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Rod-type cyclic nucleotide-gated cation channel is expressed in vascular endothelium and vascular smooth muscle cells

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Abstract

Objectives: Ca^{++}-permeable nonselective cation channels mediate the entry of extracellular Ca^{++} in vascular endothelium. They are also partly responsible for Ca^{++} entry in vascular smooth muscle cells (SMCs). The molecular identities of these channels have not been identified. The aim of this study is to examine whether rod-type nucleotide-gated nonselective cation (CNG1) channel, a channel which has been molecularly cloned, is related to the nonselective channels in vascular cells. **Methods:** We used RT-PCR, molecular cloning, northern Blot and in situ hybridization to examine the expression of CNG1 mRNA in a variety of guinea pig and rat blood vessels with different diameters and in cultured vascular endothelial cells and vascular smooth muscle cells. **Results:** We have cloned a 402-bp partial cDNA of CNG1 channel from guinea pig mesenteric arteries. RT-PCR and southern blot results indicate that the CNG1 mRNA is expressed in both cultured vascular endothelial and cultured vascular SMCs. Northern blot revealed the transcripts of ~3.2 kb, ~5.0 kb, and ~1.8 kb in cultured endothelial cells. In situ hybridization yielded strong labeling in endothelium layer of aorta, medium-sized mesenteric arteries, and small mesenteric arteries. **Conclusion:** Our findings suggest a potential role of CNG protein for Ca^{++} entry in vascular endothelium and vascular smooth muscles. The high expression of CNG1 mRNA in the endothelium of medium-sized arteries and small-sized arteries implicates a possible involvement of CNG1 protein in the regulation of blood supply to different regions and in the regulation of arterial blood pressure.

Keywords: CNG1 channel; Ca^{++} entry; Mesenteric arteries; Vascular endothelium; Vascular smooth muscle cells

1. Introduction

Cyclic nucleotide-gated (CNG) channels are originally thought to be unique in sensory signal transduction of retinal and olfactory cells [1,2]. Now, it appears that these channels have fairly widespread tissue distribution including heart, kidney, aorta, spleen, etc. [3–5]. Up to three types of CNG channels have been cloned, the rod-type photoreceptor channel (CNG1), olfactory-type channel (CNG2), and nonsensory cell-type channel (CNG3). CNG1 and CNG2 channels are not restricted in sensory systems. The mRNA for CNG1 channel has been detected in heart, kidney, pineal gland, aorta and spleen [4,5]. A CNG2 like channel has also been cloned from a rabbit aorta library [6].

The finding that CNG channels are expressed in such a variety of tissues not involved in sensory signal transduction suggests that they play functional roles in these tissues as well. CNG channels are a group of nonselective cation (NSC) channels permeable to both Ca^{++} and monovalent cations. The activities of these channels are regulated by cyclic nucleotides (cAMP and cGMP). In some cells such as retinal and olfactory cells, CNG channels are activated by cyclic nucleotides [1,2]; in others such as kidney, they are inhibited by cyclic nucleotides [7]. Regardless the mechanism by which CNG channels are regulated by cyclic nucleotides — i.e., activation or inhibition, these channels could provide a casual link between the cyclic nucleotides and $[Ca^{++}]i$.

Vascular endothelium plays an important role in modulating smooth muscle tone and blood pressure. Stimulation of endothelial cells with vasoactive agents (e.g. bradykinin, histamine, ATP, and acetylcholine) causes an increase in

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intracellular Ca^{++} concentration as a key signal triggering the formation of two autacoids, prostacyclin (PGI2) and endothelium-derived relaxing factor (EDRF, NO), which subsequently act on neighboring smooth muscle cells (SMCs) and cause vasorelaxation and a decrease in blood pressure [8–10]. Since voltage-gated calcium channels are not present in the vascular endothelium, Ca^{++}-permeable NSC channels are believed to be the sole Ca^{++} entry pathway in the vascular endothelium [11,12]. The Ca^{++} influx through NSC channels is regulated by the resting membrane potential, which determines the electromotive force for ion permeation through the channels [11,12]. Besides mediating the Ca^{++} entry elicited by vasoactive agents, NSC channels also mediate stretch-activated Ca^{++} influx [13] and basal Ca^{++} influx into unstimulated endothelial cells [14]. However, up to now none of these Ca^{++}-permeable NSC channels has been cloned in vascular endothelium and we do not know whether these channels are structurally related to CNG channels.

A Ca^{++}-permeable NSC channel has recently been reported in vascular SMCs [15]. This channel may provide an alternative Ca^{++} entry pathway in addition to the well-known voltage-gated Ca^{++} channels in the vascular SMCs. Since this channel is inhibited by cGMP, it might be another important target of nitrovasodilators. Nitric oxide through a cGMP signaling pathway may reduce Ca^{++} entry by inhibiting this Ca^{++}-permeable NSC channel, resulting in vasorelaxation and a decrease in blood pressure [15]. However, this Ca^{++}-permeable NSC channel has not been cloned from vascular SMCs.

In the present study we report the cloning of a partial CNG1 cDNA from guinea pig mesenteric arteries. The 402-bp cDNA has 100% nucleotide identity with the recently published rat eye CNG1 sequence [5]. Moreover, with the use of northern blot analysis and in situ hybridization, we have found that CNG1 mRNA is abundantly expressed in the vascular endothelial cells of large, medium and small arteries. CNG1 mRNA is also expressed in vascular SMCs, but in low abundance. Since CNG1 mRNA encodes a Ca^{++}-permeable NSC channel, it is tempting to suggest a potential role of CNG1 protein for Ca^{++} entry in vascular endothelium and vascular smooth muscles and thus a possible involvement of CNG1 protein in the contraction of blood vessels. Reverse transcriptase (RT)-PCR also revealed the presence of CNG1 mRNA in a variety of tissues, including brain, heart, kidney and testis, indicating widespread distribution of CNG1 channel.

2. Methods

2.1. Preparation of RNA and cDNA

This method conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health. Male Dunkin–Hartley guinea pigs and male Sprague–Dawley rats were decapitated and various tissues were obtained. The isolated mesenteric arteries have the internal diameter ranging from 100 μm to 1 mm. Total RNA was isolated from tissues and cultured cells by the acid guanidinium thiocyanate method [16,17]. mRNA was transcribed into first strand cDNA using oligo-dT primers and M-MLV reverse transcriptase (Gibco-BRL).

2.2. Cloning and sequencing

A 402-bp partial sequence for CNG1 channel that includes the conservative regions of S5, pore region, S6, and a short C-terminal cytoplasmic domain was amplified from guinea pig mesenteric arteries with primers VCNG(+)(TCATCATCCACTGGAA) and VCNG(−)(TCCACAGGTAGTCAAA) [2,18]. PCR reactions of 50 μl contained 1 μl first strand cDNA, 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, dATP, dCTP, dGTP, dTTP 0.2 mM each, 1.0 μM primers and 2.5 Unit Taq DNA polymerase (Gibco-BRL). Fifty cycles (94°C for 1 min, 50°C for 1 min, 72°C for 1 min) were performed with a Robocycler (Strategene, San Diego, CA, USA). Control reactions without first stand DNA were included for each PCR amplification experiment.

RT-PCR products were purified from agarose gel using Elu-Quik gel-purification kit (Schleicher & Schuell). The products were end-filled using Klenow, and then cloned in a pBluescript vector (Stratagene). Restriction analyses were carried out on plasmid DNA prepared by Qiagen mini-prep kit. The inserts with expected molecular size were sequenced with Sequenase (US Biochemicals, Cleveland, OH, USA). Sequencing was performed on both strands.

2.3. PCR-based detection of mRNA

The primers VCNG(+)(TCATCATCCACTGGAA) and VCNG(−)TCCACAGGTAGTCAAA were also used to amplify the 402-bp PCR fragment from the first strand cDNAs of different tissues and cultured cells. To detect lower abundance expression of CNG1 mRNA, we used 50-cycle RT-PCR. To confirm the identity of amplified products, PCR fragments were resolved on a 1% agarose gel, denatured, neutralized and transferred to a nylon membrane Hybond-N^+ (Amersham). A ^{32}P-labeled cDNA probe internal to the primers used in PCR was generated by digesting the cloned 402-bp fragment in pBluecripts with SmaI and then radioactively labeling the resulting 300-bp restriction fragment using ready-to-go DNA labeling kit (Pharmacia Biotech). Hybridization was done in Rapid-hyb buffer (Amersham) at 55°C overnight. Filters were washed in 50 ml 2× SSC, 0.1% SDS at room temperature for 20 min followed by 1× SSC, 0.1% SDS at 55°C. Autoradiography was performed overnight on Kodak Biomax film.

2.4. Northern blot

Poly(A)$^+$ RNA was isolated from total RNA by oligo-dT cellulose column using MessengerMaker mRNA isolation system (Gibco-BRL). 5 μg of poly(A)$^+$ RNA was denatured with formaldehyde, size fractioned by electrophoresis in agarose gels containing 2.2 M formaldehyde, and transferred to Hybond-N$^+$ nylon membranes. Hybridization was performed in Rapid-hyb buffer (Amersham) at 55°C overnight. A ^{32}P-labeled CNG1 cDNA probe was generated by digesting the pBluecripts containing 402-bp CNG1 fragment with SmaI and then radioactively labeling the resulting 300-bp restriction fragment using the ready-to-go DNA labeling kit (Pharmacia Biotech). Filters were washed in 50 ml 2×SSC, 0.1% SDS at room temperature for 20 min followed by 1×SSC, 0.1% SDS at 42°C for 20 min. Autoradiography was performed overnight on Kodak Biomax film.

2.5. Isolation of aortic SMCs

Male guinea pigs and male rats were decapitated. The thoracic aorta was removed and washed twice with sterile phosphate buffer saline (Gibco-BRL) in order to remove residual blood. Fat and connective tissues were then trimmed off. The aorta was then cut with scissors and the intima side was gently scraped with a scalpel blade to remove endothelium. The remaining aorta was cut into 2–3 mm slices, air dried for 5 min, and then incubated with intima side down in 85% DMEM (Dulbecco's modified Eagle's medium) and 15% FBS at 37°C. After 5 days, aortic slices were removed and cell incubation was continued with the change of media once every 3 days until a confluent layer of vascular SMCs was formed. These cells had a spindle-shaped morphology and attained a hill-and-valley appearance, indicative of SMCs.

2.6. Culture of endothelial cells and SMCs

A vascular endothelial cell line from human umbilical veins (ECV304) and an aortic smooth muscle cell line (A7r5) from rat were obtained from the American type culture collection (ATCC). ECV304 cell line was cultured in 90% RPMI 1640 and 10% FBS and A7r5 cell line in 90% DMEM supplemented with 10% FBS. The primary cultures of aortic SMCs were grown in 90% DMEM supplemented with 10% FBS. The cells were incubated in T-75 tissue culture flasks (Falcon) in an atmosphere of 37°C and 5% CO_2 in air. Confluent cell monolayers were passaged using trypsin containing EDTA.

2.7. In situ hybridization

A 402-bp CNG1 fragment cloned in pBluecripts was used to generate digoxigenin-labeled sense and antisense riboprobes using the T3 and T7 polymerases respectively (Boehringer Mannheim). The riboprobes were analyzed using gel electrophoresis and used for in-situ hybridization.

Thoracic aorta and mesenteric arteries with different diameters were dissected out from rat and the tissues were immediately frozen in embedding medium using isopentane. The isolated mesenteric arteries have the internal diameter ranging from 100 μm to 1 mm. Cryostat sections (8 μm) were cut on Cryostat (Shandon AS 620 Cryotome). Sections were transferred onto gelatin-coated glass slides, dried immediately at 50°C for 2 min and fixed with freshly prepared 4% paraformaldehyde at 4°C for 1 h. Sections were then digested with 1 μg/ml of proteinase K for 15 min, rinsed in 0.1 M triethanolamine, and acetylated in 0.25% acetic anhydride for 20 min at room temperature. Sections were prehybridized with 50% formamide containing 4× saline sodium citrate (SCC) for 30 min at 42°C. Subsequently, hybridization was performed overnight at 42°C in a humidified chamber with antisense or sense riboprobe (20 ng/ml) containing 50% formamide, 4× SCC, 0.25 mg/ml yeast tRNA, 0.25 mg/ml salmon sperm DNA, 100 mg/ml dextran sulfate and 1× Denhardt's solution. Post-hybridized sections were then washed with 2× SSC containing 50% formamide at 50°C. Excess probes were removed by digestion with RNase A (40 μg/ml) for 30 min at 37°C. Sections were finally washed sequentially with 2× SCC, 1× SCC, followed with 0.5× SCC at 37°C. Hybridized probes were detected with anti-digoxigenin antibody conjugated to alkaline phosphatase (1:500) and visualized by NBT/BCIP detection kit according to the manufacturer's instructions (Boehringer Mannheim).

3. Results

3.1. CNG1 cDNA is cloned from small mesenteric arteries

We used PCR to amplify cDNA sequence of rod-type CNG1 channel. cDNAs derived from tissues and cell lines were amplified using a pair of oligodeoxynucleotide primers. These primers were designed according to highly conserved sequence that includes the putative transmembrane spanning domains S5 and S6 and a short region in the C-terminal cytoplasmic domain. It does not include the C-terminal nucleotide-binding domain [2,18]. The primers will selectively amplify the cDNA for rod-type CNG1, but not olfactory-type CNG2 [19]. To detect lower abundance expression of CNG1 mRNA, we used 50-cycle RT-PCR. An amplification product of 402-bp was obtained from mesenteric arteries (Fig. 2). The 402-bp RT-PCR products from guinea pig and rat mesenteric arteries were the cloned and sequenced. The comparison of nucleotide sequence revealed 100% nucleotide identity to the recently published sequence of rat eye CNG1 channel (Fig. 1). Two additional PCR products were also amplified from vascular tissues,

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TC ATC ATC CAC TGG AAC GCG TGT GTG TAC TAC TCC ATC TCA AAA GCT ATT GGA TTT GGG
   Ile  Ile  His Trp Asn Ala Cys Val Tyr Tyr Ser Ile Ser Lys Ala Ile Gly Phe Gly

AAT GAC ACA TGG GTC TAC CCT GAT GTT AAT GAT CCT GAA TTT GGC CGT TTG GCT AGA AAA
Asn Asp Thr Trp Val Tyr Pro Asp Val Asn Asp Pro Glu Phe Gly Arg Leu Ala Arg Lys

TAC GTC TAC AGC CTT TAT TGG TCT ACC TTG ACT TTG ACG ACC ATT GGA GAA ACC CCA CCC
Tyr Val Tyr Ser Leu Tyr Trp Ser Thr Leu Thr Leu Thr Thr Ile Gly Glu Thr Pro Pro

CCC GTG CTG GAT TCC GAG TAT GTC TTT GTG GTG GTA GAC TTC TTA ATT GGA GTT TTA ATT
Pro Val Leu Asp Ser Glu Tyr Val Phe Val Val Val Asp Phe Leu Ile Gly Val Leu Ile

TTT GCC ACC ATT GTC GGT AAC ATA GGC TCC ATG ATT TCC AAT ATG AAT GCA GCC CGG GCA
Phe Ala Thr Ile Val Gly Asn Ile Gly Ser Met Ile Ser Asn Met Asn Ala Ala Arg Ala

GAA TTT CAA TCA AGA GTT GAT GCT ATC AAA CAG TAC ATG AAT TTT CGA AAT GTG AGC AAA
Glu Phe Gln Ser Arg Val Asp Ala Ile Lys Gln Tyr Met Asn Phe Arg Asn Val Ser Lys

GAC ATG GAA AAG AGA GTT ATT AAA TGG TTT GAC TAC CTG TGG A
Asp Met Glu Lys Arg Val Ile Lys Trp Phe Asp Tyr Leu Trp
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Fig. 1. Nucleotide and deduced amino acid sequence of a partial CNG1 cDNA clone isolated from guinea pig mesenteric arteries. It has 100% nucleotide identity to rat eye CNG1 cDNA [5]. The amino acid sequence was deduced based on the reading frame of rat eye CNG1 channel.

one with the molecular weight at ~600-bp and another at ~500-bp. We cloned and sequenced both fragments. BLAST searches with the resulting cDNA sequences did not reveal similarity with any known gene in the Genebank, indicating they are unknown gene products unrelated to CNG1 channel.

3.2. CNG1 mRNA are expressed in vascular tissues and a variety of other tissues

To study the distribution of CNG1 mRNA in vascular tissues, we performed RT-PCR from mRNA isolated from aorta, mesenteric arteries, and portal vein in rat and guinea pig. The RT-PCR amplified a 402-bp cDNA from all the vascular tissues we used (Fig. 2). The 402-bp PCR products can also be amplified from a variety of nonvascular tissues including brain, kidney, heart and testis (Fig. 3). To confirm that the 402-bp RT-PCR products from different tissues also represent authentic CNG1, we performed southern blot. The 402-bp PCR products hybridized under high stringency to a radiolabeled CNG1 probe that is internal to primers used in PCR (Fig. 2b, Fig. 3b). An additional PCR product of ~600-bp was amplified from many tissues and cell cultures (Fig. 2a). Another product of ~500-bp is also amplified from guinea pig mesenteric arteries and guinea pig aorta. These fragments did not hybridize with CNG1 probe in northern blot analysis (Fig. 2b). We cloned and sequenced this fragment. The nucleotide comparison shows no sequence similarity to CNG channels, indicating that it is an unrelated gene product. We cloned and sequenced both fragments. BLAST searches with the resulting cDNA sequences revealed no similarity with any known gene in the Genebank, indicating they are unknown gene products unrelated to CNG1 channel.

3.3. CNG1 mRNA is expressed cultured vascular endothelial cells and vascular SMCs

To locate the specific cell types which express CNG1 mRNA in blood vessels, we performed RT-PCR with mRNA from an aortic SMC line A7r5 and a vascular endothelial cell line ECV304. The primary cultures of

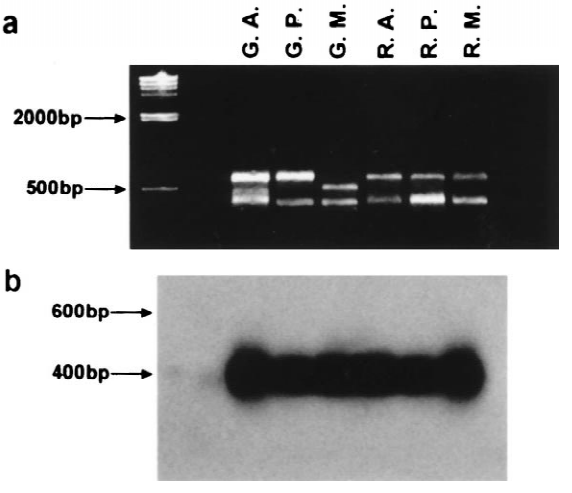

Fig. 2. RT-PCR-based detection of CNG1 mRNA in vascular tissues of guinea pigs and rats. (a) Ethidium bromide-stained agarose gel; (b) gel shown above was transferred to a nylon membrane and hybridized with a CNG1 probe. (a) and (b) G.A., guinea pig aorta; G.P., guinea pig portal vein; G.M., guinea pig mesenteric arteries; R.A., rat aorta; R.P. rat portal vein; R.M. rat mesenteric arteries.

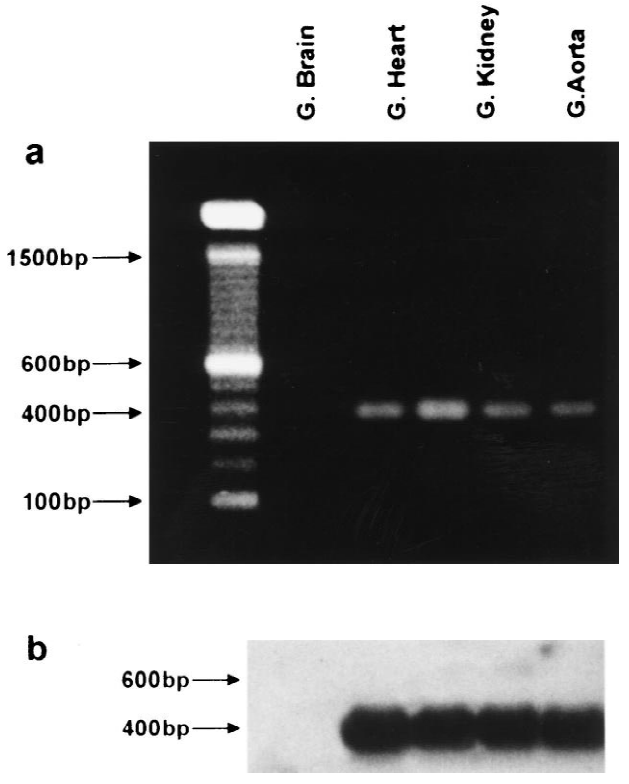

Fig. 3. RT-PCR-based detection of CNG1 mRNA in a variety of other tissues. (a) Ethidium bromide-stained agarose gel; (b) the gel shown above was transferred to a nylon membrane and hybridized with a CNG1 probe. (a) and (b): G., guinea pig.

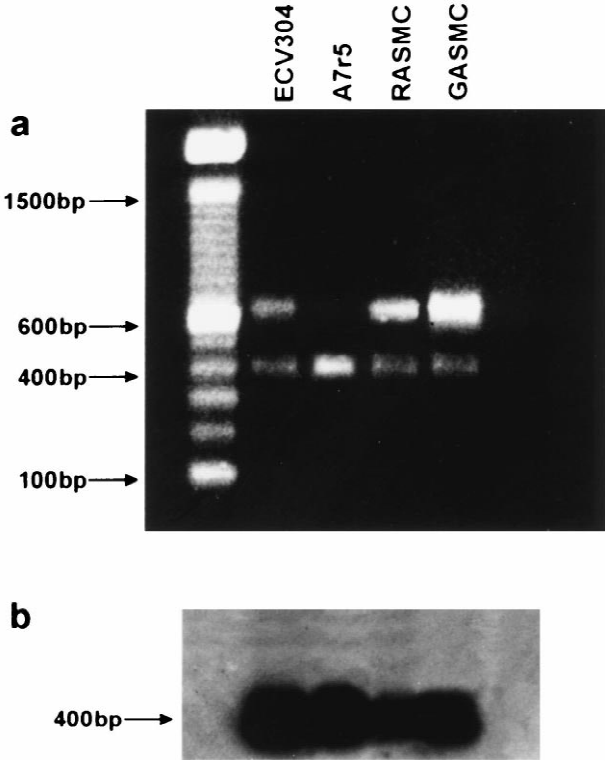

Fig. 4. RT-PCR-based detection of CNG1 mRNA in cultured vascular cells. (a) Ethidium bromide-stained agarose gel; (b) the gel shown above was transferred to a nylon membrane and hybridized with a CNG1 probe; (a) and (b); ECV304, vascular endothelial cell line ECV304; A7r5, vascular smooth muscle cell line A7r5; RASMC, primary culture of rat aortic smooth muscle cells; GASMC, primary culture of guinea pig aortic smooth muscle cells.

aortic smooth muscles were also generated from guinea pigs and rats, and RT-PCR was performed. The 402-bp PCR fragments can be amplified from vascular endothelial cell line and all the SMC cultures (Fig. 4). Southern blot analysis revealed that these 402-bp products hybridized under high stringency to a radiolabeled CNG1 (Fig. 4b). This result confirmed that the 402-bp PCR products are amplified from CNG1 mRNA. Again, a PCR product of ~600-bp was amplified from many cultured cell lines. The sequencing analysis revealed that it is the same gene product as the ~600-bp product in Fig. 2a. It belongs to a unknown gene unrelated to CNG1 channel.

3.4. Northern blot detects CNG1 transcript in cultured vascular endothelial cells and brain tissue

Northern blot analysis was performed to identify tissues or cells in which CNG mRNA is frequent. A transcript of ~3.2 kb was observed in poly(A)$^+$ RNA from the vascular endothelial cell line ECV304 and brain tissue (Figs. 5 and 6). The size of ~3.2 kb agrees reasonably with the size of CNG1 transcript from rod and kidney cell line M1CCD [7,20]. No signal was detected in cultured smooth cells, lung, heart or kidney tissues. Two additional transcripts of ~5 kb and ~1.8 kb were also detected from the endothelial cell line ECV304. Since the northern blot did not detect

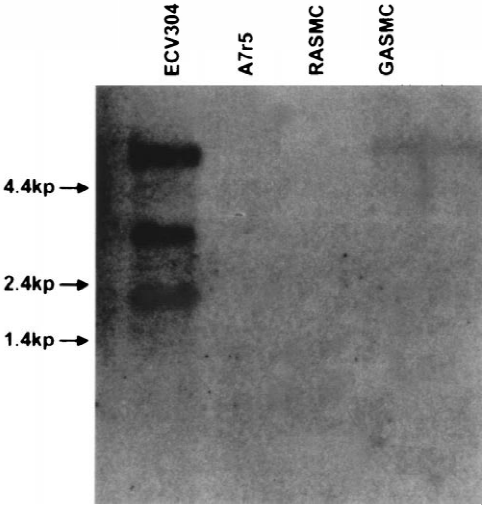

Fig. 5. Northern blot hybridization of CNG1 mRNA from cultured vascular cells. Poly(A)$^+$ RNA (5 μg) from each cultured cells were analyzed with a CNG1 probe. Position of size markers were determined by ethidium bromide staining. ECV304, vascular endothelial cell line ECV304; A7r5, vascular smooth muscle cell line A7r5; RASMC, primary culture of rat aortic smooth muscle cells; GASMC, primary culture of guinea pig aortic smooth muscle cells.

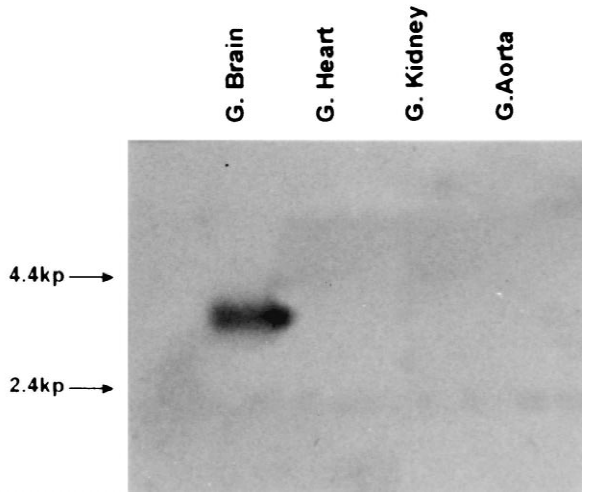

Fig. 6. Northern blot hybridization of CNG1 mRNA from a variety of tissues. Poly(A)$^+$ RNA (5 μg) from each cultured cells were analyzed with a CNG1 probe. Position of size markers were determined by ethidium bromide staining; G., guinea pig.

these transcripts in other tissues which do not abundantly express the CNG1 mRNA, they are probably related to the CNG1 channel. The larger transcript (~5 kb) may represent an unprocessed RNA and the smaller product (~1.8 kb) may be a partial breakdown product. Ding et al. also detected the similar transcripts with molecular weight of ~5 kb and ~1.8 kb in their northern blot studies of CNG1 channel in rat kidney, adrenal gland, spleen and eye.

3.5. In situ hybridization labels the endothelium layer in large and small arteries

In situ hybridization was used to further explore the cellular localization of the CNG1 mRNA in vascular tissues. Tissue sections from guinea pig thoracic aorta and mesenteric arteries were studied with sense and antisense riboprobes. We used different mesenteric arteries with the internal diameters ranging from 100 μm to 1 mm. In control experiments, hybridization with the sense riboprobe did not label any vascular structure (Fig. 7d, e and f). In contrast, hybridization of blood vessel sections with the antisense CNG1 probe yielded strong labeling in the endothelium layer but no labeling in tunica media layer which is composed of many SMCs, collagenous fibers, elastic fibers, and fibroblasts (Fig. 7a, b and c). The hybridization pattern is similar in aorta, medium-sized mesenteric arteries about 1 mm in diameter, and small-sized mesenteric arteries with the internal diameter ranging from 100 to 300 μm. Strong labeling was also observed in numerous structures in the outermost tunica adventitia layer (Fig. 7a, b and c), but we were not able to determine the cell origin of these labeled structures. Since the CNG1 probe did not label SMCs and fibroblasts in tunica media, these labeled structures in tunica adventitia might belong to other cell types.

4. Discussions

CNG channels are a group of Ca^{++}-permeable NSC channels. In retinal rods, the native CNG1 channel is a hetero-oligomer consisting of structurally different α and β subunits [21] with the α subunit encoded by CNG1 mRNA. We have performed RT-PCR with mRNA isolated from a variety of vascular tissues including aorta, mesenteric arteries and portal veins. The results indicate that CNG1 mRNA is expressed in all the vascular tissues tested. To locate specific cell types which express CNG1 mRNA in vascular tissues, we performed RT-PCR and northern blot in cultured vascular endothelial cells and vascular SMCs. RT-PCR amplification is a very sensitive method allowing the detection of only a few transcripts per cell, whereas northern blot is a much less sensitive detection method allowing the identification of tissues or cells in which CNG1 mRNA is abundantly expressed. Both RT-PCR and northern blot detected positive signals in cultured endothelial cells, indicating high abundant presence of CNG1 mRNA in cultured vascular endothelial cells. To study whether in vivo endothelium also expresses CNG1 channel, we performed in situ hybridization with digoxigenin-labeled riboprobes. In situ hybridization is another relatively insensitive method. Fig. 7 illustrates that the antisense CNG1 probe labels the endothelium layer of aorta, medium-sized mesenteric arteries about 1 mm in diameter, and small mesenteric arteries with diameter ranging from 100 to 300 μm, indicating an abundant presence of CNG1 mRNA in the endothelium layer of these arteries.

Medium-sized arteries have also been called distributing arteries because they distribute the blood to different organs and, by contraction and relaxation, aid in regulating the blood supply to different regions in response to different functional demands. Small arteries including the small mesenteric arteries belong to the resistance arteries which are the principle sites for the peripheral resistance [22]. The abundant presence of CNG1 mRNA suggests a possible role of CNG1 protein in the Ca^{++} entry in the endothelium of these arteries, therefore implicates a possible involvement of CNG1 protein in the regulation of blood supply to different tissues and in the regulation of arterial blood pressure.

Several studies have identified Ca^{++}-permeable channels in single channel recordings from cultured vascular endothelial cells [23–30]. However, none of these reported channels, with single channel conductance ranging from 8 to 120 pS, may mediate the main Ca^{++} entry pathway elicited by vasoactive agonists. The agonist-activated Ca^{++} entry may be mediated by a low conductance Ca^{++}-permeable NSC channel which has not yet been identified by single channel patch clamp recording [31]. It is difficult to determine whether any of the reported channels represents the CNG channel since in most cases only limited electrophysiological studies have been carried out and

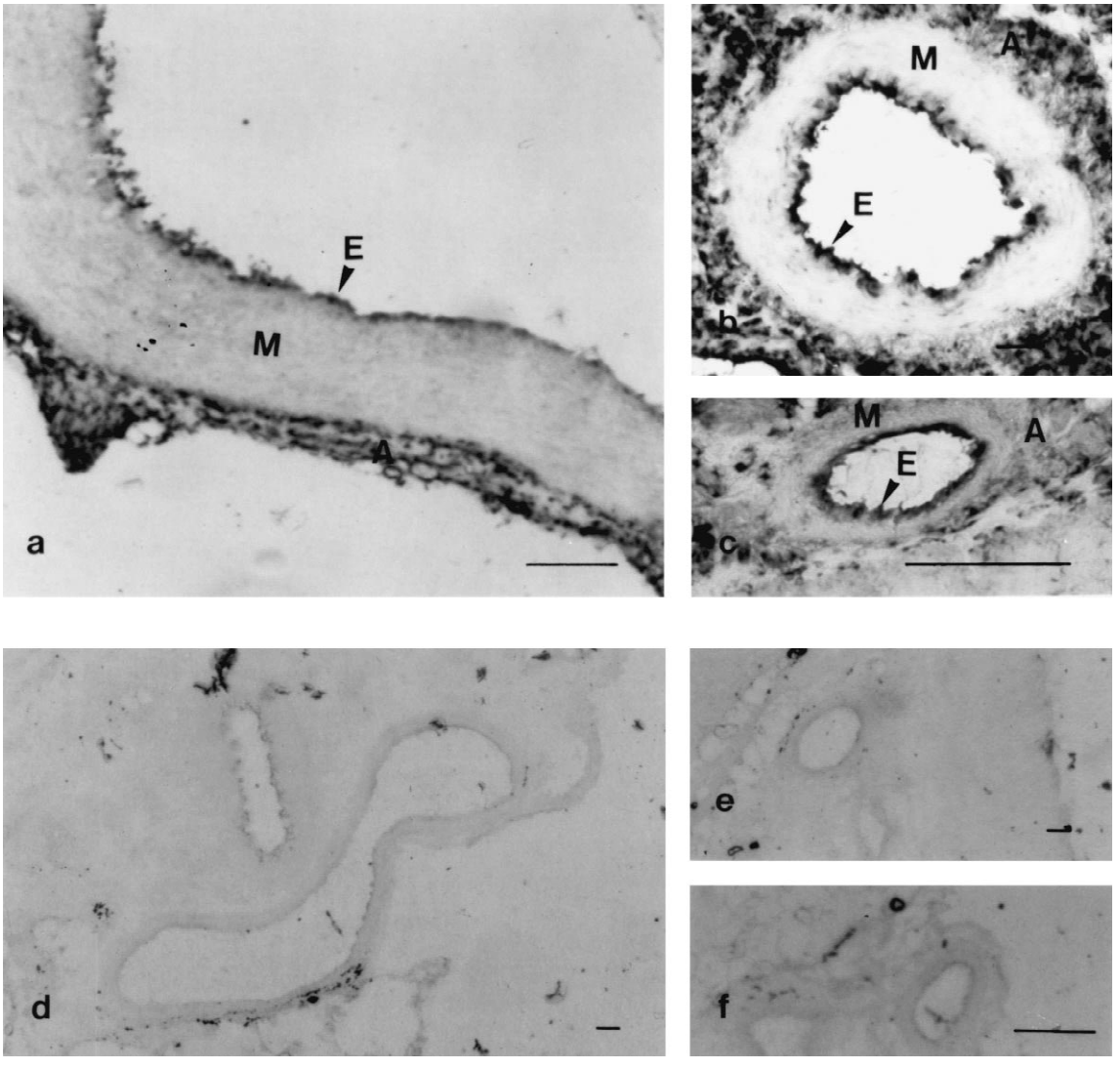

Fig. 7. Expression of CNG1 mRNA in the arteries with different diameters. Note that CNG1 mRNA was abundantly expressed in the endothelium of all-sized arteries whereas the tunica media layer was not labeled. Some unknown structures in the tunica adventitia layer were also labeled. (a) (b) and (c) were stained with digoxigenin-labeled antisense riboprobe. (d) (e) and (f) were the control stained with sense probe. (a) and (d), guinea pig aorta; (b) and (e), guinea pig medium-sized mesenteric artery; (c) and (f), guinea pig small-sized mesenteric artery. E, endothelium; E, tunica media; A, tunica adventitia. Bar=200 μm.

none of the studies tested nucleotide-sensitivity of these channels. Nevertheless, since CNG1 mRNA encodes the α subunit of CNG1 channel which is a Ca^{++}-permeable NSC channel, it is possible that CNG1 protein may constitute one or more subunit(s) in those reported channels. Moreover, CNG1 channel is a low conductance channel at physiological concentration of extracellular Ca^{++} (mM) [1], thus it might represent potential candidate for the yet unidentified NSC channel which is responsible for the main Ca^{++} entry elicited by vasoactive agonists.

Our data are also consistent with the following observations which also agree with the potential role of CNG channels in Ca^{++} entry in vascular endothelium: (1) Vasoactive agents stimulate the entry of extracellular Ca^{++} in vascular endothelial cells through the depletion of intracellular Ca^{++} store. The depletion of internal Ca^{++} store stimulates NSC channels in plasma membrane and thus promotes the entry of extracellular Ca^{++} in the endothelial cells [28,32]. These depletion-activated NSC channels resemble CNG channels in that they are regulated by cGMP at least in pancreatic acinar cells [33].(2) Following stimulation by vasoactive agents, Ca^{++} entry in vascular endothelial cells increases, and intracellular cGMP and cAMP contents are also elevated [34–37]. Since CNG channels are regulated by cyclic-nucleotides, they may provide a possible link between the elevation of cyclic-nucleotides and Ca^{++} entry.

A cGMP-inhibited Ca^{++}-permeable NSC channel has recently been identified in vascular smooth muscles by single channel patch clamp recording [15]. This channel

might be another important target of nitrovasodilators in addition to the well known voltage-gated Ca^{++} channels in these cells. We performed RT-PCR with mRNA isolated from a variety of cultured vascular SMCs which include a commercial cell line A7r5 from ATCC and two primary cultures of aortic SMCs from rats and guinea pigs respectively. CNG1 mRNA was detected in all three cultures of vascular SMCs. Based on the fact that CNG1 mRNA encodes a cGMP-sensitive Ca^{++}-permeable NSC channel, it is likely that CNG1 protein may constitute one or more subunit(s) in this cGMP-inhibited Ca^{++}-permeable NSC channel in vascular SMCs, thus contributing to the vasoregulation. However, our northern blot analysis did not detect any signal in cultured SMCs. Furthermore, in situ hybridization analysis did not label the tunica media layer that contains many SMCs. These results suggest that CNG1 protein is probably only expressed at a low level in the vascular SMCs, at least in the basal unstimulated condition.

Besides rod-type CNG1 channel, an olfactory-type CNG2 channel has been reported in rabbit aorta, though its tissues distribution remains unsolved [6]. Ding et al. proposed that CNG1 is distributed in endothelial tissues, whereas CNG2 may be restricted to the muscular layer [5]. Since our data clearly show the presence of CNG1 mRNA in both the vascular endothelium and vascular SMCs, a more reasonable hypothesis is that CNG1 and CNG2 are coexpressed in vascular endothelial and/or vascular SMCs. Hetero-oligomerization of CNG1 and CNG2 proteins may confer additional properties to the NSC channels in vascular cells.

We were also able to observe CNG1 mRNA in a variety of other tissues including brain, heart, kidney and testis by the use of PT-PCR. However, northern blot analysis did not detect any signal except in the brain tissue. These results suggest that CNG1 protein is abundantly expressed in brain, but less abundantly expressed in most nonsensory tissues. Since electrophysiological studies in most nonsensory tissues have not revealed any channel which exhibits identical electrophysiological properties to those of rod-type CNG1 channel, it is probable that CNG1 protein may form hetero-oligomer with other proteins and resulting functional channel may display electrophysiological properties which are different from rod-type CNG1 channel.

In conclusion, we have cloned a partial cDNA for CNG1 channel in mesenteric arteries, and demonstrated the expression of the CNG1 mRNA in medium-sized arteries, small-sized arteries, aorta and veins. Within the vascular tissues, CNG1 mRNA is abundantly expressed in vascular endothelial cells, and less abundantly expressed in vascular SMCs. The presence of CNG1 mRNA suggests a potential role of CNG1 protein for the Ca^{++} entry in vascular endothelial and vascular SMCs and thus a possible involvement of CNG1 protein the regulation of blood distribution in different regions and in the regulation of arterial blood pressure.

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