified and religated, and the vector was digested with *Hin*dIII to access the T7 promoter. The probe sequence was verified by DNA sequencing to be cgtggctgctcctcctattaaaacca tttttggtcccatggtcaatgagaatacgaggatgtacattccagaggaaaaccaccaaggttccaa ctatgggagcccacgccccgcccatgccaacatgaatgccaatgcggcagcggggctggcccctg agcacatccccacccc.

In RPAs, this probe generates a longer protected fragment of 173 bp for human α_{1C} transcripts containing exon 1b and a shorter 92 bp protected fragment for human α_{1C} transcripts not containing exon 1b.

Promoter constructs and transfections. Promoter constructs were prepared by PCR amplification of human genomic DNA (Roche, Indianapolis, IN) partially digested with *Hin*dIII (Promega, Madison, WI) using the FailSafe PCR kit (Epicentre, Madison, WI). The PCR product was subcloned into pDrive Cloning Vector with the PCR Cloning^{plus} kit (Qiagen, Valencia, CA) and SURE 2 supercompetent cells (Stratagene, La Jolla, CA) and subsequently transferred to the luciferase expression plasmid pGL2-Basic Vector (Promega, Madison, WI).

Transfections of all cells were carried out in triplicate, with all transfections on a particular cell type performed on the same day, with cells harvested 48–72 h later. Human colonic myocytes were prepared according to Shi and Sarna [19]. Cells were grown in RPMI media with 25 mM Hepes, and L-glutamine, and 10% FBS (Gibco-BRL, Life Technologies, Grand Island, NY). Cells in passage 6 were seeded into 6-well plates and grown to 85% confluency one day before transfection. Transfections were carried out using 20 μl Effectene reagent and 6.4 μl Enhancer (Qiagen, Valencia, CA), 2 μg pGL2 construct, and 0.5 μg pSEAP2-Control Vector (BD Biosciences Clontech, Palo Alto, CA) co-transfected for purposes of normalization. These cells were harvested after 48 h. Human coronary artery smooth muscle myocytes (CASMC, passage 2) were obtained from Clonetics (Bio-Whittaker, Walkersville, MD) and cultured to 90% confluency utilizing their SmGM-2 BulletKit. Transfections of 1 μg pGL2 constructs into CASMC in passage 3 were carried out using 3 μl Fugene 6 (Roche, Mannheim, Germany) with 0.4 μg pSEAP2-Control Vector and cells were harvested after 72 h. Rat A7r5 cells derived from fetal rat aorta [20] were cultured according to ATCC instructions. Transfections of 1.5 μg pGL2 constructs into A7r5 cells (passage 16) were carried out using 6 μl Fugene 6 with 0.5 μg pSEAP2-Control Vector and cells were harvested after 72 h.

Results

Ribonuclease protection assays were utilized to assess the proportion of exon 1a- and exon 1b-containing transcripts in different smooth muscle-containing tissues. A probe, which protects a ~350 bp fragment when exon 1a is present and a ~212 bp fragment when it is absent [10], was used to ascertain the expression of exon 1a. The longer protected fragment was in excess over the shorter protected fragment in assays involving human heart RNA (Fig. 1). Some longer protected fragment was also detected with human bladder and fetal aorta RNA (Fig. 1), but in both cases it was not as much as the shorter protected fragment. With human small intestine and lung RNA, there was no detectable longer protected fragment and only the shorter protected fragment was obtained (Fig. 1).

A probe which protects a 173 bp fragment when exon 1b is present and a 92 bp fragment when it is absent was used for additional RPAs. Human heart RNA protected very little 173 bp fragment compared to the 92 bp frag-

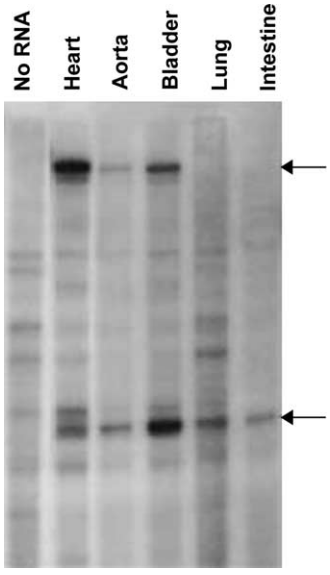

Fig. 1. Ribonuclease protection assay demonstrating presence of exon 1a and alternative first exon in RNA from human bladder, intestine, and fetal aorta. Left to right, assays with: no RNA, 10 μg human heart RNA, 10 μg human fetal aorta RNA, 10 μg human bladder RNA, 10 μg human lung RNA, and 10 μg human small intestine RNA. Riboprobe hybridizing with part of exon 2, all of exon 1a, and part of its 5′ utr. Larger protected fragments indicate presence of exon 1a; shorter protected fragments presence of transcripts not expressing exon 1a. Experiment representative of two separate determinations with all these samples.

ment, but RNA from human fetal aorta, bladder, colon, lung, and uterus all protected more 173 bp fragment than the 92 bp fragment (Fig. 2A). We have obtained similar results with RNA from human lung and small intestine (not shown).

Since all these tissues contain cells other than smooth muscle cells, RNA samples from primary cultures of human colonic smooth muscle cells and human coronary artery smooth muscle cells were also tested for the proportion of the two transcripts. The results are indicated in Fig. 2B, where it is apparent that RNA from each of the primary cultures exhibited much greater expression of exon 1b-containing transcripts, in complete contradistinction to the greater expression of exon 1a-containing transcripts in human heart seen in Fig. 2A. Furthermore, there were hardly any detectable transcripts without exon 1b in RNA from coronary artery smooth muscle cells and a still greater proportion of exon 1b-containing transcripts in human colonocyte RNA (Fig. 2B) than in RNA from the colon as a whole (Fig. 2A).

The 1.6-kb region immediately upstream of exon 1b was cloned to assess its ability to function as a promoter. This putative promoter fragment was subcloned into a pGL2 luciferase construct and transfected into primary coronary artery smooth muscle cells (CASMCs), primary human colonocytes, and rat A7r5 cells. Results were normalized by secreted alkaline phosphatase produced by co-transfected pSEAP2-Control (Clontech).

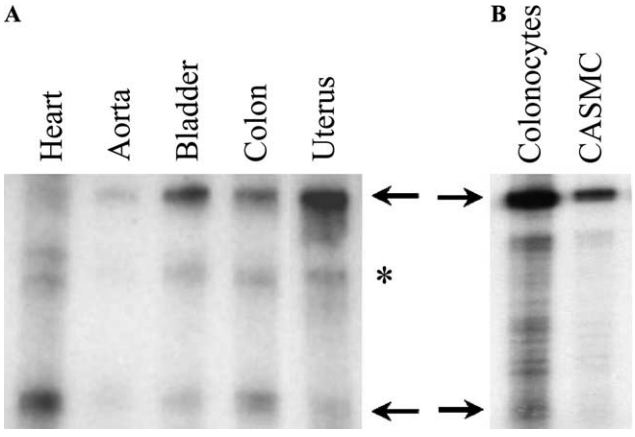

Fig. 2. Ribonuclease protection assay demonstrating presence of exon 1b and alternative first exon in RNA from human tissues and cells. (A) Left to right, assays with: 10 μg human heart RNA, 10 μg human fetal aorta RNA, 10 μg human bladder RNA, 10 μg human colon RNA, and 10 μg human uterine RNA. Riboprobe hybridizing with part of exon 2, all of exon 1b, and part of its 5′utr. Two protected fragments are indicated by arrows at right and * indicates failure of ribonuclease to fully digest the shorter protected fragment. Longer protected fragments indicate presence of exon 1b; shorter protected fragments presence of transcripts not expressing exon 1b. Representative of three separate experiments with these samples. (B) Primary cultures of smooth muscle from colon and coronary artery also demonstrate greater expression of exon 1b-containing transcripts than exon 1a-containing ones. CASMCs, human coronary artery smooth muscle cells.

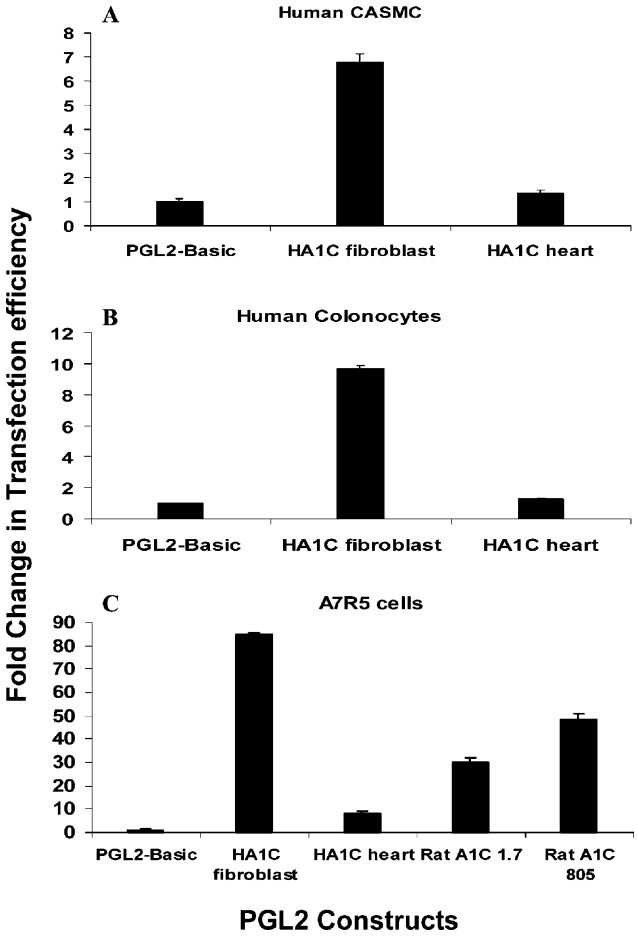

Fig. 3. Putative human promoter demonstrates functional activity in both A7r5 cells derived from rat aorta and primary cultures of human coronary artery smooth muscle and human colonic circular smooth muscle cells. (A) Results in human coronary artery smooth muscle cells (CASMCs) transfected separately with putative human heart and fibroblast promoters. (B) Results in human circular colonic smooth muscle cells (Human colonocytes) transfected separately with putative human heart and fibroblast promoters. (C) Results in A7r5 cells transfected separately with putative human heart and fibroblast promoters as well as with two lengths (1700 and 805 bp) of the rat heart promoter just upstream of exon 1a.

As seen in Fig. 3, the promoter upstream of exon 1b exhibited functional promoter activity in all three types of cells. In all three cell types, the fibroblast promoter displayed significantly greater activity than the human heart promoter upstream of exon 1a. In A7r5 cells, the rat heart promoter [12,13] was several times more active than the human heart promoter, but still not as active as the human fibroblast promoter.

Discussion

The work described here demonstrates that most of the transcripts found in smooth muscle-rich tissues begin with exon 1b rather than exon 1a. Although some

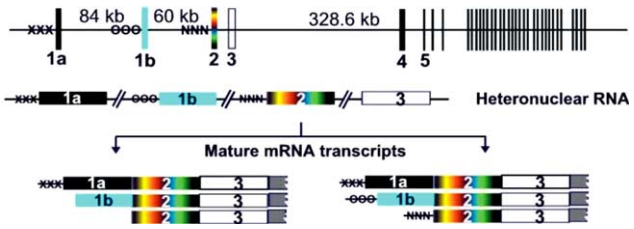

Fig. 4. RT-PCR discrimination of single vs multiple promoters. Downstream 5′ utrs (ooo and NNN) will be spliced out of transcripts generated using only one promoter just upstream of xxx (left), whereas transcripts generated by multiple promoters will each retain the 5′ utr associated with the promoter driving its expression (right).

transcripts begin with exon 1a in human aorta and bladder, these are clearly in the minority. Some expression of transcripts beginning with exon 1b was found in heart, but this could have involved fibroblasts or vascular smooth muscle. Thus, in addition to fibroblasts [1], brain [21], bone [22], testis [23], and lung [16], other smooth muscle-containing tissues express transcripts beginning at exon 1b. Highly purified smooth muscle cells cultured from coronary artery and colon expressed almost exclusively exon 1b-containing transcripts, raising the speculation that other transcripts found in tissues may have arisen from other cell types. Vascular smooth muscle cells are not the only smooth muscle cells

Fig. 5. Alignment of putative human and mouse promoters for transcripts expressing exon 1b. Mouse sequence was obtained from the mouse genome project.

that expressed transcripts beginning with exon 1b; visceral smooth muscle tissues do the same.

Transcripts beginning with exon 1b could be regulated by a promoter far upstream, such as that cloned from rat heart [12,13] or human heart [10] and then alternatively spliced so as to delete exon 1a. Alternatively, exon 1b-expressing transcripts could be regulated by a second promoter immediately upstream of exon 1b. Indeed, Lau and Eby [24,25] have reported cloning such a human promoter and demonstrating its ability to serve as a promoter in kidney cells.

Two lines of evidence suggest that smooth muscle transcripts are regulated by a promoter immediately upstream of exon 1b. First, RT-PCR demonstrated that exon 1b-containing transcripts also express the 5′ utr that is unique for exon 1b. Had the transcripts been alternatively spliced, this 5′ utr would have been removed from the mature mRNA (Fig. 4). Second, the putative human promoter immediately upstream of exon 1b showed promoter activity in luciferase reporter constructs in all three kinds of smooth muscle cells examined. Therefore, we conclude that vascular smooth muscle expression of this channel, at least in humans, is controlled principally by a different promoter from that shown to drive expression in rat PAC1 cells [12,14].

Since truncation of the amino terminus of exon 1a-containing transcripts results in channels with higher activity [26,27], exon 1b-containing channels may not only be regulated differently, but might also exhibit higher single channel open probability. In addition, PKC sensitivity of $Ca_V1.2$ channels might be affected by different N-termini [9,27–34].

The new human promoter and its mouse counterpart are both TATA-less promoters. Since the new human promoter displays high homology (80%) to its mouse counterpart (Fig. 5) higher than that observed comparing mouse and human introns or utrs but comparable to other promoters [35], it is likely that many response elements have been conserved. Furthermore, in view of the lack of availability of human smooth muscle cell lines, the results reported here demonstrate that this human promoter can be studied conveniently in the rat smooth muscle A7r5 cell line, which also can activate the heart promoter upstream of exon 1a.

Acknowledgments

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