PKC-dependent coupling of calcium permeation through transient receptor potential canonical 3 (TRPC3) to calcineurin signaling in HL-1 myocytes

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Cardiac transient receptor potential canonical (TRPC) channels are crucial upstream components of Ca^{2+} /calcineurin/nuclear factor of activated T cells (NFAT) signaling, thereby controlling cardiac transcriptional programs. The linkage between TRPC-mediated Ca^{2+} signals and NFAT activity is still incompletely understood. TRPC conductances may govern calcineurin activity and NFAT translocation by supplying Ca^{2+} either directly through the TRPC pore into a regulatory microdomain or indirectly via promotion of voltage-dependent Ca^{2+} entry. Here, we show that a point mutation in the TRPC3 selectivity filter (E630Q), which disrupts Ca^{2+} permeability but preserves monovalent permeation, abrogates agonist-induced NFAT signaling in HEK293 cells as well as in murine HL-1 atrial myocytes. The E630Q mutation fully retains the ability to convert phospholipase C-linked stimuli into L-type ($\text{Ca}_v1.2$) channel-mediated Ca^{2+} entry in HL-1 cells, thereby generating a dihydropyridine-sensitive Ca^{2+} signal that is isolated from the NFAT pathway. Prevention of PKC-dependent modulation of TRPC3 by either inhibition of cellular kinase activity or mutation of a critical phosphorylation site in TRPC3 (T573A), which disrupts targeting of calcineurin into the channel complex, converts cardiac TRPC3-mediated Ca^{2+} signaling into a transcriptionally silent mode. Thus, we demonstrate a dichotomy of TRPC-mediated Ca^{2+} signaling in the heart constituting two distinct pathways that are differentially linked to gene transcription. Coupling of TRPC3 activity to NFAT translocation requires microdomain Ca^{2+} signaling by PKC-modified TRPC3 complexes. Our results identify TRPC3 as a pivotal signaling gateway in Ca^{2+} -dependent control of cardiac gene expression.

Ca^{2+} homeostasis | NFATc1 transactivation | transient receptor potential canonical | divalent permeation

As a universal and versatile second messenger, calcium (Ca^{2+}) governs a multitude of cellular effector functions in the heart including transcriptional programs and cellular remodeling processes (1). Coordinated control of cardiac functions by Ca^{2+} requires efficient segregation of Ca^{2+} signals into regulatory microdomains, resulting in specificity of coupling between Ca^{2+} sources and Ca^{2+} -dependent effector systems. So far, the molecular composition and architecture of Ca^{2+} signaling microdomains for control of cardiac transcriptional programs is incompletely understood. Cation channels of the transient receptor potential canonical (TRPC) family constitute a ubiquitous signal transduction machinery for Ca^{2+} entry and have recently been identified as ion channels that trigger pathophysiological activation of nuclear factor of activated T-cell (NFAT)-mediated gene transcription and hypertrophic remodeling in the heart (2–4). TRPC proteins form Ca^{2+} permeable plasma membrane channels that are typically activated in response to hormonal stimuli linked to phospholipase C signaling (5). These channels lack, or display only modest, selectivity for Ca^{2+} over monovalent cation (6) and are able to generate increases in cytosolic Ca^{2+} via multiple mechanisms including indirect initiation of Ca^{2+} entry via voltage-gated Ca^{2+} channels (7) or the sodium calcium exchanger (NCX) because of modulation of

membrane potential and/or local Na^{+} gradients (8). TRPC channels are expected to contribute divergently to Ca^{2+} signaling in nonexcitable and in excitable cells, which provide a certain repertoire of voltage-dependent Ca^{2+} transport systems.

TRPC3 is a lipid-regulated member of the TRPC subfamily and a potential player in cardiac pathophysiology (9). For homomeric TRPC3 channels, a $\text{Ca}^{2+}/\text{Na}^{+}$ permeability ratio of ≈ 1.6 was determined (10) and functional crosstalk of TRPC3 channels with cardiac voltage-gated Ca^{2+} channels and NCX1 has been suggested (7, 11). Representing a typical nonselective cation channel, TRPC3 controls cellular processes by either Ca^{2+} permeation through its pore and generation of a local Ca^{2+} signal at the TRP channel signalplex or by remote effects on voltage-gated Ca^{2+} channels or electrogenic transporters. According to a paradigm of cardiac (patho)physiology, beat-to-beat Ca^{2+} cycling (E-C coupling) in the heart is separated from Ca^{2+} signaling events that control gene expression (12). Interestingly, TRPC3 has been suggested to govern gene transcription by mechanisms involving a linkage to voltage-dependent Ca^{2+} entry (7), which is also essential for E-C coupling. However, the Ca^{2+} entry mechanism, which links cardiac TRPC3 activity to gene expression is elusive and it is unclear whether nonselective TRPC channels serve as dual Ca^{2+} signaling units that control segregated Ca^{2+} pools. To address these questions, we set out to engineer the TRPC3 cation permeation pathway and generated a single point mutation (E630Q) that lacks divalent- but retains monovalent permeability and, thus, the potential to control voltage-dependent Ca^{2+} entry. This mutant was found capable of functional coupling to cardiac $\text{Ca}_v1.2$ signaling but not to NFAT activation. We present evidence for a tight link between TRPC3 channel activity and NFAT nuclear translocation based on Ca^{2+} permeation through the TRPC3 pore, generating a local Ca^{2+} signaling event that is sensed by the downstream effector calcineurin (CaN), which is targeted to cardiac TRPC3 channels. Moreover, we demonstrate that protein kinase C-dependent modulation of the channel enables switching between transcriptionally active and inactive TRPC3-signaling modes.

Results and Discussion

Identification of E630 as a Critical Residue in the TRPC3 Selectivity Filter. In an attempt to obtain TRPC mutants with altered ion selectivity, we initially generated a structural model of the TRPC3 pore region by using a recently developed alignment strategy (13)

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and structure information available for KcsA as well as a Kv1.2-Kv1.3 chimeric pore. Our hypothetical pore model is illustrated in Fig. S1A along with the sequence comparison of TRPC3 and template pore structures (Fig. S1B). Three of the five negative residues were predicted to be accessible and exposed to the permeation pathway. According to our molecular model, only one glutamate (E630) was localized within the central part of the permeation pathway, whereas the other two residues (E616 and D639) were predicted as part of the extracellular vestibule. Mutagenesis and functional analysis confirmed a critical role of the central glutamate in position 630. Charge inversion (E630K) yielded a nonfunctional channel (Fig. S2), whereas neutralization (E630Q) produced a channel that displayed moderately altered current to voltage (I-V) relation in normal extracellular (Na^+ plus Ca^{2+} containing; Fig. 1A and C) solution with a relative increase in the conductance at neutral potential. The I-V relation of the TRPC3-E630Q mutant was virtually insensitive to changes in extracellular Ca^{2+} (Fig. S3). Inspection of I-V relations with Ca^{2+} as the sole extracellular cationic charge carrier and BAPTA in the pipette solution to eliminate indirect, Ca^{2+} -mediated currents revealed that the E630Q mutation profoundly reduced Ca^{2+} permeation although the channel complex (Fig. 2B and D). Inward currents were essentially small or lacking with Ca^{2+} as a charge carrier even at large hyperpolarizing potentials, and reversal potentials were difficult to determine in most experiments. Nonetheless, from six experiments a mean of -79.6 ± 6.3 mV was calculated, demonstrating a substantial shift in reversal potential compared with wild-type channels (1.9 ± 1.4 ; $n = 7$). The $\text{Ca}^{2+}/\text{Cs}^+$ permeability ratio was reduced from 4.2 to less than 0.02 in the mutant. Thus, our experiments identified a key amino acid within the cation permeation structure of TRPC3. The negative charge in position 630 is apparently essential for divalent permeation but not for transition of monovalent cations through the TRPC3 pore. Our finding is in line with previous reports on the role of negatively charged residues in Ca^{2+} transition through TRP pores (14–17) and, specifically, with a prediction of critical residues in the TRPC selectivity filter obtained by Liu et al. in a study with the prototypical *Drosophila* TRP channel (14). It is of note that the identified negatively charged residue is conserved in the TRPC3/6/7 subfamily but absent in more distant relatives of TRPC3. Therefore, Ca^{2+} permeation in within the TRPC family of cation channels may involve distinctly different molecular

mechanisms. Our results support the notion that monovalent and divalent permeation through nonselective TRP cation channels may involve separate, specific interaction sites within the pore, which combine to a “nonselective” pathway that conducts both types of charge carriers. Based on our observation that a single point mutation within the TRPC3 pore causes specific elimination of Ca^{2+} permeation, we set out to use this genetically engineered cation channel to explore the cellular impact of the TRPC3-mediated monovalent conductance and to identify downstream signaling pathways that are specifically linked to either the monovalent transport or to Ca^{2+} entry through the channel pore. Because the TRPC monovalent conductance is considered of particular importance in excitable cells, and because recent studies have demonstrated the relevance of TRPC channels in cardiac pathophysiology (2, 4, 7, 18) we focused on the cardiac system, using the HL-1 murine atrial cell line. Initially, we compared basic properties of TRPC3 signaling in HL-1 cells with those in the well characterized electrically nonexcitable HEK293 system.

TRPC3 Mediates Agonist-Induced Ca^{2+} Signals in HEK293 and HL-1 Cells by Divergent Mechanisms. So far, the relative contribution of direct Ca^{2+} permeation through the TRPC3 pore and indirect mechanisms involving TRPC-mediated changes in membrane potential and voltage-dependent signaling partners such as NCX1 has not been evaluated in HEK293 cells. Expression levels of endogenous voltage-gated Ca^{2+} channels are below the detection threshold, and NCX1 expression is typically moderate to low. Hence, only a minor fraction of the TRPC3-mediated Ca^{2+} signal is expected to involve indirect mechanisms in HEK293. Pharmacological characterization of the TRPC3-mediated Ca^{2+} entry pathway in HEK293 and HL-1 cells, determined by using a classical Ca^{2+} readdition protocol, revealed that global Ca^{2+} signals were based on distinctly different mechanisms (Fig. S4). Electrophysiological experiments confirmed that TRPC3 channels were active when Ca^{2+} was elevated after activation by agonist administration in Ca^{2+} -free solution (Fig. S5). TRPC3 overexpressing HEK293 as well as HL-1 displayed Ca^{2+} entry that was highly sensitive to inhibition by the TRPC3 blocker Pyr3 (19). KB-R7943, an inhibitor of NCX reverse mode operation, suppressed Ca^{2+} entry only moderately in HEK293 cells and lacked inhibitory effects in HL-1 cells. Block of voltage-gated, L-type Ca^{2+} channels by nifedipine strongly suppressed Ca^{2+} entry into endothelin-stimulated HL-1 cells but had no effect on the Ca^{2+} signal in HEK293 cells (Fig. S4). These results indicate that TRPC3 is effectively linked to voltage-gated Ca^{2+} signaling in cardiac cells and are able to produce large global cytosolic Ca^{2+} rises via promotion of Ca^{2+} entry through $\text{Ca}_v1.2$ channels. The observed lack of NCX-mediated Ca^{2+} entry into HL-1 cells may be explained by predominant forward mode operation in these cardiac cells, based on a tight functional coupling to voltage-gated Ca^{2+} entry channels as well as more negative membrane potentials compared with HEK293 cells. Thus, HL-1 myocytes represent an electrically excitable cell type that displays functional cross-talk and signaling partnership between nonselective TRPC conductances and voltage-gated Ca^{2+} channels. Thereby, TRPC3 signaling in HL-1 cells is distinctly different from that in the nonelectrically excitable HEK293 cell system.

As a next step, we aimed to delineate the cellular role of direct Ca^{2+} permeation through TRPC3 channels in these cells by characterizing coupling between Ca^{2+} entry and the NFAT downstream effector system for wild type and pore mutants (E630Q and E630K) of TRPC3.

Ca^{2+} Permeation Through the Pore of TRPC3 Channels Is Essential for Activation of the NFAT Pathway. Carbachol-induced Ca^{2+} entry as well as NFAT translocation was strongly reduced by expression of either the Ca^{2+} permeation-deficient mutant (E630Q) or a pore-dead mutant (E630K) (Fig. 2). Basal Ca^{2+} entry into nonstimulated cells was reduced when expressing either of the TRPC3 pore mutants to the level of vector-transfected controls (Fig. 2B). As expected from the proposed NCX1-mediated Ca^{2+} entry contri-

Fig. 1. Neutralization of E630 in TRPC3 (E630Q) eliminates Ca^{2+} but not monovalent permeability. Representative ramp protocol recordings from HEK293 cells transfected by either TRPC3-WT (A and B) or the mutant channel E630Q (C and D) in the presence of 140 mM extracellular Na^+ and 2 mM Ca^{2+} (A and C), or in absence of extracellular Na^+ and presence of 10 mM Ca^{2+} using 10 mM BAPTA in the pipette solution (B and D), before (black) and after stimulation with 100 μM carbachol (+CCh, red).

Fig. 2. Receptor-stimulated Ca^{2+} influx as well as NFAT translocation are impaired in HEK293 cells expressing the Ca^{2+} impermeable TRPC3-E630Q or the impermeant TRPC3-E630K, compared with cells expressing wild-type TRPC3. (A) Representative traces of fura-2 Ca^{2+} -imaging experiments. Cells were stimulated by 100 μM carbachol (arrow). (B) Mean Δ ratio values ($\pm$ SEM, $n > 40$) derived from fura-2 Ca^{2+} -imaging. Black bars indicate the basal (unstimulated) Ca^{2+} entry at indicated transfections. (C) Mean nuclear/cytosol fluorescence intensity ratio ($\pm$ SEM, $n > 11$) of HEK293 cells expressing GFP-NFAT and the respective channel protein after stimulation and application of the same protocol as used in fura-2 experiments. Black bar (basal) represents mean nuclear/cytosol fluorescence intensity ratio in HEK293 cells transfected with GFP-NFAT only. (B and C) Asterisks indicate statistically significant difference to TRPC3-WT-expressing cells. (D) Representative fluorescence images recorded in GFP-NFAT translocation experiments shown at Left. Positions of nuclei are indicated by arrowheads. Nucleus/cytosol fluorescence ratios of example images are indicated.

bution, TRPC3-E630Q did not fully eliminate the Ca^{2+} entry signal. Ca^{2+} entry into cells expressing TRPC3-E630Q remained significantly higher than in cells transfected to express TRPC3-E630K. Nonetheless, NFAT translocation in HEK293 cells was completely suppressed with either pore mutation of TRPC3 (Fig. 2 C and D). This finding indicated that Ca^{2+} permeation through the pore is essential to initiate NFAT translocation, whereas indirect NCX-mediated signaling was barely involved because the NCX inhibitor KB-R7943 (5 μM) failed to prevent NFAT translocation (Fig. S6).

A possible dual Ca^{2+} signaling function of TRPC3 was further investigated in the electrically excitable cardiac HL-1 cell line. As illustrated in Fig. 3, Ca^{2+} entry into endothelin-stimulated cells was slightly enhanced compared with vector-transfected controls (Fig. 3A) by expression of either wild-type TRPC3 (Fig. 3B) or TRPC3-E630Q (Fig. 3C) but reduced down to basal (non-stimulated) level with TRPC3-E630K (Fig. 3D). Expression of wild-type TRPC3 (Fig. 3B) or TRPC3-E630Q (Fig. 3C) generated a Ca^{2+} signal that was mainly based on voltage-gated $\text{Ca}_v1.2$ channels as evident by its sensitivity to nifedipine (3 μM). In clear contrast to the observed Ca^{2+} signals, cells expressing TRPC3-E630Q lacked endothelin-stimulated NFAT translocation (Fig. 3C). Thus, the TRPC3 mutant with impaired divalent conductance (E630Q) is able to initiate a large global Ca^{2+} signal via promotion of voltage-gated Ca^{2+} entry, but this signal is not translated into NFAT activation. Importantly, even endogenous TRPC3 channels appear sufficient to exert a significant impact on NFAT translocation as evident from vector-transfected controls displaying higher translocation than cells expression the pore-dead dominant-negative E630K mutant. It is of note that, in contrast to HEK293 cells, NFAT translocation in HL-1 cells was not significantly promoted in response to depletion of intracellular stores with thapsigargin (Fig. S7), indicating that the channels involved in NFAT signaling of HL-1 are not classical store-operated Ca^{2+} entry channels. Opening of TRPC3 channels resulted in NFAT activation, but also generation of an additional intracellular Ca^{2+} signal mediated by voltage-gated Ca^{2+} channels that was fairly well segregated from the NFAT pathway. Notably, TRPC3-mediated NFAT activation can occur at barely detectable global Ca^{2+} changes such as in the presence of nifedipine (3 μM) in control cells or cells overexpressing TRPC3 (Fig. 3 A and B), therefore we hypothesized that the triggering Ca^{2+} elevation is likely to take place in a restricted signaling microdomain at the TRPC3 channel complex, containing essential downstream signaling components such as calmodulin and calcineurin (CaN) to allow specific transduction of this local Ca^{2+} signal. Indeed, direct association of TRPC3 with CaN have been demonstrated (20, 21)

and the existence of a dynamic TRPC/CaN signaling complexes have been proposed.

Coupling of Cardiac TRPC3 Signaling to Activation of the NFAT

Pathway Involves PKC-Dependent Phosphorylation. Previous investigations demonstrated assembly of CaN along with immunophilins (FKBP12) into TRPC6 signalplexes and dependency of this process on protein kinase C-mediated phosphorylation (21). PKC-mediated phosphorylation of TRPC3 appears essential for both recruitment of CaN into TRPC complexes and inhibitory regulation (22, 23). This result prompted us to hypothesize that suppression of PKC phosphorylation may disrupt the functional TRPC3/CaN signaling unit without preventing channel function. Consequently, we set out to test whether TRPC3 linkage to NFAT nuclear translocation depends on regulation of the channel complex by PKC. To suppress TRPC3 phosphorylation by PKC isoenzymes, we performed experiments with GF109203X, a compound that inhibits conventional PKC isoforms including PKC- γ , as one essential player in the control of TRPC3 channels (23). Because CaN has been shown to associate with TRPC3/6 channels in a manner dependent on phosphorylation by PKC (21), we speculated that prevention of PKC phosphorylation may disrupt TRPC/CaN complexes. Indeed, immunoprecipitation experiments in HEK293 cells confirmed that the PKC inhibitor prevents association of CaN into TRPC3 complexes along with reduction of threonine phosphorylation of the channel protein (Fig. 4). Alternatively, a mutant that is defective in PKC- γ -mediated inhibitory modulation, i.e., T573A corresponding to the murine TRPC3-moonwalker (Mwk) mutation, was expressed in HEK293 and HL-1 cells. It is of note that overexpression of the phosphorylation-deficient TRPC3-Mwk by itself was barely tolerated by the cells, presumably due to a gain in function leading to Ca^{2+} overload. Therefore, we transfected cells to overexpress the T573A mutant along with wild-type TRPC3 (DNA ratio 3:1). This transfection resulted in a heteromeric TRPC3 conductance larger than that generated by wild-type TRPC3 alone (Fig. S8) but essentially tolerated by the host cells. The derived heteromers appeared correctly targeted into the membrane as indicated by fluorescence microscopy. Interestingly, currents through TRPC3-Mwk/TRPC3-WT channels were rather stable during continuous agonist stimulation, most likely due to lack of inhibitory regulation of the channels by PKC and recordings in Na^+ -free extracellular solution confirmed Ca^{2+} permeability of the channels.

Either treatment of cells expressing TRPC3-WT with GF109203X (2 μM) or transfection of cells with TRPC3-Mwk/TRPC3-WT produced a similar cellular phenotype, displaying

A

WB: Phospho-Threonine

B

WB: HA-TRPC3

C

WB: Calcineurin

Fig. 4. GF109203X inhibits phosphorylation of TRPC3 and its association with calcineurin. HEK-293 cells expressing HA-tagged TRPC3 were incubated with or without 2 μ M GF109203X (GFX) and lysed and subjected to SDS/PAGE and immunoprecipitation. (A Left) Total HEK-cell lysates were immunoprecipitated by using an anti-HA antibody and immunoblotted with an anti-phospho-threonine antibody. (A Right) Bars representing the densitometric analysis of phospho-threonine-immunoreactivity. Mean values are given for carbachol-stimulated cells in the absence and presence of GFX ($\pm$ SEM, $n = 4$). Asterisk indicates statistically significant differences. (B) Stripped membranes were immunoblotted again by using an anti-HA antibody (Lower). Proteins detected in total cell lysates (lane 1, Input), immunocomplexes (lane 2, IP-HA-C3) and lysates precipitated only with beads (lane 3, Ctrl.). (C) Coimmunoprecipitations of cell homogenates using antibodies against the HA-tag and calcineurin, and immunoblotted against calcineurin. Proteins detected in total cell lysates (lane 1, Input), immunocomplexes (lane 2, IP-HA-C3; lane 3, IP-CN), and lysates precipitated only with beads (lane 4, Ctrl.).

PKC-dependent phosphorylation of the channel or as a consequence of the TRPC3-Mwk mutation. This fact may be considered as part of the molecular mechanism underlying the moonwalker pathophysiology (23).

Impaired PKC regulation of cardiac TRPC3 is shown to result in uncoupling from the NFAT pathway without disrupting the linkage between TRPC3 and global myocyte Ca^{2+} via voltage-gated Ca^{2+} entry. We provide evidence that TRPC3-Ca N /NFAT signaling takes place in a restricted microdomain and requires both direct Ca^{2+} permeation through the TRPC3 pore as well as Ca N targeting into the signal complex. Cardiac TRPC3 complexes are shown to produce Ca^{2+} signals both via direct Ca^{2+} transport and by control of voltage-dependent Ca^{2+} entry. Our results demonstrate the ability of cardiac TRPC3 channels to switch in a phosphorylation-dependent manner between a transcriptionally active and a transcriptionally silent signaling mode (Fig. 6). Because TRPC3 is likely to change in expression along with other TRPC species during pathophysiologic stress, the formation of divergent

heteromeric TRPC3 channel complexes may be anticipated. The observed dominant negative effect of the phosphorylation-deficient moonwalker mutation on Ca N /NFAT activation suggests a prominent role of phosphorylation in maintenance of transcriptionally active TRPC complexes in the heart. Situations of hampered PKC phosphorylation or promoted dephosphorylation may convert TRPC complexes into a cardiac Ca^{2+} signaling unit that is functionally isolated from the Ca N /NFAT activation pathway.

Our findings highlight the key role of nonselective TRPC channels in the control of transcriptional programs and extend this concept by demonstrating a pivotal role of Ca^{2+} transport through the TRP pore structure along with a unique phosphorylation-dependent molecular switch that allows efficient control of cardiac gene transcription by neurotransmitters and hormones.

Materials and Methods

Homology Modeling. For details on sequence alignment, homology modeling and model evaluation, see *SI Materials and Methods*.

Fig. 5. Phosphorylation of threonine 573 of the TRPC3 channel protein is essential for the activation of the calcineurin/NFAT pathway in HL-1 atrial myocytes. (A) Representative traces of fura-2 Ca^{2+} -imaging experiments in cells transfected with either TRPC3-WT or TRPC3-T573A and TRPC3-WT (DNA ratio 3:1, MWK/TRPC3-WT), stimulated by 100 nM endothelin (arrow) and in the absence or presence of 3 μ M nifedipine (+ Nif) or 2 μ M GF109203X (+ GFX), as indicated. (B) Mean Δ ratio values ($\pm$ SEM, $n > 30$) of fura-2 Ca^{2+} -imaging experiments. Asterisks indicate statistically significant nifedipine-induced inhibition. (C) Mean nucleus/cytoplasm fluorescence intensity ratio ($\pm$ SEM, $n > 9$) in HL-1 cells expressing GFP-NFAT and the respective (mutant) channel protein after stimulation and application of the same protocol as used in fura-2 experiments. (B and C) Asterisks indicate statistically significant difference to TRPC3-WT-expressing cells in the absence of inhibitors (white bar). (D) Representative fluorescence images recorded in GFP-NFAT translocation experiments. Individual nucleus/cytoplasm fluorescence ratios are given, and positions of nuclei are indicated by arrows.

Fig. 6. Phosphorylation of TRPC3 at position 573 (via PLC) enables Ca²⁺/calmodulin/calcineurin (Ca²⁺, Cm, CaN)-dependent activation upon receptor-activated Ca²⁺ entry through TRPC3. Dephosphorylation of position 573 turns TRPC transcriptionally silent while enhancing its general activity. L-type channels are controlled by TRPC3-induced changes in membrane potential, providing only negligible effects on Ca²⁺-induced NFAT activation in HL-1 atrial myocytes.

DNA and Mutagenesis. Site-directed mutagenesis was performed with standard protocols. Details on cDNA constructs and cloning procedures are provided in *SI Materials and Methods*.

Cell Culture and Transfection. Cell lines were cultured at 37 °C and 5% CO₂. For HEK293 cells DMEM (Invitrogen) supplied with 10% FCS and for HL-1 atrial myocytes Claycomb medium (Sigma) supplied with 100 μM norepinephrine, 4 mM L-glutamine and 10% FCS were used. Lipofection was used for gene transfer; for details on DNA amounts and reagents, see *SI Materials and Methods*.

Electrophysiology. Standard patch clamp protocols were used (*SI Materials and Methods*). Standard bath solutions contained 140 or 0 mM NaCl, 0 or 140 mM NMDG, 2 mM MgCl₂, 10 mM glucose, 10 mM Hepes, 2 or 0 mM CaCl₂, and 0 or 2 mM BaCl₂ at pH adjusted to 7.4 with NaOH or NMDG. Pipette solution contained 120 mM cesium methanesulfonate, 20 mM CsCl, 15 mM Hepes, 5 mM MgCl₂, and 3 mM EGTA, at pH adjusted to pH 7.3 with

CsOH. For delineation of Ca²⁺ permeability of TRPC3 mutants, a bath solution containing 132 mM NMDG, 2 mM MgCl₂, 10 mM Glucose, 10 mM Hepes, 3 mM CaCl₂, 7 mM Ca-Gluconate, at pH adjusted to 7.4 with methanesulfonic acid and a pipette solution composed of 140 mM cesium methanesulfonate, 15 mM Hepes, 5 mM MgCl₂, and 10 mM BAPTA at pH 7.3 was used.

Measurement of NFAT-Translocation. Cells were transfected to express an N-terminally GFP-tagged NFATc1 fusion (15) and plated on coverslips. For buffers and solutions see *SI Materials and Methods*. Agonists as well as inhibitors (Pyr3, GFX109203, nifedipine, or KB-R7943) remained present continuously after administration. GFP-NFAT translocation was monitored (488 nm excitation) with standard fluorescence microscopy (Zeiss Axiovert equipped with Coolsnap HQ). GFP-NFAT and YFP-TRPC3-WT/-mutant fluorescence were discriminated by specific cellular localization. Nuclear/cytosol fluorescence intensity ratios of cells were calculated with ImageJ software.

Measurement of Intracellular Ca²⁺ Signaling. For details on fura-2 calcium imaging experiments see *SI Materials and Methods*.

Immunoprecipitation. In short, protein-A- or protein-G-bead-precleared supernatants of lysates from stimulated HEK293 cells were incubated with precipitating antibody overnight. After the addition of respective protein-A- or protein-G-beads, washing, and denaturation in Laemmli buffer, the immunocomplexes were separated by SDS/PAGE and subjected to Western blotting. See *SI Materials and Methods* for details.

Reagents. Chemicals, reagents, and antibodies were purchased from Sigma Aldrich. KB-R7943 and GF109203X were from Tocris Biosciences. The TRPC3 pore blocker Pyr3 was synthesized as published (16).

Statistics. Data are presented as mean values ± SEM and was tested for statistical significance by using the Student *t* test (**P* < 0.05).

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