CAMP Activates TRPC6 Channels via the Phosphatidylinositol 3-Kinase (PI3K)-Protein Kinase B (PKB)-Mitogen-activated Protein Kinase Kinase (MEK)-ERK1/2 Signaling Pathway*

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CAMP is an important second messenger that executes diverse physiological function in living cells. In this study, we investigated the effect of CAMP on canonical TRPC6 (transient receptor potential channel 6) channels in TRPC6-expressing HEK293 cells and glomerular mesangial cells. The results showed that 500 μM 8-Br-cAMP, a cell-permeable analog of CAMP, elicited [Ca2+]i increases and stimulated a cation current at the whole-cell level in TRPC6-expressing HEK293 cells. The effect of CAMP diminished in the presence of the PI3K inhibitors wortmannin and LY294002 or the MEK inhibitors PD98059, U0126, and MEK inhibitor I. 8-Br-CAMP also induced phosphorylation of MEK and ERK1/2. Conversion of serine to glycine at an ERK1/2 phosphorylation site (S281G) abolished the CAMP activation of TRPC6 as determined by whole-cell and cell-attached single-channel patch recordings. Experiments based on a panel of pharmacological inhibitors or activators suggested that the CAMP action on TRPC6 was not mediated by PKA, PKG, or EPAC (exchange protein activated by CAMP). Total internal fluorescence reflection microscopy showed that 8-Br-CAMP did not alter the trafficking of TRPC6 to the plasma membrane. We also found that, in glomerular mesangial cells, glucagon-induced [Ca2+]i increases were mediated through the CAMP-PI3K-PKB-MEK-ERK1/2-TRPC6 signaling pathway. In summary, this study uncovered a novel TRPC6 activation mechanism in which CAMP activates TRPC6 via the PI3K-PKB-MEK-ERK1/2 signaling pathway.

Canonical TRPC6 (transient receptor potential channel 6) channels are Ca2+-permeable non-selective cation channels. The channels are ubiquitously expressed and play diverse functional roles, including vascular smooth muscle contraction, cell proliferation, and kidney glomerular filtration (1–3). Mutation in TRPC6 causes familial focal segmental glomerulosclerosis, which is characterized by proteinuria and a progressive decline in renal function (4, 5). TRPC6 is activated by diverse cellular signals, including agonists at Gαq protein-coupled receptors and diacylglycerol (6). Various protein phosphorylations have been reported to activate the channel (1, 9, 10). PKA can also phosphorylate TRPC6, although the phosphorylation does not appear to affect cation permeation (11). In general, the mechanisms of TRPC6 activation and regulation are still not well understood.

CAMP is an important second messenger (12). Numerous physiological factors, including hormone, cytokine, autocrine, and paracrine agents, can activate membrane-bound adenyl cyclases, resulting in cytosolic CAMP elevation. The elevated CAMP then acts on multiple downstream targets to regulate cellular responses (13). Some well known downstream targets of CAMP include PKA, PKG, and EPAC (exchange protein activated by cAMP) (14), and PKC (15, 16). An accumulating amount of evidence shows that CAMP can also initiate another signaling cascade by activating PI3K-PKB, which subsequently stimulates MEK and ERK1/2 (17–20). Interestingly, this signaling cascade of CAMP-PI3K-PKB-MEK-ERK1/2 appears to play a key role in glucagon-induced proliferation of renal mesangial cells (21, 22). Glucagon is a major hormone that can bind to specific membrane receptors to activate glycogenolytic and gluconeogenic pathways, causing blood glucose levels to increase. Elevated fasting glucagon levels (hyperglucagonemia) may contribute to the development of diabetes (23). High glucagon may also stimulate the growth and proliferation of glomerular mesangial cells, resulting in subsequent mesangial expansion, glomerulosclerosis, and glomerular injury (22, 24). Recently, Li et al. (21, 22) reported that glucagon-induced proliferation of mesangial cells is mediated by a signaling cascade involving CAMP, ERK1/2, and [Ca2+]i increases. However, it is not clear whether the [Ca2+]i increases are related to extracellular Ca2+ influx and, if so, which Ca2+-permeable channels mediate the Ca2+ influx.

In this study, we investigated the effect of CAMP on TRPC6-mediated Ca2+ influx and cation current in TRPC6-expressing HEK293 cells. Our results demonstrate for the first time that CAMP activates TRPC6-mediated Ca2+ influx and cation current and that the action is mediated through the PI3K-PKB-MEK-ERK1/2 signaling pathway. Furthermore, we show that this mechanism plays a key role in glucagon-induced [Ca2+]i increases in renal glomerular mesangial cells.

EXPERIMENTAL PROCEDURES

Cell Culture, cDNA Expression, and siRNA Delivery—HEK293 cells were obtained from the American Type Culture
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FIGURE 1. 8-Br-cAMP-induced [Ca\textsuperscript{2+}]\textsuperscript{i} increases in HEK293 cells. A and B, representative traces showing 500 μM 8-Br-cAMP-induced [Ca\textsuperscript{2+}]\textsuperscript{i}, increases in the absence (A) and presence (B) of 4 μM thapsigargin in TRPC6-expressing HEK293 cells. C and D, summary of data showing the percentage of [Ca\textsuperscript{2+}]\textsuperscript{i}-responsive cells (C) and the peak amplitude of the first [Ca\textsuperscript{2+}]\textsuperscript{i}, transient among the responding cells (D) to 500 μM 8-Br-cAMP. TG, TRPC6-expressing cells; WT, wild-type HEK293 cells; Vec, vector-transfected control; TG, thapsigargin, 0Ca\textsuperscript{2+}-PSS, Ca\textsuperscript{2+}-free bath solution. Results are the mean ± S.E. (n = 5–12 independent experiments, 15–40 cells/experiment). *p < 0.05 compared with TRPC6-expressing HEK293 cells.

Collection. All cDNA constructs were transiently transfected into HEK293 cells using Lipofectamine 2000. The cells were used for experiments 48–72 h post-transfection. The cells were cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin G, and 0.1 mg/ml streptomycin. Cells were grown at 37 °C in a 5% CO\textsubscript{2} humidified incubator.

Glomerular mesangial cells were isolated from male Sprague-Dawley rats (260–280 g) using the graded sieving technique based on the protocol described previously (25, 26). Briefly, isolated glomeruli were digested by collagenase (2 mg/ml) for 45 min at 37 °C. After several washes, cells were grown in RPMI 1640 medium supplemented with 17% FBS, 100 IU/ml penicillin G, and 0.1 mg/ml streptomycin at 37 °C in a 5% CO\textsubscript{2} humidified incubator. In this study, glomerular mesangial cells from passages 3–5 were used. For siRNA studies, TRPC6-specific siRNA or its scrambled control was transfected into glomerular mesangial cells using electroporation with Nucleofector II (Lonza Group, Ltd.) following the procedure recommended by the manufacturer. The cells were used for Ca\textsuperscript{2+} measurement and immunoblot experiments 40 h after electroporation. The nucleotide sequence of TRPC6-specific siRNA is GCAGCAUCAUCAUGCAAGAUAUA (27). See supplemental “Materials and Methods” for additional information.

RESULTS

cAMP Induces [Ca\textsuperscript{2+}]\textsuperscript{i}, Oscillations in TRPC6-expressing HEK293 Cells—Mouse TRPC6 was transiently expressed in HEK293 cells. 8-Br-cAMP (500 μM), a cell-permeable analog of cAMP, elicited oscillatory [Ca\textsuperscript{2+}]\textsuperscript{i}, increases in TRPC6-expressing cells but not in wild-type HEK293 cells or in vector-transfected cells (Fig. 1, A, C, and D). Although 8-Br-cAMP is membrane-permeable, its membrane permeability is poor (15); therefore, a relatively high concentration of 500 μM was employed. If TRPC6-expressing cells were placed in a Ca\textsuperscript{2+}-free bath solution (0Ca\textsuperscript{2+}-PSS), 8-Br-cAMP failed to elicit such [Ca\textsuperscript{2+}]\textsuperscript{i}, increases (Fig. 1, C and D), suggesting an obligated requirement for Ca\textsuperscript{2+} entry in the [Ca\textsuperscript{2+}], oscillations. Because [Ca\textsuperscript{2+}], oscillations are also known to be related to Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores (28, 29), the role of intracellular Ca\textsuperscript{2+} release was explored. It was found that, after depletion of intracellular Ca\textsuperscript{2+} stores using thapsigargin (4 μM) for 10 min, 8-Br-cAMP (500 μM) was able to induce only a single [Ca\textsuperscript{2+}], transient without further [Ca\textsuperscript{2+}], oscillations (Fig. 1B). However, thapsigargin treatment had no effect on the percentage of cells that could respond to cAMP and the peak magnitude of the first [Ca\textsuperscript{2+}], transient (Fig. 1, B–D). These data suggest that, although the first [Ca\textsuperscript{2+}], transient is due to Ca\textsuperscript{2+} influx but not intracellular Ca\textsuperscript{2+} store release, the subsequent [Ca\textsuperscript{2+}], oscillations may be related to Ca\textsuperscript{2+} store release. Because we were interested in TRPC6-mediated Ca\textsuperscript{2+} influx, we examined only the cAMP effect on the first [Ca\textsuperscript{2+}], transient.

cAMP Stimulates a Whole-Cell Cation Current in TRPC6-expressing HEK293 Cells—Note that the [Ca\textsuperscript{2+}], transient is affected not only by Ca\textsuperscript{2+} movement across the plasma membrane but also by Ca\textsuperscript{2+} movement across the membrane of intracellular Ca\textsuperscript{2+} stores. Thus, the [Ca\textsuperscript{2+}], transient could not faithfully reflect the Ca\textsuperscript{2+} influx. Next, we used whole-cell patch clamp to examine the effect of cAMP on TRPC6 activity on the plasma membrane. Application of 500 μM 8-Br-cAMP...
caused a marked increase in whole-cell cation current density in both inward and outward directions in TRPC6-expressing cells but not in the vector-transfected group (Fig. 2, A–C). The time course of cAMP activation was recorded at ±80 mV, and the results showed that cAMP activation of TRPC6 followed a relatively slow time course. It peaked at ~10 min after 8-Br-cAMP application (Fig. 2D). We reasoned that two factors might have contributed to the difference in the time kinetics between the cAMP-induced \([Ca^{2+}]_i\) transient (Fig. 1A) and the cation current (Fig. 2D): 1) the opening of a small percentage of TRPC6 might be enough for global \([Ca^{2+}]_i\) increases, and 2) the falling phase of the \([Ca^{2+}]_i\) transient was not related to the inactivation of TRPC6. In this aspect, it has been well documented that the falling phase of the \([Ca^{2+}]_i\) transient reflects
mainly Ca\(^{2+}\) sequestration into intracellular stores, as well as Ca\(^{2+}\) extrusion into the extracellular medium (30, 31).

**Participation of the PI3K-PKB-MEK-ERK1/2 Signaling Pathway**—One downstream target of cAMP is PI3K (17–20), the activity of which stimulates the PKB-MEK-ERK1/2 pathway. In [Ca\(^{2+}\)]\(_i\), measurement studies with TRPC6-expressing cells, two PI3K inhibitors, wortmannin (100 nm) and LY294002 (30 \(\mu\)M), both markedly reduced the percentage of cells responding to 8-Br-cAMP (500 \(\mu\)M), dibutyryl cAMP (300 \(\mu\)M), and forskolin (10 \(\mu\)M) (Fig. 3, A and B, and supplemental Fig. S1, A and B). These two inhibitors also reduced the peak amplitude of the first [Ca\(^{2+}\)]\(_i\) transient among the [Ca\(^{2+}\)]\(_i\)-responding cells (Fig. 3B and supplemental Fig. S1B). Three MEK inhibitors, PD98059 (20 \(\mu\)M), U0126 (10 \(\mu\)M), and MEK inhibitor I (1 \(\mu\)M), had a similar inhibitory effect on 8-Br-cAMP-, dibutyryl cAMP-, and forskolin-induced Ca\(^{2+}\) influx in TRPC6-expressing cells (Fig. 3, A and B, and supplemental Fig. S1, A and B).

The effect of PI3K and MEK inhibitors on TRPC6-mediated cation currents was also examined. As shown in Fig. 3C, 8-Br-cAMP (500 \(\mu\)M)-stimulated whole-cell cation currents, which were recorded at ±80 mV, were significantly inhibited by the PI3K inhibitors wortmannin (100 nm) and LY294002 (30 \(\mu\)M). The MEK inhibitors PD98059 (20 \(\mu\)M), U0126 (10 \(\mu\)M), and MEK inhibitor I (1 \(\mu\)M) had a similar inhibitory effect (Fig. 3C).

We tested whether 8-Br-cAMP can enhance MEK and ERK1/2 phosphorylation. Fig. 4 shows that 8-Br-cAMP (500 \(\mu\)M) induced a rapid phosphorylation of MEK and ERK1/2 within 2 min and lasting for 10 min (Fig. 4, A and B). The total MEK and ERK1/2 protein levels were not affected by 8-Br-cAMP. PI3K is an upstream signaling molecule of MEK and ERK1/2. As expected, in the presence of the PI3K inhibitor LY294002 (30 \(\mu\)M), cAMP was unable to stimulate MEK and ERK1/2 phosphorylation (Fig. 4, C and D). Likewise, inhibition of MEK (by 10 \(\mu\)M U0126), which is an upstream signal molecule of ERK1/2, prevented the cAMP-induced phosphorylation of ERK1/2 (Fig. 4D).

**Direct ERK1/2 Phosphorylation of TRPC6**—There are four ERK1/2 consensus sites (Pro-X-Ser or Thr-Pro) in TRPC6 proteins. A point mutation was made at each of these four sites. These constructs were transfected into HEK293 cells. One mutant, S281G, abolished the cAMP activation of TRPC6 in whole-cell patch recording (Fig. 5, A and B). Other mutants of TRPC6, including S194G, T220A, and S768G, did not alter the cAMP response (data not shown). A cell-attached single-channel patch recorded a cAMP-activated channel in TRPC6-expressing cells (Fig. 5C), whereas cAMP failed to activate this channel in S281G-expressing cells (Fig. 5, C and D). These results suggest that ERK1/2 may directly activate TRPC6 by phosphorylation at Ser-281.

**Lack of Involvement of PKA, PKG, and EPAC**—In [Ca\(^{2+}\)]\(_i\), measurement experiments, the PKG inhibitor KT5823 (1 \(\mu\)M) and the PKA inhibitors KT5720 (1 \(\mu\)M) and H89 (10 \(\mu\)M) had no effect either on the percentage of cells responding to 8-Br-cAMP (supplemental Fig. S2A) or on the peak amplitude of the first [Ca\(^{2+}\)]\(_i\) transient among the responding cells (supplemental Fig. S2B). We also utilized the TRPC6 mutant T69A/S321A, in which effective PKG phosphorylation sites were mutated (10). This mutant displayed cAMP-induced [Ca\(^{2+}\)]\(_i\), responses similar to those of wild-type TRPC6 (supplemental Fig. S2, A and B). EPAC is another possible downstream target of cAMP. However, the selective EPAC agonist 8-(4-chlorophenylthio)-2'-O-Me-cAMP had only a very small stimulating action on the [Ca\(^{2+}\)]\(_i\), increase (supplemental Fig. S2, A and B), suggesting that the involvement of EPAC, if any, is small.

Whole-cell patch clamp studies were used to verify the above findings. The results showed that KT5720 and KT5823 treatments had no effect on the 8-Br-cAMP-stimulated whole-cell cation current in TRPC6-expressing cells (supplemental Fig. S2D). Furthermore, 8-(4-chlorophenylthio)-2'-O-Me-cAMP failed to stimulate the whole-cell cation current in TRPC6-expressing cells (supplemental Fig. S2C). Taken together, these data suggest that the stimulating action of cAMP on TRPC6 does not involve PKA, PKG, and EPAC.

**Lack of cAMP Effect on TRPC6 Translocation**—One report has suggested that cAMP may be a key signal that facilitates TRPC6 trafficking to the plasma membrane (32). We used total internal fluorescence reflection microscopy, a powerful technique for studying protein movements within the periplasmic space, to monitor the movement of GFP-tagged TRPC6 toward the plasma membrane (33). GFP-tagged TRPC6 was transfected into HEK293 cells, and the cells were treated with 8-Br-cAMP (500 \(\mu\)M) for 5–10 min. 8-Br-cAMP had no effect on the TRPC6 fluorescence change in the plasma membrane region (supplemental Fig. S3, A and D). pMEM-YFP, which is a fluorescent marker of the plasma membrane, was used as a time control. pMEM-YFP fluorescence did not change in 10 min (supplemental Fig. S3, B and D). This control excluded the possibility of
plasma membrane movement in the axial direction during the experiments. A positive control was also included, in which thapsigargin (4 μM) was shown to stimulate the translocation of heteromeric TRPV4-C1 channels to the plasma membrane (supplemental Fig. S3, C and D) as reported previously (33). Used as another control, GFP-tagged TRPC6 could also be activated by 8-Br-cAMP in whole-cell recordings (supplemental Fig. S4).

Taken together, these data demonstrate that cAMP does not alter the translocation of TRPC6 to the plasma membrane.

**cAMP-PI3K-PKB-MEK-ERK1/2-TRPC6 Signaling Cascade in Glomerular Mesangial Cells**—Recent studies have shown that glucagon binds specific receptors to increase cAMP production and to cause \([\text{Ca}^{2+}]_i\) increases in glomerular mesangial cells and that the MEK-ERK1/2 pathway is involved in the pro-
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The major findings of this study are as follows. 1) cAMP and forskolin evoked Ca\(^{2+}\) influx and elicited a whole-cell cation current in TRPC6-expressing HEK293 cells. 2) The effect of cAMP on TRPC6 was inhibited by pharmacological antagonists of PI3K and MEK. Furthermore, cAMP could induce the phosphorylation of MEK and its downstream target ERK1/2. Importantly, point mutations at ERK1/2 phosphorylation sites on TRPC6 proteins (S281G) abolished the cAMP activation of the TRPC6-mediated cation current in whole-cell and single-channel recordings. 3) Both glucagon and cAMP could induce [Ca\(^{2+}\)], increases in primary cultured rat glomerular mesangial cells, the effect of which was abolished by TRPC6-specific siRNA and inhibitors of PI3K and MEK. These data provide strong evidence that cAMP acts through PI3K-PKB-MEK-ERK1/2 to activate TRPC6 in both TRPC6-expressing HEK293 cells and native glomerular mesangial cells (Fig. 7). It is likely that ERK1/2 directly phosphorylates Ser-281 of TRPC6 to activate the channels. These results add ERK1/2 to the list of protein kinases that can directly regulate TRPC6 via phosphorylation. This list includes Fyn, calcium/calmodulin-dependent protein kinase II, PKC, and PKG (7, 8) and now ERK1/2.

Two other studies have investigated the effect of cAMP and PKA on TRPC6 (11, 32). One report showed that PKA had no effect on TRPC6 activity (11), which is in agreement with our study. The second report suggested an involvement of cAMP in agonist-induced translocation of TRPC6 proteins to the plasma membrane in vascular endothelial cells (32). This is in contrast to the results of this study, in which we showed that cAMP did not stimulate TRPC6 translocation to the plasma membrane by total internal fluorescence reflection microscopy. The reason for this discrepancy is not clear, but it could be due to difference in cell types and/or methods used for monitoring translocation.

We used total internal fluorescence reflection microscopy, whereas the other investigators used conventional confocal imaging (32). It is relatively easier to quantify the protein trafficking by total internal fluorescence reflection microscopy than by conventional confocal imaging.

The finding that the cAMP-PI3K-PKB-MEK-ERK1/2 signaling pathway activates TRPC6 may have important physiological or pathological implications. It is well documented that the MEK-ERK1/2 pathway and TRPC6 each play important roles in regulating cell proliferation and differentiation (34–36). However, the relationship between MEK-ERK1/2 and TRPC6 has never been established. In kidney, it was reported that the major hormone glucagon, through a signaling cascade involving cAMP, ERK1/2, and [Ca\(^{2+}\)], increases (21, 22), can stimulate the growth and proliferation of glomerular mesangial cells, resulting in glomerular injury (22, 24). However, the role of TRPC6 in the process is unclear. In this study, we confirmed that glucagon and cAMP can elicit [Ca\(^{2+}\)], increases in the primary cultured glomerular mesangial cells via the PI3K-MEK-ERK1/2 pathway. More importantly, we found that suppressing TRPC6 expression levels using TRPC6-specific siRNA abolished the [Ca\(^{2+}\)], increases in response to glucagon and cAMP. Taken together, these data strongly suggest that the glucagon-induced [Ca\(^{2+}\)], increases in glomerular mesangial cells are mediated by TRPC6 through the cAMP-PI3K-MEK-ERK1/2 axis. This Ca\(^{2+}\) influx is expected to stimulate the growth and proliferation of renal mesangial cells.
We used the compounds LY294002 and wortmannin to demonstrate the role of the PI3K pathway in the cAMP activation of TRPC6. It has been shown that LY294002, in addition to its action on PI3K, can directly inhibit the activity of Ca$^{2+}$ channels (Ca1,12) (37). It is unknown whether also LY294002 has a direct inhibitory effect on TRPC6. However, in our study, another PI3K inhibitor (wortmannin) and multiple MEK inhibitors also exerted a similar effect on the cAMP activation of TRPC6. Furthermore, a point mutation at the ERK site prevents the cAMP activation of TRPC6. Therefore, it is safe to conclude that the PI3K-PKB-MEK-ERK1/2 pathway is involved.

In conclusion, we have identified the cAMP-PI3K-MEK-ERK1/2 axis as a novel signaling pathway for TRPC6 activation. ERK1/2 phosphorylates at Ser-281 to activate TRPC6. Furthermore, a point mutation at the ERK site prevents the cAMP activation of TRPC6. It has been shown that LY294002, in addition to demonstrating the role of the PI3K pathway in the cAMP activation of TRPC6, can directly inhibit the activity of Ca$^{2+}$ channels. Therefore, it is safe to conclude that the PI3K-PKB-MEK-ERK1/2 pathway is involved.

REFERENCES

1. Dietrich, A., and Gudermann, T. (2007) Handb. Exp. Pharmacol. 179, 125–141
2. Zhou, J., Du, W., Zhou, K., Tai, Y., Yao, H., Jia, Y., Ding, Y., and Wang, Y. (2008) Nat. Neurosci. 11, 741–743
3. Graham, S., Ding, M., Sours-Brothers, S., Yorio, T., Ma, J. X., and Ma, R. (2007) Am. J. Physiol. Renal Physiol. 293, F1381–F1390
4. Winn, M. P., Conlon, P. J., Lynn, K. L., Farrington, M. K., Creazzo, T., Hawkins, A. F., Daskalakis, N., Ebersviller, S., Burchette, J. L., Pericak-Vance, M. A., Howell, D. N., Vance, J. M., and Rosenberg, P. B. (2005) Science 308, 1801–1804
5. Nilius, B., and Owsianik, G. (2010) Pflugers Arch. 460, 437–450
6. Shibatani, Y., Nakamura, K., Inoue, T., Nakamura, T., Watanabe, Y., Mizutani, T., and Mikoshiba, K. (2004) J. Biol. Chem. 279, 18887–18894
7. Shi, J., Mori, E., Mori, Y., Moroi, M., Li, J., Ito, Y., and Inoue, R. (2004) J. Physiol. 561, 415–432
8. Kim, Y., and Saffern, D. (2005) J. Biol. Chem. 280, 32035–32047
9. Takahashi, S., Lin, H., Geshi, N., Mori, Y., Kawarabayashi, Y., Takami, N., Morii, M. X., Honda, A. and Inoue, R. (2008) J. Physiol. 586, 4209–4223
10. Hassock, S. R., Zhu, M. X., Trost, C., Flockerzi, V., and Authi, K. S. (2002) Blood 100, 2801–2811