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Effects of siRNA knock-down of TRPC6 and $InsP_3R1$ in vasopressin-induced Ca^{2+} oscillations of A7r5 vascular smooth muscle cells

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ABSTRACT

We used post-transcriptional gene silencing (with small interfering RNA) to examine specifically the roles of Type 1 inositol tris-phosphate receptors ($InsP_3R1$) and transient receptor potential channel 6 (TRPC6) in Ca^{2+} oscillations induced by arginine vasopressin (AVP), a typical G-protein coupled receptor agonist. Ca^{2+} oscillations were observed in individual A7r5 cells with confocal imaging of fluo-4 fluorescence, and SR-releasable Ca^{2+} was assessed by exposure to cyclopiazonic acid (CPA). In control cells, both AVP (100 nM) and a direct activator of TRPC6 (OAG, L-oleoyl-2-acetyl-glycerol, 100 μM) caused Ca^{2+} oscillations in the majority of cells (e.g. AVP: 85%, 0.97 ± 0.05/min; OAG: 83%, 1.00 ± 0.07/min). Partial knock-down of TRPC6 (to <27% protein expression) was more effective than partial knock-down of $InsP_3R1$ (to <30% protein expression) in reducing the fraction of cells that produced Ca^{2+} oscillations in response to AVP or OAG (22% and 83% of cells showing oscillations, respectively, in response to AVP; 31% and 72% of cells showing oscillation, respectively, in response to OAG). CPA-induced SR Ca^{2+} release was unaffected by siRNA transfection. Inhibition of $InsP_3R$ with Xestospongin C abolished both AVP and OAG-induced Ca^{2+} oscillations. Nifedipine (10 μM) had no effect. The key results, including the effects of partial (as opposed to complete) knock-down of $InsP_3R1$ and TRPC6, and the (unexpected) finding of OAG-induced Ca^{2+} oscillations, are predicted by a canonical mathematical model of Ca^{2+} oscillations in which $InsP_3R1$ functions as the SR Ca^{2+} release channel and TRPC6 as the receptor-operated Ca^{2+} influx channel. These results indicated that TRPC6 functioning as a major type of receptor-operated Ca^{2+} channel played a critical role in Ca^{2+} oscillations of A7r5 cells' response to AVP or OAG, and partial knock-down of TRPC6 was more effective than partial knock-down of $InsP_3R1$ in reducing Ca^{2+} oscillations.

1. Introduction

Oscillations of intracellular [Ca^{2+}] ('Ca^{2+} oscillations') occur in a wide variety of cell types and are involved in control of numerous cell functions [1]. In many types of cells, Ca^{2+} oscillations are generated by the periodic, pulsatile, release of Ca^{2+} from intracellular stores (sarcoplasmic or endoplasmic reticulum, SR or ER). The essential functional elements for such store-dependent Ca^{2+} oscillations are well understood [2], involving (i) a positive feed-back system controlling release of stored Ca^{2+}, (ii) re-uptake of Ca^{2+} into the store, and (iii) Ca^{2+} influx (if Ca^{2+} efflux is appreciable). The use of post-transcriptional gene silencing, through the use of small interfering RNAs (siRNAs) would seem to offer a method of unprecedented specificity for examining the roles of specific molecules in each of these functions, particularly when (i) specific pharmacological inhibitors are not available, (ii) attempting to determine the involvement of a particular subtype (isoform) of a molecule [3], or (iii) attempting to distinguish the contributions of closely related members of a single family of ion channels (such as the family of the transient receptor potential channels or TRPC). Complications in the use of siRNA, however, are that gene silencing is not complete, transfection of cells is not uniform, and off-target effects can occur [4–6].

In smooth muscle, Ca^{2+} oscillations are produced through activation of a G protein coupled receptor (GPCR), which in turn stimulates phospholipase C (PLC) and thus generating inositol tris-phosphate ($InsP_3$) and diacylglycerol (DAG) [7]. The former leads to the release of Ca^{2+} from SR through inositol tris-phosphate receptor ($InsP_3R$), and the latter is thought to stimulate Ca^{2+} entry from extracellular space via unknown receptor-operated channels (ROCs) [8,9]. However, the molecular identity of ROCs leading to Ca^{2+} entry and so maintaining Ca^{2+} oscillation in vascular smooth

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muscle cells (VSMCs) is not fully understood. Several members of transient receptor potential channel (TRPC) family are likely to play a role in ROCs mediated Ca^{2+} entry [10–13]. TRPC6 is a member of the canonical TRPC subfamily, which is expressed in a variety of tissues and cells including brain, kidney, lung, heart, ovary, testis, VSMCs, lymphocytes, and platelets [14–16]. Several groups have reported that TRPC6 functions as a ROC to mediated Ca^{2+} influx in VSMCs [17–20]. A recent study by Maruyama et al. (2006) suggests that TRPC6 may function with TRPC7 to form a heteromultimeric ROC to mediate Ca^{2+} entry in VSMCs. TRPC6 channel is activated by stimulation of GPCR linked to PLC and generation of DAG [21], and not by depletion of SR stores [20,22]. TRPC6 has also been shown to be involved in myogenic tone [23,24]. $InsP_3Rs$ provide a principal route for Ca^{2+} release from internal Ca^{2+} store. Three isoforms of $InsP_3R$ has been documented, and $InsP_3R1$ is a dominant one in VSMCs [25,26].

Here, we used siRNA to examine the putative roles of a particular subtype of SR Ca^{2+} release channel $InsP_3R1$, and that of a specific Ca^{2+} entry channel, TRPC6, in the generation of agonist-induced Ca^{2+} oscillations in A7r5 cells. A7r5 cells are derived from embryonic rat thoracic aorta and are often used as a model system for vascular smooth muscle. Such cell lines present an advantage for the use of siRNA, as, at present, siRNA mediated gene knockdown is much more readily achieved in cell lines than in arteries (in 'organoid' culture) or even in primary smooth muscle cells in culture. A7r5 cells however certainly express a somewhat different set of genes than do the smooth muscle cells of intact arteries. Thus the present study was intended to evaluate the use of siRNA in a 'model' system of smooth muscle, but which was optimal (with present techniques) for the use of siRNA.

2. Material and methods

2.1. Cell culture

A7r5 embryonic rat aorta smooth muscle cells (American Type Culture Collection, A.T.C.C.) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% FBS (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin at 37 °C, 5% CO_2 in a humidified incubator. Medium was changed every two days. For Ca^{2+} imaging study, cells were grown on glass coverslips. All experiments were carried out with cells between passages 6–9.

2.2. siRNA transfection

To silence the expression of $InsP_3R1$ or TRPC6 protein, A7r5 cells were transfected with small interfering RNA (siRNA) (SMART-pool reagent for $InsP_3R1$, TRPC6, Dharmacon) using SilenceMag reagent (OZ Biosciences). Non-targeting siRNA pool was used as a negative control. The working concentration of siRNA applied was 30 nM. Briefly, cells were cultured till 80–90% confluence, siRNA was diluted in serum free DMEM and mixed thoroughly with SilenceMag reagent proportionally. Incubate mixture (siRNA/SilenceMag) for 20 min at room temperature, and then add the complexes directly onto cells. Place the cell culture plate upon the magnetic plate for 15 min at room temperature. Remove the magnetic plate and continue to culture cells in an incubator at 37 °C and 5% CO_2, for 48 h. The effect of protein silencing was analyzed by Western blot.

2.3. Immunoblotting

A7r5 cells were lysed in 50 mM Tris–HCl, pH 7.4, 1% Nonidet P-40 (NP-40), 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 0.5% sodium-deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 1 mM NaF, and proteinase inhibitors. Lysates were centrifuged at 13,000 rpm at 4 °C for 15 min and supernatant was collected as total protein. Membrane fraction was isolated by spinning down the supernatant at 30,000 × *g* for 30 min at 4 °C. Pellets (membrane fractions) were re-suspended in the same lysis buffer as above. Protein concentration was determined with a BCA protein assay kit (Pierce, Inc.). Protein was separated on Novex Tri-Glycine Gel (Invitrogen) and transferred onto Trans-Blot Nitrocellulose membrane (Bio-Rad). Polyclonal antibodies against TRPC6 (Alamone), $InsP_3R1$ (Millipore), $InsP_3R2$ (Millipore), β-actin (Cell Signaling), Na-K ATPase (Cell Signaling) and monoclonal antibody against $InsP_3R3$ (BD Bioscience) were used following manufacturer protocols. Horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG was used as secondary antibodies (Sigma). Reactions were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Inc.) and exposure to autoradiographic film. Signaling was quantified from scanned films using Scion NIH Image software (Scion).

2.4. Calcium oscillations

A7r5 cells on coverslips were incubated in DMEM with membrane permeable Fluo-4 AM (15 μM, 1.5%, v/v, DMSO, and 0.03%, v/v, Cremaphore) for 30 min at room temperature. Cells were washed with HEPES solution consisting of 135 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 11.5 mM glucose, 11.6 mM Hepes, 1.5 mM $CaCl_2$ with pH adjusted to 7.35. Cells were transferred to a cell perfusion chamber and perfused at a rate of 2 ml/min. A7r5 cells were stimulated with AVP (100 nM) to induce Ca^{2+} oscillation and recorded for a 7 min interval. Cells were initially perfused by HEPES solution without AVP for 1.5 min, followed by 4 min perfusion of AVP, then switch to AVP free HEPES solution. Nifedipine (10 μM, Sigma) and Xestospongin (3 μM, Sigma) were used to block L-type VDCCs and $InsP_3R$, respectively. L-Oleoyl-2-acetylglycerol (OAG, 100 μM, Sigma) was used as an analogue of DAG. At the end of each experiment cells were subject to cyclopiazonic acid (10 μM, CPA, Sigma) to ensure that the sarcoplasmic reticulum (SR) Ca^{2+} stores were intact. Ca^{2+} oscillation was recorded under inverted microscope (Nikon) by 'real-time' confocal imaging system (Solamere Technology Group, SLC, UT, USA) consisting of a Yokogawa confocal scanner (model CSU10) and an intensified CCD camera (model XR/Mega-10). This produced 30 images/sec and images of 75 μm × 50 μm were collected. The calcium images were recorded at a rate of 1 frame/2 s. The fluorescence obtained from these cells was referred to as "Ca^{2+} image" were simply of Ca^{2+}-dependent fluo-4 fluorescence. Auto-fluorescence was negligible in these cells. Images were analyzed using custom software written with IDL (Interactive Data Language, Research systems, Inc., v6.3, Boulder, CO). This software was used to obtain average fluorescence from areas-of-interests (AOIs) within the images. F/F_0 was calculated for all measurements of Ca^{2+} oscillations. Microsoft Excel 2003 and IDL software were used to graph the data.

2.5. One-pool model of Ca^{2+} oscillations

AVP or OAG-induced Ca^{2+} oscillations were simulated by use of the one-pool model for Ca^{2+} oscillation designed by Dupont and Goldbeter [2]. In this mathematical model, Ca^{2+} rise is assumed from a single pool sensitive to Ca^{2+} as well as IP_3, and cytosolic Ca^{2+} decreases due to pumping into the Ca^{2+} store and the extrusion from the cells. This model is based on the four equations with