Involvement of chloride channels in IGF-I-induced proliferation of porcine arterial smooth muscle cells

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Abstract

Objective: The existence of Cl− channels in vascular smooth muscle cells (VSMCs) has been increasingly investigated, but the biological functions are not yet clear. Insulin-like growth factor (IGF)-I affects proliferation and migration of VSMCs, and dysregulation of this axis may be involved in atherogenesis and intimal hyperplasia. We examined the effects of Cl− channel blockers on IGF-I-induced proliferation in porcine VSMCs. The siRNA approach was used to support the role of ClC-2, a member of the volume-regulated Cl− channel family, in cell proliferation of VSMCs.

Methods and results: The IGF-I-induced VSMC proliferation was significantly suppressed by the Cl− channel blockers NPPB and IAA94 but not by DIDS. IGF-I-induced cell proliferation parallels a significant increase in the endogenous expression of ClC-2 mRNA and protein. Inhibitors of PI3-kinase, LY294002 and wortmannin, significantly attenuated the IGF-I-upregulated ClC-2 expression and cell proliferation. We observed ClC-2-like Cl− current, and this current was augmented by IGF-I. SiRNA specifically targeted to ClC-2 abolished IGF-I-induced cell proliferation.

Conclusion: Our data demonstrate that ClC-2 plays a role in IGF-1-induced regulation of VSMC proliferation in cardiovascular diseases.

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Keywords: Atherosclerosis; Voltage-gated Cl− channels; Cl− channel blocker; Proliferation; Vascular smooth muscle cells

1. Introduction

Abnormal vascular smooth muscle cell (VSMC) proliferation and directed migration from the media into the intima play a major role in the pathogenesis of atherosclerosis [1]. These cellular events are regulated by a number of peptide growth factors including insulin-like growth factor-I (IGF-I), which is a polypeptide growth factor that binds to the type I IGF-I receptor present on many cell types, including VSMC and endothelial cells [2,3]. IGF-I plays a role in cellular growth and survival in cardiovascular tissue, especially in pathological states [4]. It has been recognized that some of the Cl− channel blockers affect the proliferation of a variety of cell types such as endothelial cells, glioma cells, intestinal enterocytes, hepatocytes and peripheral T lymphocytes [5–9]. However, it is not known whether the IGF-induced growth of VSMC can be affected by Cl− channel blockers.

Several Cl− channels have been electrophysiologically demonstrated in VSMCs. Volume-regulated Cl− channels (ClC-2, ClC-3) were expressed in human VSMCs [10]. Proliferating VSMCs have higher rates of mitosis and migration, both of which require change in cell volume and shape. It is reasonable to speculate that volume-regulated Cl−
channels might play an important role in the proliferation of VSMCs. IGF-I is a mitogen and strong chemoattractant for cultured VSMCs. IGF-I mediated dephosphorylation pathways control the activity and the pharmacological properties of skeletal muscle chloride channels [11,12]. We hypothesized that volume-regulated chloride channel might contribute to IGF-I-induced VSMC proliferation and survival. To test our hypothesis, we investigated the expression and functional role of volume-regulated chloride channels in IGF-I-induced VSMC proliferation. Our findings demonstrated that the Cl− channel blockers, NPPB and IAA94, inhibited both IGF-I and des-IGF-I-induced cell proliferation. ClC-2 expression was upregulated by IGF-I stimulation. We also recorded whole-cell Cl− currents that showed an inward rectification related to ClC-2, and this current was increased by IGF-I. IGF-I-induced cell proliferation and Cl− current was inhibited after being pretreated with PI3-kinase inhibitor. Furthermore, knockdown of ClC-2 by siRNA prevented IGF-I-induced cell proliferation. These findings have major implications for linking a specific Cl− channel to VSMC proliferation and apoptosis.

2. Methods

2.1. Tissue dissection and cell culture

Pig hearts and external iliac artery were excised from large-White pigs (22–28 kg, male or female) and placed in ice-cold sterilized PBS solution. Left anterior descending coronary arteries were dissected free of surrounding tissue while maintained in ice-cold PBS solution. Both coronary and iliac arteries were rinsed in PBS supplemented with antibiotics. The endothelial layer of each artery was removed by scraping the luminal surface with a scalpel, and the adventitia was separated from the medial layer. The media was further dissected into small explants ≈3×3 mm in diameter, which showed outgrowth of VSMC after a few days in culture. Cells were cultured in 75-mm flasks using medium 199 with 10% FBS and 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Invitrogen) in a humidified 5% CO2 environment at 37 °C. Cell cultures were identified as pure VSMCs by uniform immunostaining with an antibody to smooth muscle-actin and were used for experimentation at passage 2 or 3. Cell viability was determined by trypan blue exclusion.

The research protocol of this study was approved by the Institutional Animal Care and Use Committee of Creighton University. The investigation also conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Electrophysiological recordings

The whole-cell patch-clamp technique was employed to record Cl− currents in porcine vascular smooth muscle cells.

![Fig. 1. Concentration-dependent stimulation of VSMC proliferation induced by IGF-I. (A) VSMCs were exposed to serum free media in the presence or absence of chosen concentrations of IGF-I. (B) Effect of Cl− channel blockers on VSMC proliferation. Both NPPB and IAA94 inhibited IGF-I-induced VSMC proliferation, whereas DIDS had no effect. #p<0.05 compared with corresponding control group. *p<0.05 compared with corresponding IGF-I group. Data represent mean±SEM (n=5).](https://academic.oup.com/cardiovascres/article-abstract/73/1/198/315775)

![Fig. 2. Measurement of porcine VSMC DNA synthesis using bromodeoxyuridine (BrdU). VSMCs were stimulated with IGF-I (100 ng/ml) in the presence and absence of Cl− channel blockers for 48 h, and BrdU incorporation was analyzed by photometric immunoassay. Data represent mean±SEM (n=5). #p<0.05 vs. control, *p<0.05 vs. IGF-I stimulation.](https://academic.oup.com/cardiovascres/article-abstract/73/1/198/315775)
The data were analyzed with PCLAMP, and whole-cell recordings were performed using an Axopatch 200B patch-clamp amplifier, pClamp 8.2 software, and a Digidata 1322A interface (Axon Instruments, Foster City, CA). The whole-cell recordings frequency response was set at 5 kHz with the 8-pole Axopatch amplifier Bessel filter. The series resistance and cell capacitance were compensated for through the use of the internal circuit of the amplifier. Ag–AgCl wires were immersed in the bath as well as the pipette solutions, and were connected to the patch-clamp amplifier. The bath and the Ag–AgCl reference electrodes were used to minimize changes in liquid junction potential during some experiments. To obtain Cl− voltage–current correlations, whole-cell currents were recorded during voltage pulses 500 ms in length that were applied from the holding potential of 0 mV. Potential values were corrected for the liquid-junctional potential between the bath and the patch pipette solutions. Electrophysiological experiments were performed at room temperature (20–25 °C).

The electrical resistances of the pipettes, when filled with pipette solution, ranged from 3.0 to 4.5 MΩ. For the whole-cell mode of patch-clamp, the external bathing medium was CsCl saline consisted of (in mM): 140 CsCl, 1 CaCl2, 1 MgCl2, and 10 HEPES (pH 7.4). The pipette solution was CsCl saline consisting of (in mM): 140 CsCl, 1 EGTA, 0.05 CaCl2, 1 MgCl2, and 10 HEPES (pH 7.4). All reagents were purchased from Sigma Chemical Company (St Louis, MO, U.S.A). Osmolality was set to 285±5 mOsm with sucrose. For the whole-cell recording, solution osmolality...
was monitored using a freezing point osmometer (Micro- 
osmette, Precision systems, Natick, MA). The bathing 
medium was exchanged by continuous perfusion.

### 2.3. Cell proliferation assay

VSMCs were plated on 48-well culture plates with 
complete media. After 24 h, the media was removed and 
replaced with serum-free media for 24 h to achieve 
synchronous growth. Cells were then incubated for 48 h 
after experimental stimulation, and the final cell number 
in each well was determined.

VSMCs were also plated at a density of 50,000 cells/ well 
in 96-well culture plates with complete media. Forty-eight 
hours after the experimental stimulation, VSMC bromo- 
deoxyuridine (BrdU) uptake was determined using the cell 
proliferation ELISA BrdU assay (Roche; Penzberg, Ger-
many). This assay has been previously demonstrated to be a 
marker for VSMC proliferation [13]. BrdU incorporation 
within the cell lysates utilizing antibody specific for CIC-2. Bar 
graphs correspond to representative immunoblots. Data represent mean±SEM (n=5). #p<0.05 vs. control, *p<0.05 vs. IGF-I stimulation.

### 2.4. RT-PCR analysis

Total RNA was extracted from VSMCs using Trizol 
(Invitrogen, CA) and treatment with DNase (Ambion) using 
the manufacturer’s protocol. The total RNA was reverse 
transcribed with an OmniScript RT-PCR System (QIAGEN, 
CA). PCR was performed with a thermal cycler (PE2400, 
Applied Biosystems) under the following conditions: 1 
cyte at 94 °C for 5 min, followed by 30–36 cycles consisting of 
1 min denaturation at 94 °C, 1 min annealing at 60 °C, and 
2 min extension at 72 °C. The last cycle was extended to 5 min 
at 72 °C. Products were analyzed by 2% (w/v) agarose gel 
electrophoresis. The sequences of the 5′ sense primers and the 
3′ antisense primers used in this study were as follows: CIC-2, 
sense, 5′-AGCAGCAAATAGATGAGCACTGTCA-3′; and 
antisense, 5′-TGATAAGCTTCCGGAGCTCTTTT-3′ (product 
size, 193 bp); and CIC-3, sense, 5′-CATGTCATGGGG- 
GAGG-3′, and antisense, 5′-GCAAGAAAGCAAAACT-3′ 
(product size, 164 bp).

### 2.5. Real-time PCR analysis

Reverse transcription was performed using 1 μg of total 
RNA and oligo(dT) primers in a 20-μl reaction according to 
the manufacturer’s protocol (PE Applied Biosystems, Foster 
City, CA). Primers for CIC-2, CIC-3 and GAPDH were 
designed using Primer Express software (PE Applied Biosys-
tems). The following are sequences for ClC-2 forward 
and reverse primers, 5′-GCAAGAAAGCAAAACT-3′; 
and reverse primer, 5′-TGATAAGCTTCCGGAGCTCTTTT-3′; for 
CIC-3 forward primer, 5′-ATCGTCAACATGGCAGG-CATG- 
GAGTAT-3′; and reverse primer, 5′-GCAAGAAAGCAAAACT-3′; 
and for GAPDH forward 
DNA was measured by photometric analysis using a 
standard 96-well plate reader. However, there was no change 
in LDH activity, suggesting no cytotoxic effect of the drugs.

![Fig. 5. LY294002 and wortmannin inhibit IGF-I-induced CIC-2 protein expression in cultured VSMCs.](image-url)

![Fig. 6. Effect of PI3-kinase and MAP kinase inhibitors on IGF-I-induced proliferation in cultured VSMCs.](image-url)
primer, 5′- AGGTC GGAGTGAACGGATTTGG-3′; and reverse primer, 5′-TCGCTCCTGGAAGATGGTGATG-3′.

Real-time PCR was performed on the ABI Prism 7000 sequence detection system (PE Applied Biosystems) by using SYBR green (Applied Biosystems) as a dsDNA-specific binding dye. The PCR was cycled 40 times after initial denaturation (95 °C, 2 min) with the following parameters: denaturation, 95 °C, 15 s; and annealing and extension, 60 °C, 1 min. The threshold cycle (CT) was recorded for each sample to reflect the mRNA expression level. A validation experiment proved the linear dependency of the CT value for both ClC-2 or ClC-3 and GAPDH concentration and consistency of ΔCT (ClC-2 or ClC-3 average CT minus GAPDH average CT) in a given sample at different RNA concentration. Therefore, ΔCT was used to reflect the relative ClC-2 or ClC-3 expression levels. To determine the effects of different stimuli on CIC-2 or CIC-3 gene expression as compared with unstimulated cells, ΔΔCT was calculated (ΔΔCT = ΔCT stimulus − ΔCT non-stimulated cells). CIC-2 or CIC-3 mRNA was indexed to the GAPDH using the following formula: 1/(2ΔCT) × 100%. The value of 2ΔΔCT was calculated to demonstrate the fold changes of CIC-2 or CIC-3 gene expression in stimulated cells as compared with unstimulated cells.

2.6. Western immunoblots

Cells were lysed using eukaryotic membrane protein extraction reagent kit (Pierce, Rockford, IL) supplemented with protease inhibitor mixture. Homogenates were centrifuged for 10 min at 12,000×g at 4 °C. Protein quantification was performed on the supernatant using a DC protein assay kit from Bio-Rad (Hercules, CA). Protein was boiled for 5 min in Laemmli-SDS sample buffer containing 600 mM β-mercaptoethanol. Equal amounts of protein were loaded into each lane of SDS-PAGE gel (Bio-Rad) and resolved at 120 V constant. Gels were transferred onto PVDF membrane (Bio-Rad) at 400 mA constant for 1 h at room temperature, and membranes were blocked in blocking buffer [13]. Blots were incubated in CIC-2 antibody and rinsed three times followed by incubation with HRP-conjugated mouse anti-rabbit secondary antibodies for 90 min. After washing blots were developed with enhanced chemiluminesence on Hyperfilm (Amersham Biosciences, Arlington Heights, IL). CIC-2 and secondary HRP-conjugated antibodies were obtained from Santa Cruz Biotechnology. GAPDH antibody was purchased from Novus Biologicals. Densitometric analysis was performed directly from the blotted membrane using a Bio-Rad Molecular Imager system.

Fig. 7. Effect of IGF-1 on whole-cell Cl− currents in porcine VSMCs. Whole-cell Cl− currents were recorded at different voltage before in control (A) and after (B) perfusion of IGF-I (100 ng/ml) in coronary VSMCs. (C) Whole-cell Cl− currents were also recorded from CIC-2 siRNA transfected coronary SMCs. The I−V curve (D) showed a relationship between control (A) and after (B) perfusion of IGF-I (100 ng/ml) in pig coronary artery smooth muscle cells. Whole-cell Cl− currents in control cells (A) showed an inward rectification. Representative recording of Cl− currents from individual experimental groups is shown (n=7). The bathing medium and the pipette solution contained CsCl saline. Holding potential was 0 mV. Voltage pulses, 500 ms in duration, were applied at 20 mV increments from −100 to +100 mV.
2.7. RNA interference and cell transfection

We used small interfering RNA (siRNA), an effective tool to downregulate the expression of target genes in cultured mammalian cells. Two different high-performance purity grade small interfering RNAs (siRNA) to knock down ClC-2 were synthesized by Ambion Inc. One of the siRNA sequence was 5′GGGCCCUUUUGUGCAUAUCtt3′ and its corresponding complementary strand 5′GAUAUGCACAAAAGGGCCtc3′ -siRNA. Another was 5′GCCAUUACUGCUGUA UA Gtt3′ and its corresponding complementary strand 5′CUAUCAG- CAGUAUGG Ctg3′-siRNA. Non-silencing oligonucleotide sequence (non-silencing siRNA) that does not recognize any known homology to mammalian genes was also generated as a negative control. Lipofectamine 2000 reagent (Invitrogen, CA) was used for transient transfection of VSMCs. Briefly, 4×10^5 cells were seeded into each well of a 6-well plate and cultured to 40–50% confluency. siRNAs were diluted in RNase-free water to a final concentration of 20 μM (20 pmol/μl). For each well, siRNA stock was mixed with 5 μl Lipofectamine 2000 in DMEM medium (serum- and antibiotic-free) to a final volume of 800 μl. After 30 min, the siRNA (200 nm)/Lipofectamine 2000 complex was added to the well. Forty-eight hours after transfection, gene silencing was monitored at the mRNA and protein levels by RT-PCR and Western blotting, respectively. Cell proliferation assays were also repeated in these transfected cells.

2.8. Statistical analysis

Values for all measurements are expressed as the mean±SEM. One-way ANOVA was used to determine the difference among various experimental groups. Statistical difference between two groups was performed by the Student t test. Values of p<0.05 were considered as statistically significant.

3. Results

3.1. Cl− channel blockers attenuate IGF-induced VSMC proliferation

IGF-I stimulated a dose-dependent increase in coronary VSMC proliferation after 48 h of incubation. Cl− channel blockers alone have no effect on cell proliferation but they significantly suppressed IGF-I-induced cell proliferation (p<0.05) (Fig. 1A, B).

Fig. 8. Effect of wortmannin on IGF-I-induced whole-cell Cl− current in porcine VSMCs. Whole-cell Cl− currents were recorded after perfusion of IGF-I (100 ng/ml) in VSMCs (A) and pre-incubated with 20 μM wortmannin (B). Representative recordings of Cl− currents from individual experimental groups are shown (n=7).

Fig. 9. Effect of ClC-2 siRNA on ClC-2 expression in coronary artery SMCs. (A) mRNA expression and protein abundance were assessed by RT-PCR (top) and Western blotting (bottom), respectively, in VSMC transfected with ClC-2 siRNA or non-silencing (NS) siRNA. (B) Essential role of ClC-2 in IGF-I stimulated VSMC DNA synthesis was examined. #p<0.05 vs. control, *p<0.05 vs. IGF-I stimulation.
3.2. Cl⁻ channel blockers inhibit IGF-induced DNA Synthesis

IGF-I (100 ng/ml) stimulated DNA synthesis in coronary artery VSMCs (Fig. 2). This effect of IGF-1 was attenuated by the Cl⁻ channel blockers NPPB and IAA94 (Fig. 2). However, DIDS had no effect. The effect of both NPPB and IAA94 was dose-dependent (Fig. 3) ($p<0.05$). It is known that IGF-binding proteins significantly affect the IGF-1-induced response. Therefore we used an IGF1 analog, Des-IGF-I, that binds to IGF-IR with the same affinity as IGF-1, but does not bind IGF-binding proteins, and examined the effect of various Cl⁻ channel blockers on des-IGF-induced DNA synthesis in coronary artery VSMCs. Both NPPB and IAA94 inhibited des-IGF-I-induced VSMC growth but DIDS had no significant effect (data not shown).

3.3. Expression of ClC-2 and ClC-3 mRNA in VSMCs

The mRNA expression of volume-regulated chloride channels (ClC-2 and ClC-3) in VSMCs was examined by RT-PCR. Non-stimulated coronary artery VSMCs expressed both ClC-2 and ClC-3 mRNA (Fig. 4). Real-time PCR revealed that ClC-2 mRNA expression in coronary artery VSMC was significantly upregulated by IGF-I (100 ng/ml) stimulation in a time-dependent manner (Fig. 4A). However, IGF-I stimulation did not have any significant effect on ClC-3 expression in VSMCs (data not shown).

3.4. IGF-I-induced endogenous ClC-2 expression

We examined whether IGF-I could functionally enhance endogenous ClC-2 expression, as examined by immunoblotting using a polyclonal antibody directed against ClC-2. IGF-I stimulated the expression of ClC-2 protein in a time- and dose-dependent manner (Fig. 4B, C). These results suggest that IGF-I-induced VSMC proliferation is closely associated with a corresponding increase in endogenous ClC-2 protein expression.

3.5. LY294002 and wortmannin inhibit IGF-I-induced ClC-2 expression in VSMCs

To determine whether the IGF-I-stimulated ClC-2 upregulation is mediated via the PI3-kinase or MAP-kinase signaling pathway, two structurally distinct PI3-kinase inhibitors, wortmannin and LY294002, were used to block PI3-kinase activity, and PD98059 was used to block the activation of MAPK. As shown in Fig. 5, pretreatment of coronary artery VSMCs with LY294002 (10 μM) or wortmannin (20 μM) attenuated IGF-I-induced ClC-2 protein expression. In contrast, PD98059 (40 μM) had no significant effect. We also examined the effect of PI3-kinase inhibitors on IGF-I-induced VSMC cell proliferation. Both LY294002 (10 μM) and wortmannin diminished IGF-I-induced cell proliferation (Fig. 6).

3.6. Whole-cell Cl⁻ currents in porcine VSMCs

Whole-cell Cl⁻ currents were recorded at different voltages in control cells (Fig. 7A) without IGF-I and after (Fig. 7B) perfusion of 100 ng IGF-I in pig coronary artery smooth muscle cells. The whole-cell Cl⁻ currents were also recorded from CIC-2 knock-out pig coronary artery smooth muscle cells (Fig. 7C). The whole-cell Cl⁻ currents in normal porcine vascular smooth cells (Fig. 7D) showed an inward rectification. The $I-V$ curve showed a relationship between control (Fig. 7A) and after (Fig. 7B) perfusion of 100 ng IGF-I in porcine vascular smooth cells. We also studied the effect of wortmannin on IGF-I-induced Cl⁻ current. IGF-I-induced Cl⁻ current decreased after pre-incubation with wortmannin (Fig. 7). The bathing medium and the pipette solution contained CsCl saline. Holding potential was 0 mV. Voltage pulses, 500 ms in duration, were applied at 20 mV increments from −100 to +100 mV.

3.7. Downregulation of ClC-2 abrogates IGF-I-induced cell proliferation

The functional significance of ClC-2 in the regulation of VSMC proliferation was assessed in VSMCs in which ClC-2 gene was silenced by siRNA. ClC-2 mRNA expression and protein abundance were markedly reduced in porcine coronary artery VSMCs transfected with siRNA, but not in cells transfected with non-silencing siRNA. The ClC-3 expression was not affected after CIC-2 siRNA transfection (Fig. 9A). The VSMC DNA synthesis induced by IGF-I was significantly inhibited in siRNA-transfected cells compared with non-silencing siRNA-transfected cells (Fig. 9B).

4. Discussion

We demonstrated that IGF-I-induced proliferation of porcine coronary VSMCs was significantly suppressed by NPPB and IAA94, and resistant to DIDS. We further observed that IGF-I-induced cell proliferation was accompanied by an increase in ClC-2 mRNA and protein expression in a similar time-dependent manner. PI3-kinase inhibitor reduced IGF-I-upregulated ClC-2 expression and cell proliferation. CIC-2-like chloride current was also observed and induced by IGF-I stimulation in our study. To our knowledge, this is the first report on the involvement of CIC-2 in IGF-I associated cell proliferation. Taking the data together, we conclude that CIC-2 Cl⁻ channels may be linked to IGF-I-induced VSMC proliferation.

In present study, we recorded whole-cell Cl⁻ currents and this current was abolished by siRNA specially target to ClC-2. These data indicate that CIC-2 may be a dominating chloride channel in porcine VSMCs. According to our result, we speculate that the activation of IGF-I might be increasing the number of CIC-2 channels. The absence of knowledge of the precise function of CIC-2 makes it difficult to evaluate the physiological significance of the modulation of CIC-2 by
IGF-I. However, it has been reported that the activation of CIC-2 channels requires the activation of PI3-kinase and PKC in T84 cells. We also found that PI3K inhibitor reduced IGF-I-induced CIC-2 expression as well as IGF-I promoted cell proliferation. At the same time, IGF-I-induced Cl− current was inhibited by PI3-kinase inhibitor. It is possible that the binding of IGF-I to its receptors stimulates a series of rapid responses, including activation of PI3-kinase and the activity of the latter activates PI3-kinase, which in turn triggers two events; it induces cell proliferation, and it activates chloride channel. Zhou et al. reported that CIC-3 current was observed in A10 VSMCs [14]. The clonal cell line A10 was derived from the thoracic aorta of DB1X embryonic rat and may possess different properties characteristic of other types of VSMCs. Further study to clarify the different pathways involved in the regulation of CIC channels in VSMCs is warranted.

It is well known that most cells swell during the early phase of cell proliferation probably caused by water influx that accompanies changes in cell metabolism in the cell cycle. An increase in cell volume usually initiates the so-called regulatory volume decrease (RVD) process through activation of ion (K+ and Cl−) channels and transporters, which moves K+ and Cl− out of the cell to balance the water influx and returns the cell volume to its normal size [15,16]. Therefore, activation of volume-regulated chloride channel may play an essential role in cell proliferation. Amongst the most specific chloride channel blockers available which have been tested in smooth muscle are IAA-94 and NPPB. They can depress myogenic constriction and inhibit Cl− conductance. Several studies have demonstrated that selectivity of NPPB to the chloride channel in different cell type such as portal vein and cerebral arteries is concentration-dependent [17,18]. It has been shown that IAA-94 is effective in substantially inhibiting RVD in cardiomyocytes [19]. An IC50 ranging of NPPB and IAA94 is between 5 and 100 μM. There are several NPPB and IAA94-sensitive Cl− channels including CIC-2, CIC-3, volume-regulated anion channel (VRAC), Ca2+-activated Cl− channel (CaCC) and maxi-CI− channel. However, the contribution of CIC-3, CaCC and maxi-chloride channel in porcine vascular smooth muscle cell proliferation appears less because these channels are sensitive to DIDS and in our study we did not observe any significant effect of DIDS. There are conflicting reports about the responsiveness of VRAC to DIDS, probably because this channel may not be a single entity, but instead may represent several different channels that are expressed to a variable degree in different tissues [20,21]. Another candidate of CI− channels involved in VSMC proliferation is CIC-2, which is sensitive to NPPB and IAA94 and resistant to DIDS. CIC-2 is the only one structurally identified NPPB-sensitive and DIDS-insensitive Cl− channel. We hypothesize that CIC-2 chloride channels might be the major chloride channel that is involved in cell proliferation.

IGF-I exerts its effects by interacting with IGF-IR, whose expression varies with the cellular growth status. Different expression of IGF-binding proteins may also regulate the effect of IGF-1. Des-IGF-1 is an IGF-1 analog that binds to IGF-IR with affinity similar to IGF-1 but does not bind to IGF-binding proteins [22]. Both Des-IGF-I and IGF-I-induced cell proliferation was attenuated by chloride channel blockers. Our data indicated that the function of IGF-I is linked with CIC-2 protein through IGF-IR. IGF-IR is a transmembrane tyrosine kinase that activates two primary intracellular pathways, PI3K and extracellular signal-regulated MAPK (ERK1/2). Previous reports indicate that the activation of CIC-2 requires the activation of PI3K and protein kinase C [23]. We have also shown that PI3K inhibitor can suppress IGF-I-induced CIC-2 upregulation, suggesting the possibility that CIC-2 channels are targets for IGF-IR signaling in VSMCs and PI3K pathway may play an important role in CIC-2 protein regulation.

The critical role of CIC-2 in cell proliferation is still unclear. During cell cycle progression, cells undergo a significant increase in size especially at the G1/S phase, which perturbs cell volume homeostasis and is counterbalanced by a decrease in regulatory volume [24]. Cl− channel together with the voltage-dependent K+ channel is a major player of regulatory volume decrease. Cl− permeability reportedly varies with the cell cycle phase, being low in G0 and S phases and increasing in G1/S [25]. CLH-3, an ortholog of CIC-2, is required to induce oocyte maturation [26]. The activation of CLH-3 channels occurs by serine-threonine dephosphorylation via a type 1 protein phosphatase, a feature that has also been demonstrated for rat CIC-2. These examples demonstrate intriguing functional interactions of Cl− channels with the cell cycle machinery. On the other hand, increased Cl− channel activity has been shown to coincide with entry into the cell cycle in human cervical cancer cells, and Cl− channel blockers have been shown to modulate Schwann cell proliferation [27–29]. Similarly, the proliferation of C6 glioma cells and mouse liver cells is inhibited after CIC knockdown. These studies suggest that the inability to regulate cell volume may be the underlying mechanism that leads to impaired cell proliferation. However, transgenic knock-out mice for CIC-2 has not been able to detect any defects in cell volume regulation [30]. This apparent discrepancy may be attributable to a compensatory mechanism and must be reconciled by additional studies. It has also been reported that the growth of leukemic cells was suppressed most efficiently by NPPB, but the knocking down of CIC-2 protein by antisense oligonucleotide did not affect the growth of leukemic cells [24]. However, in our study normal VSMCs were used for proliferation assay. Thus the potential reasons for discrepancy could be related to the difference in the cell type and leukemic transformed cells vs. primary human cells.

Wang and colleagues examined the effects of Cl− channel blockers on aortic smooth muscle cell proliferation and found that endothelin-1-induced aortic smooth muscle cell proliferation was inhibited by DIDS [31]. However, in this study, we did not observe any significant effect of DIDS in porcine VSMCs. This discrepancy in the responsiveness of
VSMCs to DIDS could be due to difference in species and/or the mitogen used. Wang and colleagues used rat aortic VSMCs and examined the effects of Cl− channel blockers on the proliferative response to endothelin-1. On the other hand, we used porcine coronary artery VSMCs and examined the effects of Cl− channel blockers on IGF-I-induced cell proliferation. It is of interest that Cl− channels involved in VSMC proliferation may be stimulus- and species-specific. Several growth factors such as PDGF, FGF and VEGF have been reported to be involved in vascular smooth muscle cell proliferation and remodeling in atherosclerosis [32]. PDGF was shown to increase CIC-3 expression in canine cultured pulmonary artery smooth muscle cells [33]. Proteomic analysis of vascular endothelial growth factor-induced endothelial cell differentiation reveals a role for chloride intracellular channel 4 in tubular morphogenesis [34]. FGF-responsive telencephalic precursor cells also express functional GABA(A) receptor/Cl− channels [35]. We have not investigated the effect of these growth factors on cell proliferation and chloride channel activity. These studies may help to unveil the relationship between the growth factors and chlorides and the involvement of growth factors in the pathogenesis of atherosclerosis.

In summary, Cl− channel blockers inhibited IGF-I-induced cell proliferation. CIC-2 expression was upregulated by IGF-I stimulation. Although it is still not clear how the CIC-2 channels are involved in VSMC proliferation, our findings suggest that CIC-2 channels are important modulator of cell cycle in VSMCs and may play a role in pathological processes including intimal hyperplasia, restenosis and atherosclerosis where IGF-I-induced VSMC proliferation is important in the development of vascular remodeling. Clearly, further studies are necessary to elucidate the role of Cl− channels in cell proliferation and apoptosis in general.

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References


