行政院國家科學委員會專題研究計畫　成果報告

CLC-3 氯離子通道在老鼠陰莖海綿體平滑肌之分佈與分子生物表現

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中英文摘要

（一）計畫中文摘要。

關鍵字：氯離子通道、海綿體平滑肌

氯離子通道在血管平滑肌的電生理學上扮演重要的角色。在包括血管的許多組織上給予 Agonist，可以誘發出氯離子電流。最近的研究顯示無論使用干擾氯離子穿越細胞膜或阻斷氯離子通道的方法，都可以降低新腎上腺素對血管平滑肌產生的收縮。而陰莖海綿體擁有獨特的膨脹與消脹的能力，基於陰莖海綿體與血管在構造與藥理學上的相似性，我們可以預測氯離子對調節陰莖海綿體平滑肌張力〔也就是勃起功能〕扮演一定的角色。由先前的計畫得知 CLC-3 氯離子通道蛋白質是存在於老鼠陰莖海綿體平滑肌細胞及組織上的，進一步的免疫組織化學分析與 Northern Blot 分析將有助於進一步了解 CLC-3 氯離子通道在海綿體平滑肌上的分子生物學表現。實驗的內容包括

1. 免疫組織化學分析
   探測老鼠的海綿體平滑肌組織上 CLC-3 氯離子通道蛋白質的分佈情形。
2. 探針的製備
   使用 PCR 放大的方法來製備探針.
3. Northern Blot 分析
   探測老鼠的海綿體平滑肌組織與細胞上 CLC-3 氯離子通道基因的表現.

實驗結果得知使用免疫組織化學的分析的確可以在老鼠的海綿體平滑肌組織上偵測到 CLC-3 氯離子通道蛋白質的存在。而使用 PCR 放大的方法亦可以製備出要用的探針。

（二）計畫英文摘要。

KEYWORDS: ClC-3, Chloride channel, Corpus cavernous smooth muscle

Chloride ion currents play a critical role in vascular smooth muscle electrophysiology. Agonist-induced, inward chloride currents have now been characterized in numerous tissues, including vessels. Recent studies demonstrate that interfering with either the distribution of chloride across the membrane or the ability of chloride channels to open markedly suppresses contractile responses of vascular smooth muscle to norepinephrine. Penile corpora own the unique ability to inflate and deflate itself. Based on the obvious similarities in the structural and pharmacological properties between cavernous myocytes and vascular smooth muscle cells, Cl^- might be expected to also play a role in the regulation of the cavernous smooth muscle tone, and thus, the erectile function. Our previous studies showed that the CLC-3 chloride channel proteins could be detected in rat corpus cavernous smooth muscle tissue and cells via western blot. This result means more extensive molecular studies of chloride channels in erectile tissue may be necessary to distinguishing its possible physiological role. We therefore conducted a strategy involving:

1. Immunohistochemistry
   Detection of the distribution of CLC-3 chloride channel protein in rat corporal tissues and culture cells.
2. Cloning and preparation of probes
   The probes are synthesized by the primers by PCR amplification.
3.. Northern blot analysis
   Detection of CLC-3 chloride channel gene expression in rat corporal tissues and culture cells.

The results revealed that the method of immunohistochemistry stain could identify the distribution of CLC-3 chloride channel protein in could be detected on rat corporal tissues. The probes could be synthesized by the primers by PCR amplification.

目錄

報告內容

（一）前言、研究目的及文獻探討

It has been proved that chloride ion currents play a critical role in vascular smooth muscle electrophysiology. Agonist-induced, inward chloride currents have now been characterized in numerous tissues, including vessel (1). Chloride currents can be activated by norepinephrine in myocytes from portal vein, mesenteric vein and ear artery (2-4). A similar current is elicited by endothelin in coronary, aortic and mesentery vascular smooth muscle cells (5,6) as well as by vasopressin in cultured aortic cells (6,7). Recent studies demonstrated that interfering with either the distribution of chloride across the membrane or the ability of chloride channels to open markedly suppresses contractile responses of vascular smooth muscle to norepinephrine (8-10). It indicated that chloride channels were of functional importance in the regulation of vascular smooth muscle tone.

During the past years, several types of chloride channels (11-15) have been proposed on vascular smooth muscle cells by using electrophysiologic methods although the exact molecular structure and its correlation with physiological function remain uncertain. Calcium-dependent chloride channel is found to be involved in the agonist-induced activation of vascular smooth muscle (9) but it has not been identified at the molecular level. A cloned chloride ion channel from bovine tracheal epithelial cells is calcium-sensitive but its expression is limited to epithelium (16). Volume-activated chloride current (IClvol) is suggested to be possessed by virtually every cell and is activated in response to cell swelling (14, 15) or a reduction in intracellular ionic strength (17). In dispensable tissues such as blood vessels it is unclear if its function is purely related to the detection of osmotic stress or if it can be activated by other mechanisms of membrane stretch. The swelling-induced current has been linked to a number of gene products. ICln (18) has been suggested to code for the volume-sensitive organic osmolyte

and anion channel (VSOAC) (19), however, controversy remains regarding the function of this protein (20). More recently, the CLC family of voltage-dependent chloride channels (21, 22), of which there are currently 9 human members (CLCN1-7, CLCNKa and CLCNKb), has been documented. The CLC-3 gene expression was demonstrated in various animal tissues by using RT-PCR technique (23, 24). In NIH/3T3 cells, it has been shown to produce a volume-dependent current, which has many biophysical similarities to the native ICl_{vol} (25). Mutations in the CLCN1 gene account for both dominant and recessive forms of human myotonia (26) while mutations in CLCN5 result in nephrolithiasis (27). A spectrum analysis of chloride channels in smooth muscle cells from human aorta and coronary artery has been performed, and CLCN3 is disclosed to be the most abundantly expressed chloride channel gene (28).

Our previous studies(29) showed that the CLC-3 chloride channel protein could be detected in rat corpus cavernous smooth muscle tissue and cells via western blot method. This result means more extensive molecular studies of chloride channels in erectile tissue may be necessary to distinguishing its possible physiological role. We therefore conducted a strategy involving:

(二) 研究方法

Tissue preparation and cell culture

12-14 week old adult male S-D rats were sacrificed to get penis. For entire tissue block, the corporal tissues were put into liquid nitrogen container immediately after removal and then stored in –80 °C freezer until use.

Methods of cell culture: Surgical specimens were minced to 1mm^3 pieces, washed in Hank's balanced salt solution without calcium and magnesium and transferred to Falcon 100X20 mm tissue culture dish (Becton Dickinson Labware, Franklin Lake, NJ) in Dulbecco's minimal essential media (DMEM, containing 1000 mg. Glucose/l and supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 U/ml streptomycin and 2 mM glutamine. Smooth muscle cells migrated out from the explants in 7 to 14 days. At this time, the explants were removed, and cells were allowed to achieve confluence.

Immunohistochemistry

S-D rat penis are perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS), further fixed with paraformaldehyde for 30 min, washed (4 times for 15 min) in PBS, and cryoprotected in a graded series of sucrose solutions (5, 10, 15, and 20% wt/vol made up in PBS, 1 h each). The whole penis are then embedded in Tissue Tek embedding medium (Miles, IL) and 20% sucrose in PBS (1:2 vol/vol) and rapidly frozen in isopentane precooled in liquid nitrogen. Cryosections were cut with a Leica CM 3500 cryostat at a thickness of 10 μm and were collected on Vectabond (Vector Laboratories, Burlingame, CA)-coated microscope slides. Sections were initially blocked with 10% BSA for 1 h and then incubated overnight with anti-ClC-3 antibodies were raised in rabbit (Alomone Labs, Jerusalem, Israel), washed with PBS, and incubated for 1 h with fluorescein isothiocyanate (FITC) or Texas Red anti-rabbit secondary antibodies at 5 μg/ml (Vector Laboratories), washed with PBS (3 times for 10 min), and mounted with Aquamount (Lerner Laboratories, Pittsburgh, PA). Colocalization studies were performed by first incubating

sections with ClC-3 antibody and Texas Red anti-rabbit secondary antibody. Sections incubated *1*) without primary antibodies or *2*) with preabsorbed primary antibodies (absorbed for 1 h with appropriate antigen) were used as negative controls. Sections were examined with the use of a Bio-Rad MRC 600 confocal microscope with excitation wavelengths appropriate for FITC and Texas Red. Confocal micrographs were obtained from digital composites of two-serial scans of 10 optical sections (*Z*) through a depth of 10 µm (10 × 1 µm), *Z* series were constructed with Bio-Rad Comos software, and final images were prepared using Adobe Photoshop software.

Cloning and preparation of probes

The primers used for PCR amplification of segments of the CLCN3 chloride channels are listed as followed:

Gene	Genbank No.	Primers	Nucleotides	Product (bp)
CLCN3	X78520	F-CAAGAAGGGAGTGTTTCTTGTGC R-GCATAAGACCCGGTCACAATTA	3595-3617 3843-3822	249

Nucleotide numbers represent position of the primers within the clones described under each Genbank accession number. Accession numbers all refer to full-length clones.

Target regions were chosen for their uniqueness and lack of homology to other members of the CLCN family. The majority of the clones are from the 3'' untranslated region. Oligonucleotides were synthesized on a Model 931 DNA Synthesizer (Applied Biosystems, Norwalk, cr, USA), deprotected, desalted and quantified with a uv/VIS spectrophotometer (Beckman, Fullerton, CA, USA) using an extinction coefficient of 1 optical density = 30 ng/µl. PCR reaction contained on µl of human aortic Quick Clone cDNA (Clontech Laboratories, Inc., Palo Alto, CA, USA) as template, 1 µM of each oligonucleotide, 200 µM of each dNTP, 0.25 U of Thq DNA polymerase (Boebringer Mannheim, Indianapolis, IN, USA), 10mM tris-HCl pH 8.3, 1.5mM $MgCl_2$ and 50 mM KCl in a total volume of 10 pµ1. Usual PCR parameters consisted of an initial denaturation step at 94^0C for 2 mm followed by 35 cycles of 94^0C for 30 s,

55^0C for 30 s, and 72^0C for 30s. PCR products were cloned into the T-tailed plasmid vector pKRX (Schutte *et al.,* 1997) and sequenced at the University of Iowa DNA core facility (Director, David Moser, University of Iowa, IA, USA) using a model 373A automated DNA sequencer (Applied Biosystems, Norwalk, CT, USA).

Northern blot analysis

Radiolabelled probes for Northern-blot analysis are prepared by PCR (Mertz and Rashtchian, 1994) using the above clones as template and the same primers which are used to produce the clones. Total RNA is extracted from rat corpus cavernosal smooth muscle cells by using TRIzol. Northern blots are prepared by running 20 μg of total RNA/lane plus a 0.24– 9.5 Kb RNA ladder (Gibco BRL, Gaithersburg, MD, USA) out on a (1.85%) formaldehyde/agarose gel and then transferred onto a Nilon membrane. The concentration of target RNA is normalized to β-actin expression.

(三) 結果與討論

1. Immunohistochemistry

Immunohistochemistry revealed the distribution of CLC-3 chloride channel protein on rat corpus cavernosum. Increased fluorescent uptake could be observed within the myocytes. (Fig. 1) This finding confirms the previous result of western blot in advance and indicates that CLC-3 chloride does exist on rat corpus smooth muscle tissue.

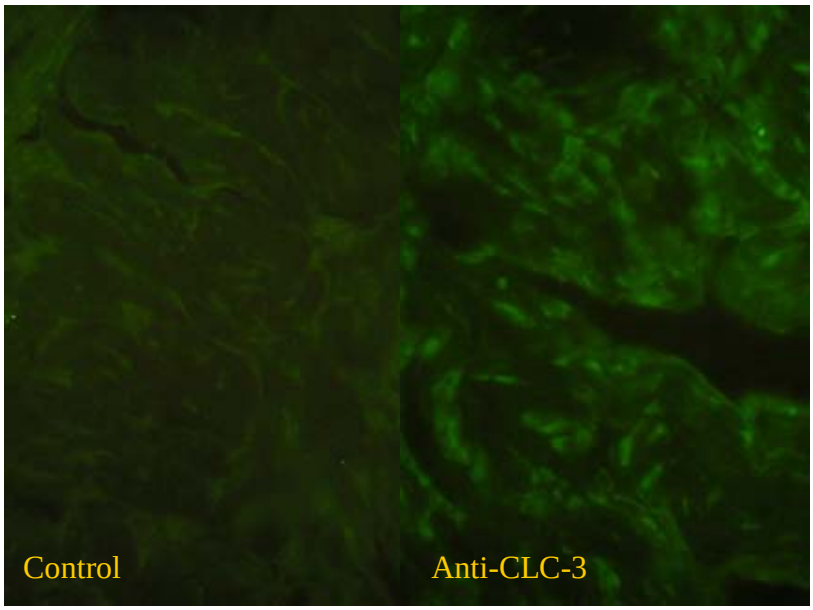

Figure 1. Immunohistochemcal stain of CLC-3 chloride channels.

2. Northern blot analysis

Although several probes has been cloned and prepared by PCR methods, northern blot analysis could not reveal good band yet. This result does not contradict the presence of CLC-3 chloride channel, but represent either the probes are not suitable or the mRNA of CLC-3 chloride channel can’t be expressed in this methods.

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計畫成果自評

在 Immunohistochemistry 方面，進行的尚稱順利。免疫螢光染色法確實可以於老鼠陰莖海棉體偵測到平滑肌上的 CLC-3 氯離子通道，與控制組有明顯的差別。

在 mRNA 的偵測上，曾製備數種 probe。但由於時間與預算的限制，尚未於 Northern blot 上偵測出來，實屬美中不足。分析其原因，可能是未能製備到合適的 probe，需要更多的時間與經費來努力才行。