Involvement of Phosphoinositide 3-Kinase and PTEN Protein in Mechanism of Activation of TRPC6 Protein in Vascular Smooth Muscle Cells* [S]

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Background: Mechanisms involved in the trafficking and activation of TRPC6 are unclear.

Results: PI3K and PTEN influence the agonist-induced translocation of TRPC6 to the plasma membrane and Ca2+ entry into cells expressing TRPC6.

Conclusion: PI3K/PTEN pathway is involved in the translocation of TRPC6 to the plasma membrane.

Significance: We provided evidence that the PI3K/PTEN pathway plays an important role in TRPC6 activation.

TRPC6 is a cation channel in the plasma membrane that plays a role in Ca2+ entry after the stimulation of a Gq-protein-coupled or tyrosine-kinase receptor. TRPC6 translocates to the plasma membrane upon stimulation and remains there as long as the stimulus is present. However, the mechanism that regulates the trafficking and activation of TRPC6 are unclear. In this study we showed phosphoinositide 3-kinase and its antagonistic phosphatase, PTEN, are involved in the activation of TRPC6. The inhibition of PI3K by PIK-93, LY294002, or wortmannin decreased carbachol-induced translocation of TRPC6 to the plasma membrane and carbachol-induced net Ca2+ entry into T6.11 cells. Conversely, a reduction of PTEN expression did not affect carbachol-induced externalization of TRPC6 but increased Ca2+ entry through TRPC6 in T6.11 cells. We also showed that the PI3K/PTEN pathway regulates vasopressin-induced translocation of TRPC6 to the plasma membrane and vasopressin-induced Ca2+ entry into A7r5 cells, which endogenously express TRPC6. In summary, we provided evidence that the PI3K/PTEN pathway plays an important role in the translocation of TRPC6 to the plasma membrane and may thus have a significant impact on Ca2+ signaling in cells that endogenously express TRPC6.

Ca2+ is an essential intracellular messenger for a wide range of important physiological functions, including cell growth, differentiation, contraction, and secretion (1, 2). An increase in intracellular Ca2+ is initiated by the activation of phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2)3 and generates the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 activates a specific receptor channel that releases Ca2+ from the endoplasmic reticulum. IP3-induced Ca2+ release is the first phase of Ca2+ mobilization. In the second phase, Ca2+ enters the cell through channels in the plasma membrane. These channels remain activated as long as the stimulus is maintained.

In non-excitable cells, transient receptor potential canonical (TRP) channels play an important role in cytosolic Ca2+ regulation (3, 4). Given their importance in Ca2+ signaling in all cell types, it is not surprising that Ca2+ channel dysfunctions may contribute to various pathologies. TRPC6 is part of the TRPC family that includes seven members (TRPC1-TRPC7). The dysregulation of TRPC expression or activity is involved in many pathologies. For example, gain-of-function TRPC6 mutations cause focal segmental glomerulosclerosis (5, 6), whereas the overexpression of TRPC6 in pulmonary artery smooth muscle cells has been observed in some cases of idiopathic pulmonary hypertension (7, 8). In hepatocytes, an up-regulation of TRPC6 has also been linked to liver cancer (9). Recent studies have provided evidence that some TRPC family members translocate to the plasma membrane after hormonal stimulation (10, 11). However, the mechanisms involved in the intracellular trafficking and activation of TRPC6 are poorly characterized.

Phosphoinositide 3-kinase (PI3K) plays a crucial role in a broad range of cellular functions in response to extracellular signals (12). PI3K is a cytoplasmic lipid kinase that phosphorylates the 3′-hydroxyl group of the inositol head groups of phosphatidylinositol and its derivatives (13). Regulated dephosphorylation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) is crucial for the proper functioning of PI3K signaling. PTEN opposes PI3K activity by dephosphorylating the 3′-hydroxyl group of PIP3 to form (PIP2) (13, 14). This phosphatase is ubiq-

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3 The abbreviations used are: PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-trisphosphate; AVP, vasopressin; [Ca2+]i, intracellular concentration of Ca2+; CCh, carbachol; GPCR, G-protein coupled receptor; PIP4, phosphatidylinositol 3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog; TRPC, transient receptor potential canonical.
uitously expressed, and its gene is one of the most commonly mutated tumor suppressor genes in human cancer (15). It has recently been shown that PI3K is involved in TRPC5 translocation to the plasma membrane after the stimulation of receptor-

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\text{tyrosine kinases (16). It was also shown that PI3K is activated by G-}
\text{protein-coupled receptors (GPCR) (17–19) and is possibly involved in Ca}^{2+}
\text{entry (20–22). Furthermore, the anchoring of PIP}_2 \text{ at the phagocytic cup is an essential step for phagosome closure and Ca}^{2+}
\text{signaling in HL60 neutrophils (23).}
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As PI3K is a key protein involved in trafficking mechanisms and in Ca\(^{2+}\) regulation, we hypothesized that it might influence the intracellular trafficking and activation of TRPC6. We showed for the first time that the inhibition of PI3K by PIK-93, LY294002, and wortmannin decreases agonist-induced translocation of TRPC6 to the plasma membrane and agonist-induced Ca\(^{2+}\) entry into HEK293 cells. Conversely, the reduction of PTEN expression did not affect the trafficking of TRPC6 but increased Ca\(^{2+}\) entry through TRPC6. We also showed that the PI3K/PTEN pathway regulates the trafficking of TRPC6 to the plasma membrane induced by vasopressin (AVP) as well as AVP-induced Ca\(^{2+}\) entry into A7r5 cells, which endogenously express TRPC6. In summary, we demonstrated that the PI3K/PTEN pathway is involved in the trafficking of TRPC6 to the plasma membrane and may thus have a significant impact on Ca\(^{2+}\) signaling in cells that endogenously express TRPC6.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture media, serum, Hepes, trypsin, Opti-

MEM I, siRNA, and Lipofectamine 2000 were purchased from Invitrogen. G418 was from Wisent (St-Bruno, QC, Canada). Nonidet P-40 was from Roche Applied Science. CCh, AVP, wortmannin, LY294002, and fura-2/AM were from Calbiochem. PIK-93 was from Cederlane (Burlington, ON, Canada). Rabbit polyclonal anti-hemagglutinin (HA)-specific antibody was from Covance (Berkeley, CA). Mouse monoclonal anti-ac-
tin, rabbit polyclonal anti-TRPC6, and rabbit monoclonal anti-
PTEN antibodies were from Chemicon (Temecula, CA). Peroxidase-conjugated donkey anti-rabbit antibody, peroxi-
dase-conjugated sheep anti-mouse antibody, and Biomax MR films were from GE Healthcare. Western Lightning Chemilu-
minescence Reagent Plus and 0.2 μm nitrocellulose mem-
branes were from PerkinElmer Life Sciences. Sulfo-NHS-SS-
biotin and streptavidin-agarose resin were from Pierce. Unless otherwise stated, all other reagents were from Sigma or Labo-

ratoire MAT (Quebec City, QC, Canada).

**Cell Cultures and Transfections**—HEK293-AT1 (HEK293 stably transfected with mouse AT1 receptor) cells and A7r5

vascular myocytes were maintained at subconfluence in Dul-
becco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO\(_2\). T6.11 cells (HEK293 stably transfected with mouse TRPC6) were cultured in the same medium supplemented with 400 μg/ml G418. T6.11 and A7r5 cells were transfected with siRNA and HEK293-AT1 cells with cDNA encoding the M5 musca-

rinic receptor as described previously (24). Briefly, 1.5 × 10\(^5\) cells were plated on poly-1-lysine-treated coverslips in 6-well culture dishes and incubated overnight at 37 °C in a humidified atmosphere containing 5% CO\(_2\).

**Protein Extractions**—The cells from six-well culture dishes were lysed with 1 ml of ice-cold lysis buffer (1.25% Nonidet P-40, 1.25% sodium deoxycholate, 2 mM EDTA, 12.5 mM sodium phosphate, pH 7.2, 1 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, 100 μM phenylmethylsulfonyl fluoride) for 30 min on ice with gentle agitation. They were then scraped from the dishes and centrifuged at 15,000 × g for 15 min at 4 °C. The samples were dissolved in 4X Laemmli buffer and heated at 60 °C for 5 min before being separated on 7% SDS-polyacryl-

amide gels. The gels were then either dried and exposed to a film for autoradiography or the protein bands were transferred to a 0.2-μm nitrocellulose membrane (400 mA for 2 h or 100 mA overnight in 150 mM glycine, 20 mM Tris-base, and 20% methanol) for immunoblotting.

**Immunoblots**—The immunoblots were stained with Ponceau S (0.1% in 5% acetic acid) to visualize the marker proteins, destained in TBST (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% Tween 20), and blocked in TBST containing 5% (w/v) nonfat dry milk for either 1 h at room temperature or overnight at 4 °C. The membranes were then washed and incubated in TBST for either 2.5 h at room temperature or overnight at 4 °C with specific primary antibodies (rabbit anti-HA or rabbit anti-
PTEN (1:1000), rabbit anti-TRPC6 (1:300), or mouse anti-actin (1:10 000)). After 3 washes with TBST, the membranes were incubated for 1.5 h at room temperature in TBST containing peroxidase-conjugated donkey anti-rabbit-IgG (1:30,000) or peroxidase-conjugated sheep anti-mouse-IgG (1:10,000). The blots were washed 3 times with TBST, and the immune complexes were detected using Western Lightning Chemilumines-
cence Reagent Plus kits according to the manufacturer’s protocol.

**Biotinylation Assays**—We used a previously described method to biotinylate cell surface proteins (11, 25). Briefly, siRNA-transfected T6.11 and A7r5 cells were grown for 40–48 h in 6-well plates. The cells were then treated with PI3K inhib-
itors for 20 min before being stimulated with CCh for 5 min. They were then placed on ice, washed twice with ice-cold PBS (137 mM NaCl, 3.5 mM KCl, 10 mM sodium phosphate buffer, pH 7.4) containing 1 mM MgCl\(_2\) and 0.5 mM CaCl\(_2\) (PBS-CM), and incubated for 60 min at 4 °C with 2 μg of NHS-SS-biotin diluted in 1 ml of ice-cold PBS. The biotinilation reaction was terminated by washing the cells 3 times with ice-cold PBS contain-
ing 20 mM glycine. The cells were then lysed with 1 ml of ice-cold lysis buffer for 30 min at 4 °C. Cell extracts were homogenized by 10 passages through a 25-gauge needle and cleared by centrifugation for 15 min at 4 °C, 14,000 × g. The supernatant (900 μl) was added to 100 μl of streptavidin-agarose beads (50% slurry, pre-equilibrated in lysis buffer) and incubated for 16 h at 4 °C. The biotinylated protein-streptavi-
din-agarose complexes were harvested by quick centrifugation and washed 3 times with lysis buffer. Biotinylated-TRPC6 was

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eluted and analyzed by immunoblotting as described under “Immunoblots,” above.

Measurement of \([\text{Ca}^{2+}]_i\)—We used a previously described method (26) to measure \([\text{Ca}^{2+}]_i\). Briefly, A7r5, T6.11, or HEK293-AT1 cells grown on poly-L-lysine-treated coverslips were washed twice with HBSS (120 mM NaCl, 5.3 mM KCl, 0.8 mM MgSO4, 10 mM glucose, 1.8 mM CaCl2, 20 mM Hepes, pH 7.4) and loaded with fura-2/AM (1.5 \(\mu\)M in HBSS) for 20 min at room temperature in the dark. After washing and de-esterifying in fresh HBSS for 20 min at room temperature, the coverslips were inserted in a circular open-bottom chamber and placed on the stage of an Olympus IX71 microscope (Olympus Canada Inc., Markham, ON, Canada) equipped with a Lambda-DG-4 illuminator (Sutter Instrument Co., Novato, CA). The \([\text{Ca}^{2+}]_i\) in selected fura-2-loaded cells was measured by fluorescence videomicroscopy at room temperature using alternating excitation wavelengths of 340 (26-nm bandpass filter) and 387 nm (11-nm bandpass filter), and emitted fluorescence was monitored through a 415–570-nm dichroic mirror and a 510-nm (84-nm bandpass) filter set. Fluorescence intensity was monitored using an Evolve EMCCD camera (Photometrics, Tucson, AZ), and the images were digitized and analyzed using MetaFluor software (Universal Imaging Corp., Downingtown, PA). Free \([\text{Ca}^{2+}]_i\) was calculated from the 340/387 fluorescence ratios using the method of Grynkiewicz et al. (27). Reagents were diluted to their final concentrations in HBSS and applied to the cells by surface perfusion. Ca\(^{2+}\)-free HBSS was supplemented with 0.5 mM EGTA to chelate any remaining extracellular Ca\(^{2+}\). For the transient transfections, the HEK293-AT1 cells were co-transfected with cDNA encoding the M5 muscarinic receptor, and only those responding to carbachol (CCh) were analyzed. \([\text{Ca}^{2+}]_i\) values were recorded every 3 s.

RESULTS

To investigate the role of PI3K in the modulation of CCh-induced Ca\(^{2+}\) mobilization in T6.11 cells, we used three PI3K inhibitors, wortmannin, LY294002, and PIK-93. To discriminate between CCh-induced Ca\(^{2+}\) release and CCh-induced Ca\(^{2+}\) entry, we used a Ca\(^{2+}\) depletion-readdition protocol. T6.11 cells were treated with the PI3K inhibitors for 20 min before depleting the intracellular Ca\(^{2+}\) stores with 5 \(\mu\)M CCh. Once the [Ca\(^{2+}\)]i, had returned to the basal level (1 min after the addition of CCh), the extracellular medium was repleted with 1.8 mM CaCl\(_2\). As shown in Fig. 1A, in the absence of extracellular Ca\(^{2+}\), CCh-induced Ca\(^{2+}\) release was similar whether T6.11 cells had been pretreated or not with the PI3K inhibitors. After adding 1.8 mM CaCl\(_2\) to the external medium, intracellular Ca\(^{2+}\) rose to a plateau level of ~125 nM under control conditions. In T6.11 cells pretreated with a concentration of inhibitors known to block mainly PI3K (100 nM wortmannin, 300 nM PIK-93, and 50 \(\mu\)M LY294002), intracellular Ca\(^{2+}\) rose to a lower plateau level between 85 to 95 nM (Fig. 1A). Net Ca\(^{2+}\) entry was calculated by subtracting the basal Ca\(^{2+}\) level from the maximum Ca\(^{2+}\) level recorded once external Ca\(^{2+}\) had been restored. In the presence of wortmannin (100 nM), PIK-93, or LY294002, CCh-induced Ca\(^{2+}\) entry decreased to 60.3 ± 15.5, 56.6 ± 10.1, and 67.0 ± 8.0%, respectively, compared with control cells (Fig. 1B). At this concentration, PI3K inhibitors did not affect the accumulation of inositol phosphates induced by 5 \(\mu\)M CCh or the cellular level of PIP\(_2\) before and after stimulation (supplemental Figs. S1 and S2). Furthermore, after a pretreatment of the cells with 10 \(\mu\)M wortmannin, a concentration known to inhibit PI4K, the basal level of PIP\(_2\) was decreased by 35%, but the accumulation of inositol phosphates induced by 5 \(\mu\)M CCh was not affected (supplemental Fig. S2). Under these conditions, CCh-induced net Ca\(^{2+}\) entry was reduced to 71.4 ± 5.0% of control cells, which was similar to the inhibition of PI3K only (Fig. 1B). These results suggest that the inhibition of PI3K, but not of PI4K, is the cause for the decreased CCh-induced Ca\(^{2+}\) entry.

To determine whether the decrease in net Ca\(^{2+}\) entry observed after the PI3K inhibitor treatments was due to a
decrease in TRPC6 activity or to another endogenous Ca^{2+} entry channel, we repeated the experiments using stably transfected HEK293 cells (HEK-AT1R) that did not overexpress TRPC6. With these cells, we observed that PI3K inhibitors did not modify CCh-induced Ca^{2+} release and Ca^{2+} entry (Fig. 2A). Under control conditions, CCh caused a net Ca^{2+} entry of 126.2 ± 6.6 nM, whereas it caused net Ca^{2+} entries of 120.6 ± 7.8 and 129.4 ± 7.6 nM after the treatments with 50 μM LY294002 and 10 μM wortmannin, respectively (Fig. 2B). These results suggested that PI3K positively influences TRPC6 activity in HEK293 cells.

A7r5 cells express high levels of TRPC6, whereas the knockdown of TRPC6 decreases AVP-induced Ca^{2+} responses in these cells (28–30). As expected, a mixture of three siRNAs against TRPC6 decreased the expression of TRPC6 to an undetectable level (Fig. 3A). Using the Ca^{2+} depletion-readition protocol, we showed that AVP (100 nM)-induced Ca^{2+} release was not affected, whereas AVP-induced Ca^{2+} entry was considerably reduced in TRPC6 knocked down cells compared with control A7r5 cells that had been transfected with scrambled siRNA (siCTL) (Fig. 3B). AVP-induced net Ca^{2+} entry

FIGURE 2. The inhibition of PI3K with LY294002 or wortmannin does not affect CCh-induced Ca^{2+} entry into HEK-AT1 cells. A, HEK293 cells overexpressing AT1R were used as a negative control for TRPC6 expression and were treated and stimulated as described in Fig. 1. The graphs represent the average of 405 cells for each condition from three independent experiments. B, net Ca^{2+} entry values were calculated and graphed as the averages ± S.D. of three independent experiments.

FIGURE 3. AVP-induced Ca^{2+} entry into A7r5 cells is inhibited by TRPC6 silencing and by the inhibition of PI3K. A, A7r5 cells were transfected with a 50 nM concentration of a mix of three siRNAs specific for TRPC6 (siTRPC6) or with a universal negative control (siCTL). After 48 h, the cells were solubilized, and the presence of TRPC6 (upper panel) or actin (lower panel) in the lysates was determined by Western blotting. B, A7r5 cells were transfected with siTRPC6 (open squares) or a universal negative control (closed squares). After 48 h, fura-2-loaded transfected cells were incubated in the absence of extracellular Ca^{2+} (in the presence of 0.5 mM EGTA) for 30 s and were then stimulated with 100 nM AVP. External Ca^{2+} (1.8 mM) was restored after 180 s. The graphs represent the average of 45 cells from a typical experiment (n = 5). C, maximum net AVP-induced Ca^{2+} entry in siTRPC6 and siCTL-transfected A7r5 cells were calculated by subtracting the basal [Ca^{2+}] values (average of three values before Ca^{2+} restoration) from the average of three maximal [Ca^{2+}] values after Ca^{2+} restoration. The histogram represents the average ± S.D. of three independent experiments. *, p < 0.01. Ctrl, control. D, maximum net AVP-induced Ca^{2+} entry in A7r5 cells treated with PI3K inhibitors was calculated by subtracting the basal [Ca^{2+}] values (average of three values before Ca^{2+} restoration) from the average of three maximal [Ca^{2+}] values after Ca^{2+} restoration. The histogram represents the average ± S.D. of three independent experiments. *, p < 0.05.
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decreased significantly to 35.5 ± 12.16% of control cells (Fig. 3C). These results confirmed that TRPC6 plays an important role in the mechanism of Ca\(^{2+}\) entry into A7r5 cells. We investigated the influence of PI3K inhibitors on AVP (100 nM)-induced Ca\(^{2+}\) entry in A7r5 cells. As shown in Fig. 3D, in the presence LY294002, PIK-93, or wortmannin (10 μM), AVP-induced Ca\(^{2+}\) entry decreased significantly to 60.7 ± 7.2, 68.3 ± 22.7, and 43.9 ± 17.2%, respectively, of control cells. To demonstrate that the inhibition on AVP-induced Ca\(^{2+}\) entry by PI3K inhibitors was due to the activity of TRPC6, we investigated the influence of PI3K inhibitors on AVP-induced Ca\(^{2+}\) entry in TRPC6 knocked down A7r5 cells. In those experiments net AVP-induced Ca\(^{2+}\) entry was 212.8 ± 63.1 nM in control A7r5 cells, whereas it was reduced to 55.6 ± 20.3 nM in TRPC6 knocked down A7r5 cells (Fig. 4, A, B, and C). When control A7r5 cells were pretreated with LY294002 or wortmannin, AVP-induced net Ca\(^{2+}\) entry decreased to 63.8 ± 23.2 and 95.8 ± 24 nM, respectively (Fig. 4, A and C). When TRPC6 knocked down A7r5 cells were pretreated with LY294002 or wortmannin, AVP-induced net Ca\(^{2+}\) entries were 0 ± 20.5 nM, 22.7, and 43.9 nM, respectively, which were not significantly different from the values measured with untreated cells (55.6 ± 20.3 nM) (Fig. 4, B and C). These results suggested that PI3K positively modulates TRPC6 activity in A7r5 cells.

A cell surface biotinylation approach was used to evaluate the decreased Ca\(^{2+}\) entry caused by PI3K inhibitors was due to a decreased externalization of TRPC6. Cell surface proteins were biotinylated with the membrane-impermeant amino reagent sulfo-NHS-SS-biotin. The proteins were then solubilized, incubated with streptavidin-agarose, and reacted with an anti-HA antibody. Under unstimulated conditions, TRPC6 was barely detectable at the plasma membrane of T6.11 cells (Fig. 5A). CCh (5 μM) markedly increased the amount of TRPC6 at the plasma membrane (3.7 ± 2.4 times the basal level). A pretreatment with LY294002 or wortmannin markedly decreased CCh-induced TRPC6 externalization (1.9 ± 1.2 and 0.7 ± 0.6 times the basal level, respectively) (Fig. 5B). These results suggested that PI3K plays an important role in the CCh-induced externalization of TRPC6 in T6.11 cells.

The influence of PI3K on agonist-induced TRPC6 externalization was also investigated in A7r5 cells. As shown in Fig. 6, under unstimulated conditions, the presence of TRPC6 at the cell surface was barely detectable, whereas AVP significantly increased the externalization of TRPC6. AVP-induced TRPC6 externalization markedly decreased after a pretreatment with 300 nM PIK-93, 100 nM wortmannin, or 50 μM LY294002. These results suggest that PI3K plays an important role in the agonist-induced externalization of TRPC6 in A7r5 cells.

PTEN is a dual lipid and protein phosphatase that is a key negative regulator of PI3K signaling. PTEN dephosphorylates the D3 inositol head group of PIP3, generating PIP2 (31, 32). We investigated whether the knockdown of PTEN would modulate CCh-induced Ca\(^{2+}\) mobilization in T6.11 cells. Fig. 7, A and B shows that siRNAs against PTEN decreased its expression by 50% and enhanced the basal level of PIP3 by 3.47 ± 1.56-fold (supplemental Fig. S3). The knockdown of PTEN did not modify CCh-induced Ca\(^{2+}\) release but significantly enhanced CCh-induced Ca\(^{2+}\) entry into T6.11 cells (Fig. 7C). In PTEN knocked down cells, net CCh-induced Ca\(^{2+}\) entry was 382.5 ± 33.8 nM, whereas it was 247.0 ± 23.3 nM in cells transfected with siCTL (Fig. 7D).

The effect of PTEN on AVP-induced Ca\(^{2+}\) entry into A7r5 cells was also investigated. Fig. 8A shows that the mixture of siRNAs against PTEN decreased the expression of PTEN below the detectable level in A7r5 cells. In PTEN knocked down A7r5 cells, net AVP-induced Ca\(^{2+}\) entry was 243.9 ± 6.9 nM, which was significantly higher than net AVP-induced Ca\(^{2+}\) entry in siCTL-transfected cells (110.5 ± 17.3 nM) (Fig. 8, B and C). To determine whether the increase in AVP-induced Ca\(^{2+}\) entry observed in PTEN knocked down cells was due to the activity of TRPC6, a double knockdown experiment was designed. As
expected, in TRPC6 knocked down A7r5 cells, net AVP-induced Ca\textsuperscript{2+} entry was 58.5 \pm 9.9 nM (Fig. 8, B and C), whereas in PTEN/TRPC6 double knocked down cells, net AVP-induced Ca\textsuperscript{2+} entry was 123.0 \pm 22.4 nM. This net Ca\textsuperscript{2+} entry was well above (by 64.5 nM) that measured in siCTL/siTRPC6-transfected cells. These results suggested that TRPC6-dependent Ca\textsuperscript{2+} entry is negatively modulated by PTEN in A7r5 cells.

The influence of PTEN on CCh-induced TRPC6 externalization was evaluated using a biotinylation approach. Unexpectedly, when T6.11 cells were transfected with a mixture of three siRNAs against PTEN, the total expression of TRPC6 decreased by 20% under basal conditions and by 40% when the cells were stimulated with 5 \mu M CCh (Fig. 9, A and C). Similar results were obtained after transfecting a single siRNA against PTEN (data not shown). The decrease in total expression of TRPC6 in cells transfected with siRNAs against PTEN did not modify the cell surface TRPC6/total TRPC6 ratio (Fig. 9B) observed under basal conditions. Similar results were obtained when siCTL- and siPTEN-transfected cells were stimulated with 5 \mu M CCh (Fig. 9B). These results suggested that PTEN decreases Ca\textsuperscript{2+} entry by a mechanism that is not related to the externalization of TRPC6.

DISCUSSION

Intracellular trafficking of TRPC channels has emerged as an important regulatory process for their activation (10). However, the mechanism that regulates agonist-induced TRPC translocation is not clear. It is known that PIP\textsubscript{3} is a key regulator of the traffic of many ion channels and transporters (33–35). In this study we investigated the role of PI3K and PTEN, two key enzymes involved in the mechanisms of activation and trafficking of TRPC6. We showed that the activity of PI3K is essential for maintaining TRPC6 at the plasma membrane when G\textsubscript{q}-PCR is stimulated in T6.11 cells overexpressing TRPC6 (T6.11) and in A7r5 cells endogenously expressing TRPC6. Our results further demonstrated that pre-treating T6.11 cells and A7r5 cells with PI3K inhibitors decreases net agonist-induced Ca\textsuperscript{2+} entry, whereas the knockdown of PTEN enhances net agonist-induced Ca\textsuperscript{2+} entry. The effect of PI3K inhibitors was attributed to TRPC6 activity as agonist-induced Ca\textsuperscript{2+} entry was unaffected in HEK293 cells that did not express TRPC6 or in TRPC6 knocked down A7r5 cells.

Our results are in agreement with those of previous studies showing that PI3K inhibitors decrease the agonist-induced...
activation of TRPC1, TRPC5, and TRPC6 (16, 36–39). However, because PI3K inhibitors can also inhibit PI4K, some of these studies (36, 38, 39) attributed the effect of PI3K inhibitors to the depletion of membrane PIP2 rather than to the inhibition of PI3K activity. In this study we used concentrations of wortmannin (100 nM) and LY294002 (50 μM) that inhibit less than 50% of PI4K activity (40, 41). It was shown that only a small amount of PI4K (as low as 15%) is sufficient to maintain the phosphatidylinositol 4-bisphosphate pool at the plasma membrane upon acute Gq-PCR activation (42). Also, the PI3K inhibitor PIK-93 was used at a concentration of 300 nM, which does not inhibit PI4K type III (43). PI4K type III is essential to generate the plasma membrane pool of phosphatidylinositol 4-bisphosphate (42), the precursor of PIP2, which leads to the formation of IP3 (44–46). It must be noted that, to mobilize Ca2+, we used agonist concentrations near their EC50 to pro-

**FIGURE 7.** PTEN silencing potentiates CCh-induced Ca2+ entry into T6.11 cells. A, T6.11 cells were transfected with 50 nM concentrations of a mix of three siRNA specific for PTEN (siPTEN) or a universal negative control (siCTL). After 48 h, the cells were solubilized, and the presence of PTEN (upper panel) or actin (lower panel) in the lysates was determined by Western blotting (IB). B, the immunoblots from A were scanned, and the PTEN bands were quantified using ImageJ software. The PTEN/actin ratios were graphed relative to siCTL control cells. The results are expressed as the means ± S.D. of three independent experiments. C, T6.11 cells were transfected with siPTEN (open diamonds) or a universal negative control (closed squares). After 48 h, fura-2-loaded transfected cells were incubated in the absence of extracellular Ca2+ (in the presence of 0.5 mM EGTA) for 30 s and were then stimulated with 5 μM CCh. External Ca2+ (1.8 mM) was restored after 180 s. The graphs represent the average of 405 cells for each condition from three independent experiments. D, net Ca2+ entry was calculated by subtracting the [Ca2+]i value determined from the average of three values measured just before adding 1.8 mM extracellular Ca2+ from the average of three [Ca2+]i values measured 12–22 s after the addition of extracellular Ca2+. Results are expressed as the means ± S.D. of three independent experiments. *, p < 0.01.

**FIGURE 8.** PTEN silencing potentiates AVP-induced Ca2+ entry into TRPC6-expressing cells. A, A7r5 cells were transfected with 50 nM of a mix of three siRNA specific for PTEN (siPTEN) or a universal negative control (siCTL). After 48 h, the cells were solubilized, and the presence of PTEN (upper panel) or actin (lower panel) in the lysates was determined by Western blotting (IB). B, A7r5 cells were transfected with siPTEN alone (gray circles), siTRPC6 alone (light gray triangles), siPTEN/siTRPC6 (open triangles), or siCTL alone (closed squares). After 48 h, the fura-2-loaded transfected cells were incubated in the absence of extracellular Ca2+ (in the presence of 0.5 mM EGTA) for 30 s and were then stimulated with 100 nM AVP. External Ca2+ (1.8 mM) was restored after 180 s. The graphs represent the average of 135 cells for each condition from three independent experiments. C, net Ca2+ entry was calculated by subtracting the [Ca2+]i value calculated from the average of three values measured just before adding 1.8 mM extracellular Ca2+ from the average of three [Ca2+]i values measured 41–51 s after the addition of extracellular Ca2+. Results are expressed as the means ± S.D. of three independent experiments. *, p < 0.02.
tect the phosphatidylinositol 4-bisphosphate pool and maintain de novo synthesis of PIP$_2$. Under these conditions, we showed that the level of PIP$_2$ or CCh-induced IPs accumulation was unaffected by PI3K inhibitors. Therefore, the effects of PI3K inhibitors may be attributed to the inhibition of PI3K and not to the depletion of membrane PIP$_2$. When T6.11 cells and A7r5 cells were stimulated with CCh and AVP, respectively, the level of TRPC6 at the plasma membrane decreased to the control (unstimulated and untreated) level. A similar result has also been observed with platelets, where glucose-induced TRPC6 translocation is significantly attenuated in the presence of PI3K inhibitors (39). We thus propose that PI3K inhibitors cause a decrease in agonist-induced Ca$^{2+}$ entry into cells expressing TRPC6 by decreasing TRPC6 levels in the plasma membrane.

These are eight isoforms of PI3K that have been divided in three classes (47). Class I regroups four isoforms (p110$\alpha$, p110$\beta$, p110$\delta$, p110$\gamma$) that are activated by tyrosine kinase receptors and GPCRs. Three isoforms (PI3K-C2$\alpha$, PI3K-C2$\beta$, PI3K-C2$\gamma$) are comprised in the class II and are activated by integrin engagement, growth factors, and chemokines. Only one isoform, VPS34, constitutes the class III. All isoforms of PI3K are involved in the intracellular trafficking (48). Therefore, we could not identify which isoform is really involved in TRPC6 exocytosis. Many studies have shown that the $\beta$γ subunits of G-proteins can activate the p110$\gamma$ isoform (49). It was shown that the p110$\gamma$ isoform is implicated in the regulated exocytosis of insulin in pancreatic $\beta$ cells and in the degranulation of mast cells (50–52). PI3K-C2$\alpha$, a class II PI3K, is also involved in the regulated insulin and neurosecretory granule exocytosis (53, 54).

In this study we used PIK93 at a concentration (300 nM) that potently inhibits the p110$\gamma$ isoform (IC$_{50}$ of 16 nM) and weakly inhibits PI3K-C2$\alpha$ (IC$_{50}$ of 16,000 nM) (43). Although these results suggest that p110$\gamma$ is the isoform involved in the translocation of TRPC6, further studies are needed to clarify that point.

Our results showed that the knockdown of PTEN in TRPC6-expressing cells enhances agonist-induced Ca$^{2+}$ entry. However, the knockdown of PTEN in T6.11 decreased the total amount of TRPC6 without affecting the agonist-induced translocation of TRPC6 to the plasma membrane. The decrease in the total amount of TRPC6 was unexpected, as its expression in T6.11 is driven by the cytomegalovirus promoter. Furthermore, our quantitative PCR data showed no significant change in TRPC6 mRNA upon depletion of PTEN (data not shown). Thus, the decrease in the total amount of TRPC6 is likely due to an increased degradation of TRPC6. It was previously shown that through a direct protein-protein interaction, PTEN can increase the activation of AKT and Mdm2 (57–60). Mdm2 is E3 ubiquitin ligase involved in the ubiquitinylation of proteins (61), an essential step for proteasomal degradation (62). Also, it was shown that the formation of phosphatidylinositol 3-phosphate mediated by the class III PI3K is required for the trafficking of membrane proteins from the endosomes to the lumen of lysosomes (48, 63). Further studies are needed to elucidate how the depletion of PTEN influences the ubiquitinylation level of TRPC6 and its degradation.

Unlike PI3K, PTEN appeared to act mainly on TRPC6 activity and not on TRPC6 translocation to the plasma membrane.
Because PTEN is a PIP$_3$ 3-phosphatase, these results suggested that the phosphorylation of phosphatidylinositol at position 3 is important for TRPC6 activity. Tseng et al. (64) showed that the exogenous application of PIP$_3$ increases Ca$^{2+}$ entry into HEK293 and Jurkat cells, an effect that is dependent on the expression of TRPC6. Interestingly, PIP$_3$ is known to bind to the C terminus of TRPC6 and increase the activity of the channel (65). The elevation of membrane PIP$_3$ also leads to the activation of AKT/ERK pathway, which has been shown to increase TRPC6 activity (66). A recent study showed that PTEN serves an important role in the activation of TRPC6. Tseng et al. showed that the phosphorylation of phosphatidylinositol at position 3 is important for TRPC6 activity. Tseng et al. showed that the phosphorylation of phosphatidylinositol at position 3 is important for TRPC6 activity. 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Role of PI3K and PTEN in Activation of TRPC6

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