日本財団補助金による
1998 年度日中医学協力事業報告書

－在留中国人研究者研究助成－

1999 年 3 月 15 日

財団法人 日中医学協会

理事長 中島章 殿

研究室で撮影した本人のスナップ写真、及び発表論文のコピーを添付

1．研究者氏名 陳潔

研究機関 東京大学医学部附属病院 研究指導者 豊岡照彦 職名 教授

所在地 〒113 東京都文京区本郷7-3-1 電話 3815-5411 内線 3074

研究テーマ 血管平滑筋細胞内イノシトールミリン酸誘発性 Ca^{2+} 放出と細胞増殖における1と3型イノシトールミリン酸受容体の異なる役割の解明

2．本年度の研究業績

(1) 学会・研究会等においての口頭発表　有 ・ 無　(学会名・内容)

1. 作者：Yuepeng Wang, Jie Chen, Wang Yue, Wee Soo Shin, Aiji Sakamoto, Teruhiko Toyo-oka.
   学会：XIII International Congress of Pharmacology , Munchen, Germany, July, 1998
   題目：Intact 1,4,5-trisphosphate-induced Ca^{2+} release and capacitative Ca^{2+} entry partially defrays the effect of autocrined nitric oxide on intracellular Ca^{2+} concentration in endothelial cells.
   雑誌：*Archives of Pharmacology* 1998; 358 (suppl I):R300 (abstr)
2. 作者：Jie Chen, Yuepeng Wang, Wang Yue, Wee Soo Shin, Aiji Sakamoto, Toshiaki Nakajima, Kuniaki Iwasawa, Teruhiko Toyo-oka.
   学会：The 71th Scientific Session of American Heart Association, Dallas, USA, Nov., 1998.
   題目：Nitric oxide buffers intracellular Ca^{2+} dynamics by its opposite autocrine actions on inositol 1,4,5-trisphosphate-induced Ca^{2+} release and capacitative Ca^{2+} entry in endothelial cells.
   雑誌：*Circulation* 1998; 96(suppl I): I-667 (abstr)
3. 作者：王岳鵬、陳潔、王岳、申偉秀、岩沢邦明、中島敏明、豊岡照彦
   学会：第６３回日本循環器学会総会　東京　1999年3月
   題目：内因性Nitric Oxideは血管平滑筋細胞(VSMC) 内I型IP_3受容体 (IP_3R_1) とIP_3-Induced Ca^{2+} Release (IICR)をDown-Regulateする事でVSMC増殖を抑制する
   雑誌：*Japanese Circulation Journal* 1999; 63(Suppl I): in press

(2) 学会誌等に発表した論文　有 ・ 無　(雑誌名・論文名)

1. 王岳鵬、陳潔、王岳、豊岡照彦　第２章 "NOと細胞間クロストーク"、in 特集：NOと治療薬、治療学 (Biomedicine & Therapeutics)、平成１１年６月出版、ライフサイエンス社。
2. 近く投稿する英文論文三つ。

3．今後の研究計画

1）速やかに今の研究成果を英文雑誌に投稿します。

2）血管平滑筋細胞の増殖におけるIP_3R_3の長期間の役割をDNA合成時の^{3}H-thymidine取り込み量で評価する。共存培養の血管平滑筋細胞では、in situ hybridizationでIP_3R_1の発現変化を確認する。内皮細胞では、ecNOS又はIP3R1の全長のcDNAを細胞に導入する。内皮細胞内のecNOSとIP3R1蛋白質をover-expressionさせた後、IICR、CCEとNOの変化をpatch-clampで解析し、NOのCCEを促進する機構を検討する。血管平滑筋細胞では、*trp/trpl*、電位依存性Ca^{2+} channel、細胞膜Ca^{2+} pump、及びcalsequestrin等を別々にantisenseでblockし、その抑制作用を細胞内Ca^{2+}動態測定又は電気生理的解析で確認した上、IICR/CCE/NOの機能変化と細胞増殖能の変化を解明する。内皮細胞内NOはどのようなCa^{2+}channelを介してCCEを促進するか解明する。血管平滑筋細胞と比較し、NOは血管平滑筋細胞に強い作用を示す原因はNOのCCEに作用機構の違いによるものか否かを確認する。更に、PDGF等の刺激後のc-fos/rasの発現変化を求める。

3）上記の培養細胞での成果を*in vivo*に応用する為、動脈硬化の動物modelを作る。Ca^{2+} 動態の修飾操作の視点から血管平滑筋細胞の増殖とrestenosisを抑制する。AAVを用いて、β-galをreporter geneとしてratの動脈硬化modelの頚動脈に遺伝子を導入する。IP_3R_1、IP_3R_3、*trp/trpl*、電位依存性Ca^{2+} channel、細胞膜Ca^{2+} pump、calsequestrin等の発現をantisense DNAでblockし、細胞増殖の抑制能力を評価し、効率の良い方法を見つける。

4．研究指導者の意見

この一年間、貴協会から研究助成を頂きまして、本当に有難うございました。陳潔さんは９４年８月から客員研究員として本内科に私費留学していました。その時、経済的困難を克服し、日本語を勉強しながら研究技能を学びました。９７年４月大学院に入学し、血管内皮細胞と血管平滑筋細胞の相互作用の分子機序の研究に従事しています。この数年間、彼女が毎週の月曜から日曜まで、研究室で自分の研究課題に没頭に取り込むでいます。

現在、彼女は：１）細胞内IP_3受容体抗体やantisenseを細胞内にﾏｲｸﾛｲﾝｼﾞｪｸｼｮﾝ法で直接注入、細胞機能に傷害を与えずに蛋白機能や発現をする実験を行い、既に一部は成功して去年の米国心臓学会で発表しました。２）又、そのﾏｲｸﾛｲﾝｼﾞｪｸｼｮﾝの欠点を見つけ、直接にantisense DNAのconstruct vectorを自分で設計と作成し、細胞内にtransfectionし、大量のantisense DNAを発現させ、細胞自身からの目的蛋白質のmRNAの翻訳を遮断する。本法は従来遮断薬剤が無ければ解析が不可能だった既法の欠点を本質的に解決する画期的な薬理学的手法となります。３）更に、研究室の同僚と協力して、内皮細胞内IP_3-induced Ca^{2+} release、capacitative Ca^{2+} entryと一酸化窒素三者間の短期間の相互調節に関する研究を完成し、現在投稿中である。４）その他、彼女は同僚と協力して、内因性一酸化窒素は血管平滑筋細胞内１型IP_3受容体とIP_3-induced Ca^{2+} releaseをdown-gegulateして血管平滑筋細胞増殖を抑制するとの新しい信号伝達系を証明しました。血管平滑筋細胞増殖における３型IP_3受容体の役割も解明しました。従って、彼女がこの一年間に従事している研究も大詰めの段階に来て、その結果を二つか三つの論文にまとめ、来月中に投稿予定であります。留学生として良く頑張りましたと思います。

研究指導者氏名　豊岡照彦　㊞

5．研究報告

別紙形式を参考に、報告本文4000字以上で報告して下さい（枚数自由・ワープロ使用）

タイトル・要旨等は日本語で、KEY WORDS以下は日本語或いは英語で記入して下さい。

研究成果の発表予定がある場合は発表原稿・抄録集等を添付して下さい。

論文発表に当っては、日中医学協会－日本財団補助金による旨を明記して下さい。

研究テーマ：血管平滑筋細胞内イノシトール三リン酸誘発性Ca^{2+}放出と細胞増殖における1と3型イノシトール三リン酸受容体の異なる役割の解明

研究者氏名: 陳　潔

中国での所属、役職: 上海中医薬大学附属龍華病院内科　医師

日本での指導者氏名、所属、役職： 豊岡照彦、東京大学医学部第二内科　教授

要旨：ホルモンと成長因子等はG-蛋白質又はtyrosine-kinaseと繋ぐ受容体を刺激する事によって細胞内Ca^{2+}　動態信号を介して細胞増殖を制御する。イノシトール三リン酸受容体チャンネルを介するイノシトール三リン酸 (IP_3)誘発性Ca^{2+}放出 (IICR)は細胞内重要なCa^{2+}動態信号です。免疫染色法で血管平滑筋細胞には、1型(IP_3R_1)と3型(IP_3R_3)イノシトール三リン酸受容体が存在する事を示した。しかし、そのIP_3R_1とIP_3R_3はCa^{2+}動態と細胞増殖における役割が不明である。血管平滑筋細胞の異常増殖は高血圧、動脈硬化、及びPTCA治療後冠動脈再狭窄の成因となっているので、IP_3R_1とIP_3R_3は細胞増殖における役割の解明は基礎医学に限らず、臨床治療法の開発にも急務である。培養の血管平滑筋細胞内にIP_3R_1とIP_3R_3に対する特異的機能抗体を微注射すると、IP_3R_1に対する抗体だけがvasopressinに対するIICR反応を抑制できた。IP_3R_1とIP_3R_3のantisense-遺伝子をvectorに差し入れ、血管平滑筋細胞に導入し、細胞自身からのIP_3R_1とIP_3R_3のmRNAの翻訳を遮断し、30-40%のreporter vector(GFP)の陽性細胞ではIP_3R_1とIP_3R_3の発現を阻止した。IP_3R_1の場合、機能的に細胞のvasopressinに対するIICR反応と細胞増殖を部分又は完全に抑制した。IP_3R_3の場合、細胞のvasopressinに対するIICR反応と細胞増殖を抑制しなかった。しかし、IP_3R_3のantisense　遺伝子を安定的に細胞内へ導入したcoloniesはthapsigarginに対する容量性Ca^{2+}流入反応は低下し、細胞増殖も減速した。血管平滑筋細胞では、IP_3R_1はIICR反応と細胞増殖に重要な役割を果し、IP_3R_3は直接IICR反応に影響せず、長期的に細胞増殖に一定の作用を有する。

KEY WORDS: Inositol 1,4,5-trisphophate receptor, Ca^{2+} dynamics, Proliferation, Vascular smooth muscle cells, Antisense DNA, Transfection

Different Role of Type-1 and Type-3 Inositol 1,4,5-Trisphosphate Receptors In Inositol 1,4,5-Trisphosphate-Induced Ca^{2+} Release and Proliferation of Vascular Smooth Muscle Cells

Jie Chen§, Yuepeng Wang§, Yue Wang, Wee Soo Shin, Yoko Matsuo-Okai, Toshiaki Nakajima, Kuniaki Iwasawa , Teruhiko Toyo-oka¶. *From the Second Department of Internal Medicine, Health Service Center, University of Tokyo, Tokyo 113-0033, Japan*

* This study was financially supported by grants-in-aid from the Ministry of Education, Science and Culture, the Ministry of Health and Welfare of Japan, the Research Foundation of the Japan Society for the Promotion of Science, the Uehara Memorial Foundation, the Sankyo Foundation of Life Science, and the Japanese-Chinese Medical Research Collaboration Foundation. § Both authors contributed equally to this study. ¶ To whom correspondence should be addressed: Second Dept. of Internal Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan. Tel.: 81-3-3815-5411, ext. 3074; Fax: 81-3-3813-2009; E-mail: toyooka-2im@h.u-tokyo.ac.jp.

SUMMARY

Stimulation of G-protein or tyrosine-kinase coupled receptor by hormones or growth factors regulates cell growth through the way of intracellular Ca^{2+} (Ca^{2+}_i) signaling. Inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release (IICR) through IP_3 receptor channel is an important trigger for the Ca^{2+}_i handling. In VSMCs, type-1 (IP_3R_1) and type-3 (IP_3R_3) IP_3 receptor are detected by immunoblotting. However, their contribution for Ca^{2+} handling and cell proliferation is unclear. In this study, only the functional antibody to IP_3R_1 (IP_3R_1Ab), but not the antibody to IP_3R_3 (IP_3R_3Ab) microinjected into A7R5 was capable to block the vasopressin-induced IICR. Transfection of the antisense-DNA coincided with IP_3R_1 and IP_3R_3 gene into A7r5 successfully blocked the expression of the receptors in 30-40% transfected cells. Functionally, expression of IP_3R_1 antisense DNA inhibited completely or partially IICR and cell proliferation. Blockade of the expression of IP_3R_3 antisense DNA neither significantly changed an agonist induced IICR, nor effected cell growth. But, the stable colonies transfected with IP_3R_3 antisense DNA demonstrated a decrease in capacitative Ca^{2+} entry response to thapsigargin and slow in cell proliferation. It is concluded that in VSMCs IP_3R_1 plays a more important role than IP_3R_3 in the agonist-induced IICR and cell proliferation.

KEY WORDS: Inositol 1,4,5-trisphophate receptor, Ca^{2+} dynamics, Proliferation, Vascular smooth muscle cells, Antisense DNA, Transfection

INTRODUCTION

In many cells, hormones, neurotransmitters, and growth factor stimulate a receptor coupling with G-protein or tyrosine-kinase and activate the phospholipase C. Phospholipase C catalyses the production of inositol 1,4,5-trisphosphate (IP_3) from membrane lipid phosphatidylinositol 4,5-biphosphate (1). IP_3 is a second messenger to cause IP_3-induced Ca^{2+} release (IICR) from the internal Ca^{2+} store. IICR forms an initial intracellular Ca^{2+} (Ca^{2+}_i) rise and is also an important trigger for the subsequent capacitative Ca^{2+} entry (2-3). The Ca^{2+}_i rise caused by IICR is the most versatile and universal signalling and is crucial for controlling fertilization (4-5), development (6), differentiation (7), and cell proliferation (8, 9). In mammals, there are at least three different IP_3 receptor gene, each of which, in turn, is spliced into three isoform respectively at least (10). In most organ, several types of IP_3 receptors are coexpressed at different ratios (11). Of three types, type-1 (IP_3R_1) is most generally distributed (10). In central nervous system, IP_3R_1 is a predominant type and is proven to be essential for the development of growth cones (10, 12). While type-3 (IP_3R_3) would play a role in store-operated or capacitative Ca^{2+} entry, insulin secretion in pancreatic islets, and initiation of Ca^{2+} signalling (13-17). However, in nonexcitable cells, their contributions to proliferation are unclear.

In vascular system, we detected only IP_3R_1 in endothelial cells and both IP_3R_1 and IP_3R_3 in smooth muscle cells (VSMCs, our unpublished data, 18). Although it is reported that both IICR and Ca^{2+}-induced Ca^{2+} release (CICR) mechanism are operated for Ca^{2+} dynamics in VSMCs, we found that only IICR is a dominant way for internal Ca^{2+} in A7r5. A few fundamental studies showed IP_3R_1 and IP_3R_3 have different affinity to their receptor and are differently regulated by Ca^{2+} (11, 19-21). In this study, we aimed to clarify the functional differences between IP_3R_1 and IP_3R_3 in Ca^{2+} handling and in cell proliferation. Since the proliferation of VSMCs is a key step in the process of arteriosclerosis, especially during coronary restenosis after angioplasty, elucidation of these differences are very meaningful (22-25).

We succeeded in blocking the vasopressin (VP)-induced Ca^{2+}_i response by microinjection of specific IP_3R_1Ab and IP_3R_3Ab in A7r5. However, it was a failure to follow the growth in the microinjected cells due to the difficulties in determining the position of an injected cell and the possible cell damage. Furthermore, we transfected two types of antisense DNA coincided with the genes of IP_3R_1 and IP_3R_3, respectively, into VSMCs to block the expression of the receptors. Under the guidance of cotransfected green fluorescent protein (GFP) which has been proven to be a stable reporter, we found the antisense DNA to IP_3R_1 gene partly or completely block IP_3R_1 expression and inhibit IICR in 40% successfully-transfected cells. In those IICR-inhibited cells, we observed the stop in cell proliferation by serial photomicrographs follow. While 35% transfected cells with the antisense DNA to IP_3R_3 gene

showed a partly or completely blocked expression of the receptor. However, the blockade had no effect on VP-induced IICR and also did not significantly change the cell proliferation. In stable transfection colonies with IP_3R_3 antisense-DNA, the cell showed a slow cell proliferation.

MATERIAL AND METHODS

Cell Culture

VSMCs (A7r5) were grown and passaged in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Gibco-BRL), as described previously (26-29). They were seeded in a dish made of fluorescence-free glass chamber for microinjection and the assay of $Ca^{2+}{}_i$. In the case of transfection and photomicrographs monitoring, the cells was seeded and cultured on a CELLocate coverslip (*Eppendorf*, 175 μm in diameter) which was placed in the chamber. In the case of stable transfection, they were cultured in a dish of 10 cm in diameter. The cells of 4~12 passages were used in the study.

Antibody Preparation and Immunoblotting

Three peptides were synthesized according to the amino acid sequence of the cytosolic *C*-terminal domain deduced from the cloned cDNA of human type-I (IP_3R_1), -II, and -III IP_3 receptor. The sequence GHPPHMNVNPQQ for type-I is preserved in most species including human, rat, mouse, and Xenopus oocyte, and is working for Ca^{2+} release (5, 6, 30). The employed amino acid sequences for type-II and -III were LGSNTPHVNHHMPPH and RQRLGFVDVQNCISR, respectively. The sequences of the corresponding domain in rats are identical except for substitutions of the underlined residues. All polypeptides were synthesized with an additional cysteine at their *N*- or *C*-terminal to facilitate the coupling reaction. The conjugated with epitope peptide polyclonal antibodies from New Zealand white rabbits (n = 6) to three types of IP_3 receptor were purified by affinity chromatography and were concentrated up to 10 mg/ml. The specificity of these antibodies was determined by immunoblotting, as we have described previously (31, 32). After solubilizing whole homogenate of rat brain, A7r5 and Chinese hamster ovary cells, sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis was performed in 6% gels.

Cell Microinjection

Dulbecco's phosphate-buffered saline (PBS) which contained 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$ (pH 7.4) was utilized as the extracellular medium. Ca^{2+} free medium consisted of PBS in which $CaCl_2$ was replaced by EGTA (1 mM). After loading fura-2AM (4 μM, Molecular Probes) for 40 min, A7r5 were rinsed and incubated in Ca^{2+}/Ca^{2+}-free PBS. To assess an adverse effect of the

microinjection (*Eppendorf* 5171 micromanipulator and 5246 transjector, Hamburg) procedure, 400 μM unesterified fura-2 (Molecular Probes) was added to the following substances and injected into perinuclear cytoplasm of VSMCs: 1) the vehicle for microinjection, containing 48 mM K_2HPO_4, 14 mM Na_2HPO_4, and 4.5 mM KH_2PO_4 (pH 6.9); 2) normal rabbit IgG; 3) IP_3R_1Ab; and 4) IP_3R_3Ab. The antibodies were dissolved in the vehicle. The injection time was fixed to 0.1 sec. A successful injection was verified by a stirring wave in cytoplasm and a further increase in cell fluorescence due to the injected fura-2. The injection volume into a cell could be controlled by changing the agent concentration and injection pressure (5~80 mmH_2O). The injected substances were estimated to be diluted to approximately 50 to 200 fold, according to the intensity increase of the fluorescence. Since multiple injections result in cell damage, no more than one injection was given to a cell. In some cells, a significant leakage of fura-2 was detected immediately after the injection by a reduction of emission fluorescence. These cells were considered to be damaged and excluded from the study. When no significant drop of fluorescence was observed in a cell within 5 min after the injection, the medium was changed to PBS containing 1 mM Ca^{2+} and incubated for additional 30 min to stabilize the injected cells.

Construction of Vector

The 100 bases (from nucleotide position -90 to +9) of the gene for rat IP_3R_1 or IP_3R_3 cDNA (according to the sequence of Genebank g204673 and L06096 , respectively) were synthesized (Takara, Japan). This region spans the translation initiation site and shares no homology between these two receptors. Two-strands DNA was generated and amplified by the polymerase chain reaction (PCR). The used upper and lower primers are 5'-caaggagctgactaca-3' and 5'-gtcagacatgtccttg-3' for IP_3R_1 and 5'-tttccgcccagcgcgcgc-3' and 5'-ttcattcatggctttg-3' for IP_3R_3. The PCR product was subcloned into the vector pOPRSVI/MCS (Stratagene) in an antisense or sense orientation. The orientation and sequence of inserts were confirmed by using an Applied Biosystems automated DNA sequencer.

To follow the VSMCs proliferation, we inserted the same fragment of IP_3R_1 or IP_3R_3 DNA into pTracer^{TM}-SV40 in an antisense or sense orientation. This vector owns no probability that above pOPRSVI/MCS insert and reporter pGreen Lantern^{TM1} were transfected into different cells and therefore is simple for identification. However, it is unsuitable for $Ca^{2+}{}_i$ measurement as an interfering excitation wavelength to fura-2.

Transient and Stable Transfection of VSMCs

The VSMCs were transfected at 40-60% confluence in serum-free medium by using the mixture of 0.67 μg of cloning vector with an insert of antisense cDNA, 0.67 μg pGreen Lantern^{TM1} plasmid, and 0.33 μl of lipofectin reagent (Gibco-BRL) per 1.2 mm-diameter dish. For all experiments, the following transfection controls were also performed: (1) sense IP_3R_1

or IP_3R_3 DNA with pGreen Lantern^{TM1} and (2) pGreen Lantern^{TM1} only as a mock transfection. Twelve~24 hours after transfection, the cells were gently rinsed with PBS for 2 times in order to remove the residual extracellular DNA. Then, the medium was changed to the DMEM containing 10% FBS and 1% antibiotic-antimycotic. The cells were used in experiment at 24-48 hours after the medium change.

In the case of stable transfection, VSMCs were transfected at 30%-40% of confluence in serum- and antibiotics-free DMEM by using 6 μg of cloning vector with an insert of antisense or sense cDNA and 15 μl of lipofectin reagent per 10 cm-diameter dish. One day after transfection, the cells were washed with and cultured in DMEM containing 10% FBS and 1% antibiotic-antimycotic. Two days after medium change, the cells were subcultured into dishes of 60-mm -diameter at the ratios 1:5-8 in selection medium which consist of DMEM with 10% FBS and 400 μg/ml G418 (Gibco-BRL). The concentration of G418 used has been previously determined as the lowest concentration which caused death of all non-transfected VSMCs within 2 weeks. The G418-resistant colonies were allowed to develop for 1~2 weeks in the G418-containing medium. Single colonies were isolated to separate dishes and passaged in the medium containing 50-200 μg/ml G418. The transfected VSMCs kept stable deficiency in IP_3R_1 or IP_3R_3 expression in several weeks, as long as the presence of G418. The cells in 2~6 generations were used for experiment.

Measurement of $Ca^{2+}{}_i$ Concentration

After microinjection, the $Ca^{2+}{}_i$ responses of injected and uninjected ECs in the same observation field were individually analyzed by the 2D $Ca^{2+}{}_i$ imaging system, as reported previously (26-29). The cells were stimulated with VP (3 μM, Sigma) or thrombin (10 U/ml, Sigma). We designated a VSMCs whose peak F_{340}/F_{380} increased more than 15% of the rest F_{340}/F_{380} as a responser, because some technical factors in measuring fluorescence intensity induced ±5% of fluctuation. In a part of the study, ionomycin (IM, Sigma) and thapsigargin (TG, Sigma) were employed at the end to confirm the preservation of normal $Ca^{2+}{}_i$ handling.

From twenty-four hours after transfection, the cells of successful transfection was recognized by their exciting blue light under FITC filter. After loading fura-2AM (4 μM) for 40 min, the VSMCs were stimulated under UV filter by VP (3 μM) or thrombin (10 U/ml) in the absence of extracellular Ca^{2+}. These two agonists evokes the rise in $Ca^{2+}{}_i$ in VSMCs in an IP_3-dependent way and therefore can be used to test the functional IICR changes in the transfected cells. The $Ca^{2+}{}_i$ responses of transfected and untransfected VSMCs in the same observation field were individually analyzed by the 2D $Ca^{2+}{}_i$ imaging system, as reported previously (11, 12).

The absolute concentration of $Ca^{2+}{}_i$ was calculated by comparing the fluorescence ratios at both wavelengths obtained at the maximum $Ca^{2+}{}_i$ (achieved by lysing the cells and saturating

fura-2 with Ca^{2+}) and minimum Ca^{2+}_i (achieved by chelating all free Ca^{2+} with EGTA) using the equation: $Ca^{2+}_i = Kd \times (R-Rmin)/(Rmax-R) \times Sf2/Sb2$. *Kd* is the dissociation constant (224 nM for fura-2) and *Rmin* and *Rmax* are the F340/F380 ratios of the Ca^{2+}-free and Ca^{2+}-bound forms. *Sf2/Sb2* is the ratio of the fluorescence values at 380 nm excitation determined at *Rmin* and *Rmax*, respectively (33).

Immunocytostaining

VSMCs, grown on CELLocate coverslips, were rinsed with PBS and then fixed in the same buffer plus 3.6% paraformaldehyde. All subsequent steps were performed in Ca^{2+}/Mg^{2+}-free PBS; cells were washed once for 2 min, permeabilized with 0.5% Triton X-100 (Sigma) for 10 min, and washed 3 times (5 min/wash). Endogenous peroxidase activity was reduced in MetOH with 0.3% H_2O_2 for 10 min. Nonspecific staining was reduced by 2% milk powder for 15 min before the cells were incubated with IP_3R_1Ab (x 200) or IP_3R_3Ab (x 1,000) in the presence of 10% goat serum for 1 hour at room temperature. Then, the cells were incubated with biotinylated goat anti-rabbit or anti-mouse IgG (Vector Labs, CA) for the IP_3R_1 or IP_3R_3 staining, respectively. Subsequently, peroxidase labeling was carried out with an avidin-biotin complex (Vector Labs, CA) and visualized by 0.05% 3, 3'-diaminobenzidine (Dojin, Japan) in PBS with 0.01% H_2O_2. The coverslips were finally counter-stained with hematoxylin, dehydrated, and mounted. To define nonspecific staining, all control studies were carried out by normal rabbit or mouse IgG in the same protein concentration.

Monitoring of VSMCs Growth by Photomicrographs:

To observe the changes in cell proliferation, the photomicrographs of GFP positive-cells and phase-control were taken every 12-24 hours, from 24 hours after transfection till 5-7 days.

RESULTS

Expression of IP_3R_1 and IP_3R_3 in VSMCs

An immunoblotting showed that IP_3R_1Ab reacted with a single 260 kD band in rat cerebellum membrane fraction and whole homogenate of cultured A7r5 (Fig. 1). Also, IP_3R_3Ab reacted with a single about 260 kD band in whole homogenate of A7r5. In contrast, type-II IP_3 receptors was not detected in A7r5, although they were positive in CHO cells. On the other hand, pharmacological study using Ca^{2+}_i dynamics of cultured VSMCs revealed that A7r5 weakly responded (7%) to caffeine (3~60 mM), with or without the pretreatment of ryanodine (10~60 μM, data not shown). These results suggest that CICR is not mainly working for the Ca^{2+}_i handling, and IICR mediated by IP_3R_1 and IP_3R_3 could be a major source of the Ca^{2+}_i release.

Ca^{2+}_i Dynamics in VSMCs Microinjected with IP_3R_1Ab or IP_3R_3Ab

Microinjection of IP_3R_1Ab (10 mg/ml), but not IP_3R_3Ab (10 mg/ml) could inhibit the VP-induced $Ca^{2+}{}_i$ responses, including both IICR and CCE (Fig. 2).

Immunocytostaining of IP_3R_1 and IP_3R_3 in Transfected VSMCs.

About 17% cells showed a successful transfection as observed as bright blue light. Of all GFP-brilliant cells, a part of the antisense-DNA transfected cells showed a partial reduction (17%) or complete disappearance of IP_3R_1 (17%, 23%) or IP_3R_3 (13%, 22%, Fig. 3). While the surrounding cells of the antisense-DNA transfected, the whole cells of sense-DNA transfected, and the only GFP-transfected cells demonstrated a normal staining, indicating the reduced expression of IP_3 receptors were specific to IP_3R_1 or IP_3R_3 antisense. This reduced expression was most evidenced during 24-48 hours, still significant until 5-7 days (data not shown).

$Ca^{2+}{}_i$ Dynamics in VSMCs Transfected with Antisense-DNA of IP_3R_1 or IP_3R_3

Of all the brilliant cells transfected with antisense-DNA of IP_3R_1, about 16% and 23% cells showed an attenuated or completely-blocked $Ca^{2+}{}_i$ response to VP (3 μM) or thrombin (10 U/ml), correctly consistly with the immunocytostaining results described above. These attenuation or blockade of $Ca^{2+}{}_i$ responses of transfected VSMCs to the agonists continued until 5-7 days follow after transfection (Fig. 4a).

While all the cells transfected with antisense-DNA of IP_3R_3 showed a normal $Ca^{2+}{}_i$ response to VP (3 μM) or thrombin (10 U/ml). These unchanged $Ca^{2+}{}_i$ responses of transfected VSMCs continued until 5-7 days follow (Fig. 4b).

Serial Photomicrographs Follow of Cell Growth in Transfected VSMCs

In the experiment of IP_3R_1, the cells cultured on a CELLocate coverslips were placed in the ZOG-2 chamber. Ca^{2+} measurement was carried out to choose the cells that was used to follow must be of efficient inhibition of IICR. The cells showing complete (<10% of response in control cell) or partial (11~95% of response in control cell) inhibition of IICR was followed. During follow, the cells with complete inhibition of IICR demonstrated a complete stop of cell proliferation, as compared with adjacent cells. The cells with a partial inhibition of IICR exhibited a retarded growth (Fig. 5). The GFP-transfected cell kept the normal ability in proliferation, as showed by the increasing number of the cells exciting a bright blue light during follow of only GFP-transfected cells. In the cell transfected with antisense DNA of IP_3R_3, no such changes in cell growth was observed.

Further Identification of Important Role of IP_3R_1 in Proliferation by Cell Selection

Compared with IP_3R_1 sense-DNA transfected cells, of the IP_3R_1 antisense-DNA transfected cells, no G418-resistant colony occurred during first week after transfection. Up to 2-3 weeks, there was a few very small colony. These colonies a showed much reduced immunocytostaining of IP_3R_1 and a weak Ca^{2+} response to the agonists (Fig . 6).

While in IP_3R_3 antisense-DNA transfected cells, many colonies occurred during first week. These subcultured cells showed much reduced IP_3R_3 staining in immunocytostaining, a little weak capacitative Ca^{2+} entry response to thapsigargin, and a significant down in cell proliferation (Fig . 7).

DISCUSSION

In *Drosophila*, that only IP_3R_1 is existed make it can be used for functional analysis after knock-out of the receptor gene. In result, knock-out of the receptor gene caused a great retard in growth and differentiation (7). In some mammal cells, for example, in neuron, IP_3R_1 is abosolutely dominant . It has been proven that lacking IP_3R_1 caused an ataxia and epileptic seizures in mice (12, 34). Recently, in the level of single culture neuron, Mikoshiba group has further proven that IP_3R_1 is essential for the development of growth cones (12). In pancreatic islet (13), it is reported that IP_3R_3 is main type and is contributed for insulin excretion.

However, it is difficult to analyze the difference in function in the cells which coexpressed several types of the receptor. By using antisense technique, it has been proven that the IP_3R_1 is crucial also for T-cell antigen receptor signaling and IP_3R_3 is increased in B and T lymphocyte apoptosis by causing capacitative Ca^{2+} entry (17, 35). VSMCs coexpresses IP_3R_1 and IP_3R_3 (25). The former is a dominant type and its expression is increased in elderly (23). We also showed that IP_3R_1 expression is increased in the neointima of VSMCs of rat arteriosclerosis model (unpublished data).

Both IP_3R_1Ab and IP_3R_3Ab we used are capable of functional blocking of C-terminal Ca^{2+} channel domain. But, only microinjected IP_3R_1Ab blocked agonist-evoked $Ca^{2+}{}_i$ response in VSMCs. Furthermore, microinjection of IP_3R_1Ab or IP_3R_3Ab has failed to discover any change in cell growth, probably due to the potential cell damage. Most monoclonal antibodies do not functionally inactivate the antigen they recognize. Recently, this limitation can be overcome by localized chromophore-assisted laser inactivation (12, 36) of specific antibody-linked proteins. However, CALI also need the delivery of antibody into cytoplasm by microinjection and thus owns the same limitation to microinjection in which a potential cell damage will confuse the real effects of CALI. Microinjection or combined with CALI may be more suitable for a short-time analysis of a protein function than for a long-time follow up of the influence on cell growth. The injected antibody into cytoplasm will be metabolized within hours and new protein is continued to be synthesized from gene expression. But, multiple microinjection and laser irradiation can not be given to a cell repetitively.

On the other hand, functional analysis in a knock-out mice might be influenced by some compensatory mechanism (34). Although no significant increases in the expression level of IP_3R_2 and IP_3R_3 in mice lacking IP_3R_1, that a gross anatomy appeared normal in many organs indicates the compensatory mechanism may be operated by unknown type of IP_3 receptor, for example, putative type-4 or type-5 (34).

Therefore, for a long-time follow up, expressed antisense technique without cell damage may be more suitable. Our study did overcome a major limitation of this technique that it is not effective in all transfected cells, by selecting the successful cells only. We selected the cell with successful transfection by confirmation of a loss of the receptor in immunocytostaining and an abolishment of functional IICR on 2D imaging of $Ca^{2+}{}_i$ response. Of course, a complete inhibition of IICR resulted from disappearance of IP_3R_1 may lead a complete stop in cell proliferation. A part of transfected cells showed a different extent in the inhibition of both receptor expression and functional IICR. The cells those with partial inhibition also show the decreased proliferation.

GFP has a compact structure which make it is very stable under a variety of conditions, including treatment with protease (37-38). In this study, we found that GFP expressed stably during 1-2 weeks in A7r5 in a control experiment. Therefore, we followed the changes in cell proliferation by the number of cell with brilliant blue light after confirming the changes in $Ca^{2+}{}_i$ handlings.

In VSMCs, $Ca^{2+}{}_i$ response to G protein-coupled agonist stimulation consists also of an IICR and the subsequent capacitative Ca^{2+} entry, as identified by microinjection. Although in VSMCs, the real action on proliferation is IICR or CICR is unclear (1, 9, 39). It is possible that CCE is more important than IICR for proliferation. As IICR is a crucial trigger for CCE, it is proper to conclude that IICR directly control the VSMCs proliferation.

Also, a few data has showed that in elderly or the neointima of atherosclerotic plaques, the IP_3R_1 expression is potentiated (23). From our results, we further get hint for control of VSMCs contraction by decreasing intracellular IP_3R_1 expression and thus decreasing $Ca^{2+}{}_i$ concentration. Also, decreasing IP_3R_1 expression may be an effective way to inhibit VSMCs proliferation in the coronary artery restenosis after PTCA (40). Especially, the cell showing a partial inhibition of IICR also resulted in the inhibition of proliferation. Physiologically, some inhibitor of IICR, such as endothelium-derived nitric oxide, also inhibit IICR partially (29). So far, L-type Ca^{2+} channel antagonists capable of block Ca^{2+} influx have been proven useful for inhibiting VSMCs growth or some refractory cancer (8). It may open a new era for controlling the proliferation of VSMCs by partially inhibiting IICR in VSMCs.

REFERENCES

1. Berridge, M. J. (1993) Inositol trisphosphate and calcium signalling. *Nature*, **361**, 315-325.
2. Berridge, M. J. (1995) Capacitative calcium entry. *Biochem. J.*, **312**, 1-11.
3. Randrlamampita, C., and Tsien, R. Y. (1993) Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. *Nature*, **364**, 809-814.
4. Miyazaki, S., Yuzaki, M., Nakada, K., Shirakawa, H., Nakanishi, S., Nakade, S. and Mikoshiba, K. (1992) Block of Ca^{2+} wave and Ca^{2+} oscillation by antibody to the inositol 1,4,5-trisphosphate receptor in fertilized hamster eggs. *Science*, **257**, 251-255.
5. Kume, S., Muto, A., Aruga, J., Nakagawa, T., Michikawa, T., Furichi, T., Nakade, S., Okano, H., and Mikoshiba, K. (1993) The Xenopus IP3 receptor: structure, function, and localization in oocytes and eggs. *Cell*, **73**, 555-570.
6. Kume, S., Muto, A., Inoue, T., Suga, K., Okano, H., and Mikoshiba, K. (1997) Role of 1,4,5-trisphosphate receptor in ventral signaling in Xenopus embryos. *Science*, **278**, 1940-1943.
7. Jairaj, K., Jalink, K., Hardy, R. W., Hartenstein, V., and Zuker, C. S. (1997) InsP3 receptor is essential for growth and differentiation but not for vision in drosophia. *Neuron*, **18**, 881-7
8. Kohn, E. C., Reed, E., Sarosy, G., Christian, M., Link, C. J., Cole, K., Figg, W. D., Davis, P. A., Jacob, J., Goldspiel, B., and Liotta, L. A. (1996) Clinical investigation of a cytostatic calcium influx inhibitor in patients with refractory cancer. *Cancer Res.*, **56**, 569-573.
9. Berridge, M. J., Bootman, M. D., and Lipp, P. (1998) Calcium - a life and death signal. *Science*, **395**, 645-648.
10. Mikoshiba, K., Furuichi, T., and Miyawaki, A. (1994) Structure and function of IP3 receptors. *Semin.Cell Biol.*, **5**, 273-281.
11. Wojcikiewicz, R. J. H. (1995) Type I, II and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. *J. Biol. Chem.*, **270**, 11678-11683
12. Takei, K., Shin, Y. M., Inoue, T., Kato, K., and Mikoshiba, K. (1998) Regulatio of nerve growth mediated by inositol 1,4,5-trisphosphate receptors in growth cones. *Science*, **282**, 1705-1708.
13. Blondel, O., Takeda, J., Janssen, H., Seino, S., and Bell, G. I. (1993) Sequence and functional chracterization of a third inositol trisphosphate receptor subtype, IP3R-3, expressed in pancreatic islets, kidney, gastrointestinal tract, and other tissues. *J. Biol. Chem.*, **268**, 11356-11363.
14. Putney, J. W. Jr. (1997) Type 3 inositol 1,4,5-trisphosphate receptor and capacitative calcium entry. *Cell Calcium*, **21**, 257-261.
15. Hagar, R. E., Burgstahler, A. D., Nathanson, M. H., and Ehrlich, B. E. (1998) Type III InsP3 receptor channel stays open in the presence of increased calcium *Nature*, **396**, 81-84.
16. Missiaen, L., Parys, J. B., Sienaert, I., Maes, K., Kunzelmann, K., Takahashi, M.,

Tanzawa, K., and Smedt, H. D. (1998) Functional properties of the type-3 $InsP_3$ receptor in 16HBE14o- bronchial mucosal cells. *J. Biol. Chem.*, **273**, 8983-8986.

17. Khan, A. A., Soloski, M. J., Sharp, A. H., Schilling, G., Sabatini, D. M., Li, S. H., Ross, C. A., and Synder, S. H. (1996) Lymphocyte apoptosis: mediated by increased type 3 inositol 1,4,5-trisphosphate receptor. *Science* , **273**, 503-507.
18. Morgan, J. M., Smedt, H. D., and Gillespie, J. I. (1996) Identification of three isoform of the InsP3 receptor in human myometria smooth muscle. *Pflugers Arch*, **431**, 697-705.
19. Cardy, T. J. A., Traynor, D., and Taylor, C. W. (1997) Differential regulation of type-1 and -3 inositol trisphosphate receptors by cytosolic Ca^{2+}. *Biochem. J.*, **328**, 785-793.
20. Smedt, H. D., Missiaen, L., Parys, J. B., Henning, R. H., Sienaert, I., Vanlingen, S., Gijsens, A., Himpens, B., and Casteels, R. (1997) Isoform diversity of the inositol trisphosphate receptor in cell types of mouse origin. *Biochem. J.*, **322**, 575-583.
21. Yoneshima, H., Miyawaki, A., Michikawa, T., Furuichi, T. and Mikoshiba, K. (1997) Ca^{2+} differentially regulates the ligand-affinity states of type 1 and type 3 inositol 1,4,5-trisphosphate receptors. *Biochem. J.*, **322**, 591-596.
22. Ross, R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*, **362**, 801-809.
23. Marks, A. R. (1992) Calcium channels expressed in vascular smooth muscle. *Circulation*, **86**, suppl-III, 61-67.
24. Somlyo, A. P. and Somlyo, A. V. (1994) Signal transduction and regulation in smooth muscle. *Nature*, **372**, 231-236.
25. Sipma, H., Deelman, L., Smedt, H. D., Missiaen, L., Parys, J. B., Vanlingen, S. Henning, R. H., and Casteels, R. (1998) Agonist-induced down-regulation of type 1 and type 3 inositol 1,4,5-trisphosphate receptors in A7r5 and DDT1 MF-2 smooth muscle cells. *Cell Calcium*, **23**, 11-21.
26. Shin, W. S., Toyo-oka, T., Masuo, M., Okai, Y., Fujita, H., and Sugimoto, T. (1991) Subpopulations of rat vascular smooth muscle cells as discriminated by calcium release mechanism from internal stores. *Circ. Res.*, **69**, 551-556.
27. Masuo, M., Toyo-oka, T., Shin, W. S., and Sugimoto, T. (1991) Growth-dependent alterations of intracellular Ca^{2+}-handling mechanisms of vascular smooth muscle cells. *Circ. Res.*, **69**, 1327-1339.
28. Shin, W. S., Sasaki, T., Kato, M., Hara, K., Seko, A., Yang, W. D., Shimamoto, N., Sugimoto, T., and Toyo-oka, T. (1992) Autocrine and paracrine effects of endothelium-derived relaxing factor on intracellular Ca^{2+} of endothelial cells and vascular smooth muscle cells. *J. Biol. Chem.*, **267**, 20377-20382.
29. Wang, Y. P., Shin, W. S., Kawaguchi, H., Inukai, M., Kato, M., Sakamoto, A., Uehara, Y., Miyamoto, M., Shimamoto, N., Korenaga, R., Ando, J., and Toyo-oka, T. (1996) Contribution of sustained Ca^{2+} elevation for nitric oxide production in endothelial cells and subsequent modulation of Ca^{2+} transient in vascular smooth

muscle cells in coculture. *J. Biol. Chem.*, **271**, 5647-5655.

30. Nakade, S., Maeda, N., and Mikoshiba, K. (1991) Involvement of the *C*-terminal of the inositol 1,4,5-trisphosphate receptor in Ca^{2+} release analysed using region-specific monoclonal antibodies. *Biochem. J.*, **277**, 125-131.
31. Okai, M. Y., Takano, O. H., Toyo-oka, T., and Sugimoto T. (1991) A novel myosin heavy chain isoform in vascular smooth muscle. *Biochem. Biophys. Res. Commun.*, **176**, 1365-1370.
32. Sakamoto, A., Ono, K., Abe, M., Jasmin, G., Eki, T., Murakami, Y., Masaki, T. Toyo-oka, T. and Hanaoka, F. (1997) Both hypertrophic and dilated cardiomyopathy are caused by mutation of the same gene, d-sarcoglycan, in hamster: A model of dystrophin-associated glycoprotein complex. *Proc. Natl. Sci. Acad. USA.* **94**, 13873-13878.
33. Grynkiewicz G., Poenie M., Tsien R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. *J. Biol. Chem.*, **260,** 3440-3450.
34. Matsumoto, M., Nakagawa, T., Inoue, T., Nagata, E., Tanaka, K., Takano, H., Minowa, O., Kuno, J., Sakakibara, S., Yamada, M., Yoneshima, H., Miyawaki, A., Fukuuchi, Y., Furuichi, T., Okano, H., Mikoshiba, K., and Noda, T. (1996) Ataxia and epileptic seizures in mice lacking type 1 inositol 1,4,5-trisphosphate receptor. *Nature*, **379**, 168-171.
35. Jayaraman, T., Ondriasova, E., Ondrias, K., Harnick, D. J., Marks, A. R. (1995) The inositol 1,4,5-trisphosphate receptor is essential for T-cell receptor signaling. *Proc. Natl. Acad. Sci. USA.*, **92**, 6007-6011.
36. Jay, D. G. and Keshishian, H. (1990) Laser inactivation of fasciclin I disrupts axon adhesion of grasshopper pioneer neurons. *Nature*, **348**, 548-550.
37. Chalfie, M., Tu, Y.,Ward, W. W., Prasher, D. C., (1994) Green fluorescent protein as a marker for gene express. *Science*, **263**, 802-805.
38. Heim, R., Cubitt, A. B., Roger Y.Tsien, R. Y. (1995) Improved green fluorescence. *Nature*, **373**, 663-664.
39. Putney, J. W. Jr. (1991) A model for receptor-regulated calcium entry. *Cell Calcium*, **11**, 611-624.
40. Popma JJ, Califf RM, Topol EJ. (1991) Clinical trials of restenosis after coronary angioplasty. *Circulation*, **84**, 1426-1436.