Selective and direct inhibition of TRPC3 channels underlies biological activities of a pyrazole compound

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Canonical transient receptor potential (TRPC) channels control influxes of Ca^{2+} and other cations that induce diverse cellular processes upon stimulation of plasma membrane receptors coupled to phospholipase C (PLC). Invention of subtype-specific inhibitors for TRPCs is crucial for distinction of respective TRPC channels that play particular physiological roles in native systems. Here, we identify a pyrazole compound (Pyr3), which selectively inhibits TRPC3 channels. Structure-function relationship studies of pyrazole compounds showed that the trichloroacrylic amide group is important for the TRPC3 selectivity of Pyr3. Electrophysiological and photoaffinity labeling experiments reveal a direct action of Pyr3 on the TRPC3 protein. In DT40 B lymphocytes, Pyr3 potently eliminated the Ca^{2+} influx-dependent PLC translocation to the plasma membrane and late oscillatory phase of B cell receptor-induced Ca^{2+} response. Moreover, Pyr3 attenuated activation of nuclear factor of activated T cells, a Ca^{2+}-dependent transcription factor, and hypertrophic growth in rat neonatal cardiomyocytes, and in vivo pressure overload-induced cardiac hypertrophy in mice. These findings on important roles of native TRPC3 channels are strikingly consistent with previous genetic studies. Thus, the TRPC3-selective inhibitor Pyr3 is a powerful tool to study in vivo function of TRPC3, suggesting a pharmaceutical potential of Pyr3 in treatments of TRPC3-related diseases such as cardiac hypertrophy.

Ca^{2+} signaling | pyrazole compounds | TRPC channels | TRPC3

Ca^{2+} signals control diverse cellular processes, ranging from ubiquitous activities like gene expression to tissue specific responses such as lymphocyte activation and cardiac diseases (1, 2). Stimulation of plasma membrane (PM) receptors that generates 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol-4,5-bisphosphate (PIP_2) via phospholipase C (PLC) elevates cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which is controlled by 2 components, IP_3-induced Ca^{2+} release from intracellular Ca^{2+} store, endoplasmic reticulum (ER), and Ca^{2+} influx across PM. Ca^{2+} influx is mediated by diverse Ca^{2+}-peameable ion channels activated by various triggers (1, 2). *Drosophila transient receptor potential* (*trp*) protein and its homologues are assembled to form cation- and Ca^{2+}-permeable channels (3). Members of the "canonical" TRPC subfamily are characterized by activation induced upon stimulation of PLC-coupled receptors (4, 5). TRPC channels have been originally proposed as store-operated channels (SOC) activated by Ca^{2+} depletion of stores, whereas closely related TRPC3, TRPC6, and TRPC7 showed activation sensitivity to the membrane-delimited action of DAG (6, 7). Hence, the exact roles of TRPCs in mediating Ca^{2+} entry in response to Ca^{2+} store depletion and messenger molecules upon receptor activation remain controversial. In native systems, genetic disruption experiments have revealed important roles of TRPC1 and TRPC4 in the formation of SOCs in different cell types (8–10), and TRPC6 in receptor-operated cationic channels in vascular smooth muscle cells (11). Specific pharmacological inhibitors greatly facilitate functional identification of native TRP channel subtypes.

TRPC channels have been implicated in diverse biological functions. In B lymphocytes, TRPC1 or TRPC3 regulates B cell receptor (BCR)-mediated Ca^{2+} oscillations that activate nuclear factor of activated T cells (NFAT), a Ca^{2+}-responsive transcription factor (8). In particular, TRPC3 is associated with PLCγ2 to control amplification of receptor-mediated signals (12, 13). TRPC3 is also important in the T cell receptor-dependent Ca^{2+} entry pathway (14). Recently, studies employing transgenic mice and RNAi-mediated knockdown or overexpression strategy in cardiac myocytes have showed that TRPC3 and TRPC6 promote cardiac hypertrophy through activation of calcineurin and its downstream effector, NFAT (15–19). These results suggest TRPC channels as new targets for the development of pharmaceutical agents to treat cardiac hypertrophy. Roles are demonstrated as well for TRPC3 in the brain (20) and skeletal muscle (21), for TRPC6 in smooth muscle (22) and kidney (23), and for TRPC4 in endothelial cells (10).

Bis(trifluoromethyl)pyrazoles (BTPs) are a class of pyrazole derivatives that act as potent immunosuppressive compounds by inhibiting cytokine release from human lymphocytes and suppressing T cell proliferation (24). The BTP derivative 4-methyl-4′-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-5-carboxanilide, BTP2 (YM-58483), was shown to block SOCs in T lymphocytes and TRPC channels in HEK293 cells (25–27). Importantly, unlike other TRP inhibitors, SK&F 96365 and 2-aminoethyldiphenylborate, BTP2 is selective to TRP channels and does not affect Ca^{2+} handling by mitochondria or ER, or K^+ channels or voltage-dependent Ca^{2+} channels (25–27). Pharmacological profiles of BTP2 have been investigated in vitro and in vivo to evaluate its potential as a therapeutic anti-asthma drug (28). However, BTP2 failed to show subtype selectivity among members of the TRPC family, inhibiting both TRPC3 and TRPC5 (27).

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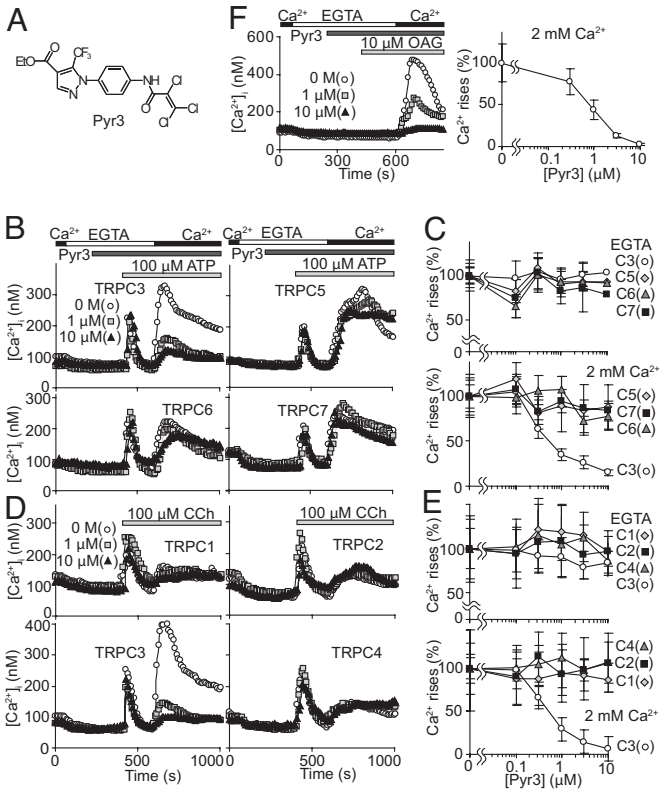

Fig. 1. Selective inhibition of TRPC3-mediated Ca^{2+} influx by Pyr3. (*A*) Chemical structure of Pyr3. (*B–E*) Concentration-dependent inhibitory action of Pyr3 on ATP receptor-induced (*B* and *C*) or mAChR-induced (*D* and *E*) induced Ca^{2+} influx via TRPCs. (*B* and *D*) Average time courses of Ca^{2+} responses induced by 100 μM ATP in HEK293 cells (*B*) or by 100 μM CCh in HEK293T cells (*D*) transfected with TRPCs at indicated Pyr3 concentrations. (*C* and *E*) Percentage peak $[Ca^{2+}]_i$ rises in Ca^{2+}-free, 0.5 mM EGTA-containing (*Upper*) or 2 mM Ca^{2+}-containing (*Lower*) external solution compared with control responses without Pyr3 (n = 33–104). (*F*) Pyr3 Inhibition of Ca^{2+} influx via OAG-activated TRPC3. Average time courses of Ca^{2+} responses induced by 10 μM OAG at indicated Pyr3 concentrations in TRPC3-transfected HEK293 cells (*Left*). Percentage peak $[Ca^{2+}]_i$ rises in 2 mM Ca^{2+} solution (*Right*) (n = 19–37).

Here, we study pharmacological properties of the pyrazole compound Pyr3 and demonstrate that Pyr3 selectively and directly inhibits TRPC3 channels among TRPC family members. Pyr3 potently inhibits BCR-induced responses and hypertrophic responses, in which importance of TRPC3 have been reported. Our findings suggest that Pyr3 is a useful tool for clarification of crucial and widespread functions of TRPC3 and for treatments of TRPC3-mediated diseases as well.

Results

Pyr3 Selectively Inhibits TRPC3-Mediated Ca^{2+} Influx in HEK293 Cells. Ethyl-1-(4-(2,3,3-trichloroacrylamide)phenyl)-5-(trifluoromethyl)-1H-pyrazole-4-carboxylate was synthesized as reported in ref. 24 (Fig. 1*A* and Fig. S1 in *SI Appendix*). It lacks the BTP group in contrast to BTP1 and BTP2. Therefore, we recategorized the compound together with BTP1 and BTP2 as pyrazole compounds (Pyrs), and abbreviated it as Pyr3 and BTP1 and BTP2 as Pyr1 and Pyr2, respectively. Effects of Pyr3 were examined on TRPC channel-mediated Ca^{2+} influx observed separately from Ca^{2+} release as prominent $[Ca^{2+}]_i$ rises upon readministration of Ca^{2+} to the extracellular solution under stimulation of native P2Y purinoceptors by ATP or UTP in HEK293 cells or muscarinic acetylcholine receptors (mAChR) by carbachol (CCh) in HEK293T cells (Fig. S2 in *SI Appendix*),

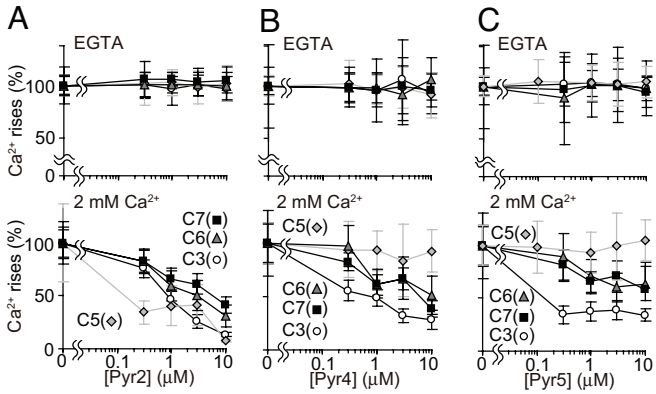

Fig. 2. Inhibition of TRPCs by Pyr2, Pyr4, or Pyr5. (*A–C*) Concentration-dependent inhibitory action of Pyr2 (*A*), Pyr4 (*B*), or Pyr5 (*C*) on Ca^{2+} influx induced by 100 μM ATP via TRPCs. Percentage peak $[Ca^{2+}]_i$ rises in Ca^{2+}-free, 0.5 mM EGTA- (*Upper*) or 2 mM Ca^{2+}-containing (*Lower*) external solution compared with control responses without drugs (n = 18–66).

which have only low endogenous Ca^{2+} influx activity (29). Application of Pyr3 inhibited TRPC3-mediated Ca^{2+} influx in a dose-dependent manner with the IC_{50} value of 0.7 μM (Fig. 1 *B–E* and Figs. S3 and S4 in *SI Appendix*): inhibitory action of Pyr3 became apparent at 0.3 μM, and was almost complete at 3 μM. Ten μM Pyr3 failed to significantly alter Ca^{2+} influx mediated by other TRPC members, Ca^{2+} responses in the absence of extracellular Ca^{2+} (Fig. 1 *B–E*), and the control basal $[Ca^{2+}]_i$ levels (data not shown). Interestingly, Ca^{2+} influx was inhibited by Pyr3 in cells coexpressing TRPC3 plus TRPC6 but not in cells coexpressing TRPC1 plus TRPC5 (Fig. S5 in *SI Appendix*). The results suggest a selectivity of Pyr3 to TRPC3 channels among TRPC family members and intactness of Ca^{2+} release and Ca^{2+} extrusion machinery after Pyr3 administration.

Because DAG has been suggested as a physiological activation trigger for TRPC3, TRPC6, and TRPC7 channels (6), we examined effects of Pyr3 on Ca^{2+} influx induced by the membrane-permeable DAG analogue, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) in TRPC3-transfected HEK293 cells (Fig. 1*F*). The OAG-induced Ca^{2+} influx via TRPC3 was inhibited by Pyr3 in a dose-dependent manner as observed for receptor-activated Ca^{2+} influx via TRPC3: IC_{50} of Pyr3 was 0.8 μM. The results support the TRPC3 channel as the main action site of Pyr3.

Structural Motif Important for TRPC3 Channel Selectivity on Pyr3. Pyr2 inhibited only TRPC5 at 0.3 μM. However, at higher concentrations, Pyr2 inhibited TRPC3, TRPC5, TRPC6, and TRPC7 (Fig. 2*A* and Fig. S6 *A* and *D* in *SI Appendix*) as reported in ref. 27. Thus, Pyr2 is different from Pyr3 in not distinguishing members of the TRPC family. We designed chimeric pyrazole derivatives, Pyr4 and Pyr5 (Fig. S1 in *SI Appendix*) to determine structural requirements for the selectivity to TRPC3 in Pyr3. Pyr4, a Pyr3 analogue with 4-methyl-1,2,3-thiadiazole-5-carboxyamide group of Pyr2 substituted for trichloroacrylic amide group, at 10 μM inhibited TRPC3, TRPC6, and TRPC7 but not TRPC5 (Fig. 2*B* and Fig. S6 *B* and *E* in *SI Appendix*). These results indicate a selectivity of Pyr4 to DAG-activated TRPC channels, suggesting that 3,5-bis(trifluoromethyl)pyrazole group is important for Pyr2 to recognize TRPC5. Pyr5, a Pyr3 analogue with 3,5-bis(trifluoromethyl)pyrazole group of Pyr2 substituted for ethyl-3-trifluoromethylpyrazole-4-carboxylate group, at 0.3 μM inhibited only TRPC3, suggesting that the trichloroacrylic amide group shared by Pyr3 and Pyr5 is critical for the TRPC3 selectivity (Fig. 2*C* and Fig. S6 *C* and *F* in *SI Appendix*). Interestingly, at higher concentrations such as 10 μM, Pyr5 enhanced Ca^{2+} response in vector-transfected cells or

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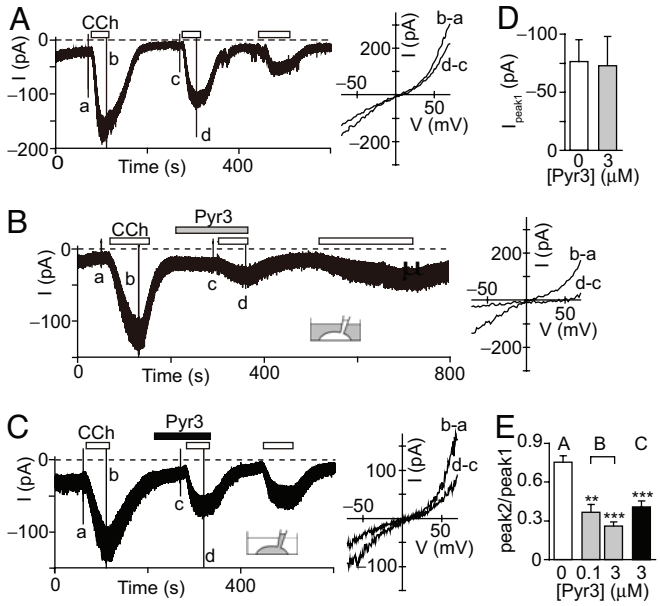

Fig. 3. mAChR-activated TRPC3 current is suppressed by extracellular application of Pyr3. (*A–C*) Traces of ionic currents induced by 60 μM CCh at a holding potential of −50 mV in TRPC3-transfected HEK293 cells (*Left*). I–V relationships obtained by subtracting the currents evoked by the voltage-ramps before activation of channels (a and c) from those after activation (current traces b and d) (*Right*). (*B*) Three μM Pyr3 is added 1.5 min before second stimulation of CCh into the external solution. (*C*) Three μM Pyr3 is added in internal solution before the recordings, and then external Pyr3 is also applied 1.5 min before second CCh stimulation. (*D*) Average current amplitudes of the first response at −50 mV in the presence ($n = 7$) or absence ($n = 8$) of 3 μM Pyr3 in the internal solution. (*E*) Concentration-dependent inhibitory action of Pyr3, using the testing paradigm depicted in *B* and *C*. The amplitude of the second response was normalized to that of the first (peak2/peak1) ($n = 4–8$). **, $P < 0.01$ and ***, $P < 0.001$ vs. 0 μM Pyr3.

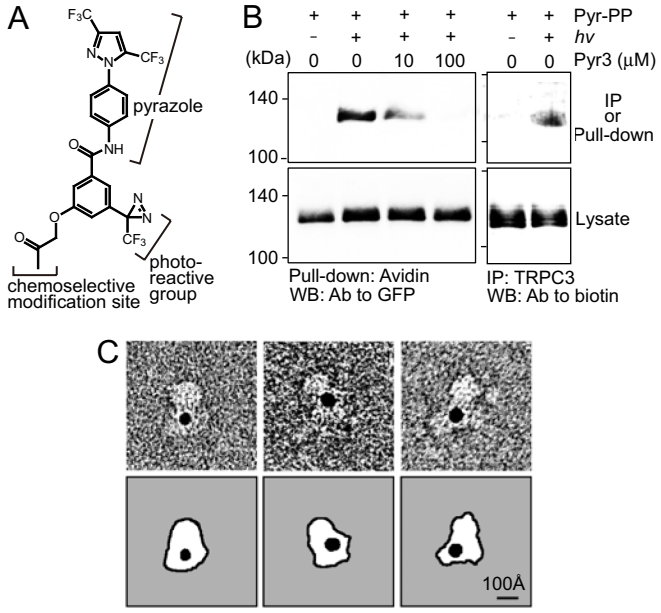

Fig. 4. Photochemical cross-linking of TRPC3 with Pyr-PP. (*A*) Chemical structure of Pyr-PP. (*B*) Pyr-PP directly binds TRPC3. After P-PALM, TRPC3-GFP proteins are detected with anti-GFP antibody by Western blot analysis (WB) in avidin pull-down samples. The photochemical Pyr-PP cross-linking of TRPC3 is inhibited by 3-min preincubation and subsequent coincubation with Pyr3 (10 or 100 μM) (*Left*). After P-PALM, the incorporation of the Pyr-PP-ARP adduct is detected with anti-biotin antibody by WB in immunoprecipitated (IP) samples with anti-TRPC3 antibody (*Right*). (*C*) Electron microscopic visualization of negatively stained TRPC3 after P-PALM with gold nanoparticles. Streptavidin-gold conjugate is attached to labeled-TRPC3 via biotin-labeling site.

at sustained phase in TRPC-expressing cells (Fig. S6*C* in *SI Appendix*), indicating a stimulatory side effect of Pyr5 on endogenous $[Ca^{2+}]_i$ regulation mechanism in HEK293 cells.

Direct Action of Pyr3 on TRPC3 Channel. Inhibitory action of Pyr3 on the TRPC3 channel was confirmed in TRPC3-transfected HEK293 cells, using the whole-cell mode of patch-clamp method (Fig. 3). When 60 μM CCh was added to stimulate endogenously expressed mAChRs, TRPC3-transfected HEK293 cells showed inward currents accompanied with an increase in the current fluctuation in the 2 mM Ca^{2+} external solution (Fig. 3*A*). Current-voltage (I–V) relationships of the currents in TRPC3-expressing cells showed a reversal potential at + 7.2 ± 3.4 mV ($n = 5$) and the prominent rectification at depolarizing potentials, corresponding well with those reported for receptor-activated TRPC3 currents (30). Ionic currents with a similar I–V characteristics were absent in control HEK293 cells (data not shown). The CCh-induced TRPC3 current was suppressed by extracellular perfusion of Pyr3 in a dose-dependent manner (Fig. 3 *B* and *E*) on top of run down in TRPC3 currents (Fig. 3*A*). TRPC3 maintains the same current level after washout of Pyr3 in contrast to control currents that gradually decrease with repeated CCh stimulation, suggesting that the Pyr3 block of TRPC3 is at least in part reversible but that the recovery from the blockade is a slow process (Fig. S7*A* in *SI Appendix*). Notably, because CCh induced indistinguishable Ca^{2+} response levels at the second and the third CCh stimulation in the presence and absence of Pyr3 (Fig. S7*B* in *SI Appendix*), mAChR desensitization induced by Pyr3 should be minimal if at all during repeated stimulation. Intracellular application of Pyr3 from the patch pipette failed to elicit significant changes in current levels or I–V relationships (Fig. 3 *C* and *D*), and, importantly, in susceptibility to inhibition by Pyr3 applied extracellularly (Fig. 3 *C* and *E*). OAG-activated TRPC3 currents were also inhibited by extracellular Pyr3 (3 μM) application, which failed to affect CCh- and OAG-activated TRPC6 currents (Fig. S8 in *SI Appendix*), TRPM4 currents (Fig. S9 in *SI Appendix*), and TRPM2 and TRPM7 currents (unpublished results). Inhibition of glycosylation by tunicamycin, however, failed to affect Pyr3 sensitivity of TRPC3 Ca^{2+} influx (Fig. S10 in *SI Appendix*). These results suggest that the action site of Pyr3 is located in the external side of the TRPC3 protein.

Photoaffinity labeling method is a powerful tool to identify target proteins of biologically active molecules. Recently, bifunctional photoaffinity probes having ligand moiety and biotin-tag were used for cross-linking studies of ligand/receptor complex (31). However, the introduction of a highly polar and sterically congested biotin-anchored tag to an affinity compound often resulted in marked impairment of intrinsic biological activity in the crucial probe design step. Therefore, we have carried out postphotoaffinity labeling modification (P-PALM), using a compact bifunctional Pyr probe, Pyr-PP, which carries a small functional group for selective modification by aldehyde/keto-reactive biotin derivative ARP (chemoselective modification site) and a photoreactive group for subsequent photoaffinity labeling (Fig. 4*A* and Fig. S11*A* in *SI Appendix*) (32). Importantly, Pyr-PP retained the activity to inhibit TRPC3-mediated Ca^{2+} influx (Fig. S11*B* in *SI Appendix*). In cells expressing the green fluorescent protein (GFP)-tagged TRPC3 protein, photoirradiation followed by biotin modification elicited incorporation of Pyr-PP into an ≈130-kDa protein band, which corresponds well with the molecular mass of an adduct of TRPC3-GFP (130 kDa) (Fig. 4*B Left*). Notably, Pyr-PP incorporation was inhibited

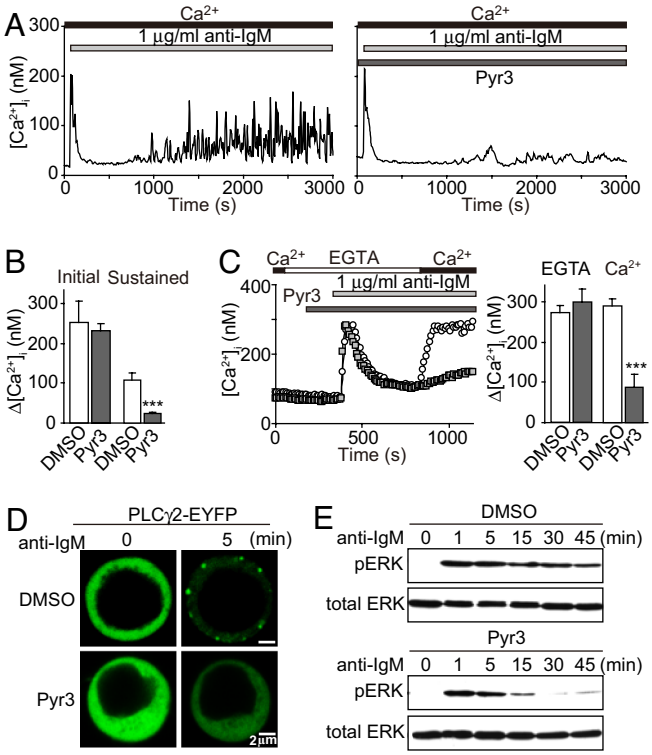

Fig. 5. Pyr3 inhibits native TRPC3 channels and downstream responses in DT40 B lymphocytes. (*A* and *B*) Inhibitory action of 0.3 μM Pyr3 on Ca^{2+} oscillation upon BCR stimulation with 1 μg/mL anti-IgM. (*A*) Representative time courses with (*Right*) or without of Pyr3 (*Left*). (*B*) Peak $[Ca^{2+}]_i$ rises at initial and sustained phases (30–40 min). (*C*) Inhibitory action of 1 μM Pyr3 on BCR-induced Ca^{2+} influx. Average time courses (*Left*). Peak $[Ca^{2+}]_i$ rises in Ca^{2+}-free, 0.5 mM EGTA- or 2 mM Ca^{2+}-containing solution (n = 42–49) (*Right*). (*D*) Confocal fluorescence images indicating PM translocation of PLCγ2-EYFP upon BCR-stimulation with 10 μg/mL anti-IgM. Three μM Pyr3 is applied 10 min before BCR stimulation. (*E*) Effects of Pyr3 on ERK activation induced by BCR stimulation (5 μg/mL anti-IgM). Three μM Pyr3 is applied 10 min before BCR stimulation. Cells are analyzed by WB, using anti-phospho-ERK2 antibody. ***, $P < 0.001$ vs. DMSO.

by coapplication of Pyr3. Furthermore, immunoprecipitation of TRPC3 followed by Western blot analysis with anti-biotin antibody strongly supports that Pyr-PP is incorporated into TRPC3 (Fig. 4*B Right*). To further confirm direct attachment of Pyr-PP on the TRPC3, biotin-labeled Pyr-PP in the negatively stained preparation was visualized with electron microscopy after incubation with streptavidin-gold conjugates (Fig. 4*C*) (33). Thus, Pyr3 selectively and directly binds to TRPC3. Interestingly, CCh failed to influence labeling of TRPC3 by Pyr-PP (Fig. S11*C* in *SI Appendix*). This is consistent with the observation that Pyr3 potently inhibited basal activity of TRPC3 but not that of TRPC6 (Fig. S12 in *SI Appendix*), excluding a possibility that receptor-induced channel activation mediates the Pyr3 action.

Pyr3 Suppresses Receptor-Activated Signaling by Inhibiting Native TRPC3 Channels in B Lymphocytes. Ca^{2+} oscillation is important for the efficiency and specificity of gene expression in lymphocytes (34). We recently reported that the Ca^{2+} oscillation is maintained by Ca^{2+} entry via TRPC3 that evokes translocation toward PM and secondary activation of PLCγ2 in DT40 B lymphocytes (13). Fig. 5*A* depicts a typical Ca^{2+} oscillation that follows initial transient Ca^{2+} responses upon stimulation of BCR in DT40 cells. Pyr3 significantly suppressed the Ca^{2+} oscillation (Fig. 5 *A* and *B*) and NFAT activity (Fig. S13 in *SI Appendix*), whereas it failed to suppress the initial Ca^{2+} responses. Pyr3 also suppressed BCR-induced Ca^{2+} influx but not Ca^{2+} release (Fig. 5*C*). In addition, observation by time-lapse confocal laser microscopy showed localization of EYFP-tagged PLCγ2 (PLCγ2-EYFP) near PM upon BCR stimulation, which was inhibited by 3 μM Pyr3 (Fig. 5*D*). Interestingly, coimmunoprecipitation of PLCγ1 and PLCγ2 with TRPC3 is unaffected by Pyr3 (Fig. S14 in *SI Appendix*), suggesting that Pyr3 acts independently of PLC-TRPC3 interaction. Thus, Pyr3 efficiently attenuates TRPC3-mediated PLCγ2 translocation and Ca^{2+} oscillation.

The activation of protein kinase C (PKC) by DAG promotes activation of extracellular signal-regulated kinase (ERK) through phosphorylation in DT40 cells (13). The PLCγ2 translocation and subsequent activation also enhance the downstream responses of DAG. In fact, the ERK phosphorylation maintained by BCR stimulation over 45 min became transient after application of 3 μM Pyr3 (Fig. 5*E*). Hence, Ca^{2+} influx via TRPC3 is required for full ERK activation in DT40 B lymphocytes.

Pyr3 Suppresses Cardiac Hypertrophy. Cardiac hypertrophy is an adaptive response of the heart to many forms of cardiac disease, including hypertension and mechanical load abnormalities (35). The importance of G protein-coupled receptors such as angiotensin II (Ang II) receptors, that activate PLC, is well established in cardiac hypertrophy (36). Recently, it has been revealed through siRNA strategy that Ca^{2+} influx through TRPC3 and TRPC6 activation is essential for Ang II-induced NFAT activation and cardiomyocyte hypertrophy (16). Pyr compounds were examined on hypertrophic responses in rat neonatal cardiomyocytes. The Ang II-induced NFAT translocation was suppressed by Pyr3, but weakly by Pyr2 in a concentration-dependent manner (IC_{50} value was 0.05 μM for Pyr3 and 2 μM for Pyr2) (Fig. S15 *A* and *B* in *SI Appendix*). Therefore, compared with Pyr2, Pyr3 is more potent in inhibiting NFAT signaling of cardiac myocytes. Pyr3 also suppressed the mechanical stretch-induced NFAT activation (Fig. S15*C* in *SI Appendix*). Notably, the Ang II-induced hypertrophic responses, such as actin reorganization, brain natriuretic peptide (BNP) expression, and protein synthesis were completely suppressed by Pyr3, but weakly by Pyr2 (Fig. S15 *D–F* in *SI Appendix*).

We further examined Pyr3 in pressure overload-induced cardiac hypertrophy in vivo. Importantly, systolic and diastolic blood pressure, heart rate, mortality, body weight, and weight for liver, lung, and heart were unaffected by chronic treatment with Pyr3 (0.1 $mg \cdot kg^{-1} \cdot day^{-1}$) in sham operated mice (Fig. S16*A* and Tables S1 and S2 in *SI Appendix*). In addition, transverse aortic constriction (TAC) operation significantly increased left ventricular end-systolic pressure (ESP) in mice treated with vehicle or Pyr3 (Tables S1 and S2 in *SI Appendix*), suggesting that pressure overload was equally induced in these mice. Strikingly, increased size of the heart by 1-week TAC operation was significantly attenuated by Pyr3 (Fig. 6 *A* and *B* and Fig. S16*B* in *SI Appendix*). The Pyr3 effect refers to concentric hypertrophy, because the ratio of internal ventricular radius at end diastole (r) to ventricular wall thickness (h) was significantly decreased in echocardiography in mid transverse heart sections (Fig. 6*A* and Fig. S16*C* in *SI Appendix*), in contrast to fractional shortening (FS) and the right ventricle unaffected by TAC (Fig. S16 *D* and *E* in *SI Appendix*). The TAC-induced increase in expression of atrial natriuretic peptide (ANP) mRNA, a reliable marker for cardiac hypertrophy, was also suppressed by Pyr3 (Fig. 6*C*). Six-weeks TAC operation induced an r/h ratio increase characteristic of dilated hypertrophy (Fig. S16*F* in *SI Appendix*), and deterioration of FS and elevation of weight-to-tibia length ratio of the left ventricle (LVW/TL) in good correlation with systolic pressure gradient. These symptoms were suppressed by Pyr3 (Fig. 6 *D* and *E*). Thus, Pyr3 is potent against concentric and dilated cardiac hypertrophy.

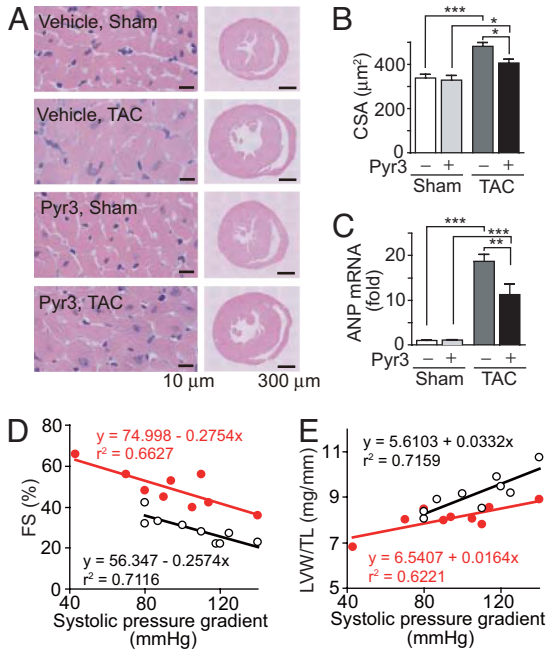

Fig. 6. Potent suppressive effects of Pyr3 on cardiac hypertrophy induced by pressure overload in mice. (*A–C*) Effects of Pyr3 on 1-week TAC-induced concentric hypertrophy. (*A*) H&E-stained mid transverse sections of hearts isolated from sham- and TAC-operated mice. (*B* and *C*) Effects of Pyr3 on the increase in cross-sectional areas (CSA) (*B*) and ANP mRNA expressions (*C*). *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$. (*D* and *E*) Effects of Pyr3 on 6-weeks TAC-induced dilated hypertrophy. Scattergram of systolic pressure gradient vs. FS, a surrogate of systolic function (*D*) and LVW/TL (*E*) in TAC-operated mice with (red) and without (black) treatment with Pyr3. Pyr3 significantly shifts relationships upward in FS ($P < 0.001$) and downward in LVW/TL ($P < 0.01$).

Discussion

The present investigation demonstrates a potent inhibitory action of Pyr3 on both recombinant and native TRPC3 channels. Photoaffinity labeling with Pyr-PP reveals direct action of Pyr3 on the TRPC3 channel. Pyr3 efficiently suppressed biological responses in which critical involvements of TRPC3 have been reported. In B lymphocytes, Pyr3 eliminated the BCR-induced Ca^{2+} oscillation regulated by TRPC3-mediated Ca^{2+} influx. In the cardiac system, Pyr3 attenuates NFAT activation and hypertrophic growth in myocytes and pressure overload-induced hypertrophy in vivo.

BTPs were originally identified as inhibitors of T lymphocyte activation (24). Several reports have suggested that BTP2 (Pyr2) is a potent inhibitor for both Ca^{2+} release-activated Ca^{2+} (CRAC) channels and TRPC channels and for NFAT-driven IL-2 production (25–27). Structure-function relationships in BTPs proposed that 4′-[3,5-bis(trifluoromethyl)pyrazol-1-yl]-carboxanilide moiety is useful for discovering potent inhibitors for CRAC channels (37). However, here we demonstrate that the 3,5-bis(trifluoromethyl)pyrazole group is not required for the inhibition of TRPC3, because Pyr3 without this group selectively inhibited TRPC3 channel, and is more potent than Pyr2 in inhibiting NFAT of cardiac myocytes. Moreover, our structure-function relationship study using Pyr4 and Pyr5 demonstrates that the 3,5-bis(trifluoromethyl)pyrazole or trichloroacrylic amide group is critical for the selectivity of Pyr4 or Pyr5 to TRPC5 or TRPC3, respectively. Thus, pyrazole group provides a molecular skeleton to invent potent inhibitors for each TRPC.

It has been suggested that Pyr2 activates the Ca^{2+}-activated nonselective cation channel TRPM4 that decreases Ca^{2+} influx by depolarizing membrane potential and reducing the Ca^{2+} entry driving force in lymphocytes (38). This contradicts with the report that Pyr2 failed to alter membrane potential in Jurkat cells (25). In addition, when external Na^+ ions were completely replaced by the nonpermeant organic cation *N*-methyl-D-glucamine, Sr^{2+} influx activated by CCh still showed an intact sensitivity to Pyr2, leading to an idea that the action of Pyr2 to block TRPC3 channels is independent of the membrane-depolarizing action of TRPM4 (27). This idea is consistent with our results. Importantly, if Pyr3 activates TRPM4-mediated membrane depolarization, it should also inhibit receptor-activated Ca^{2+} influx via TRPC channels other than TRPC3. However, this is not the case. Hence, the action of Pyrs is mainly attributable to inhibition of TRPCs.

Pyr2 unlikely acts from the cytosolic side of CRAC channels, for instance, by inhibiting the activation mechanism or the intracellular part of the channel pore (26). It has been also proposed that Pyr2 inhibits TRPC3 by preventing channel interactions with DAG or by compromising the conformational change in the channels that leads to opening (27). We have confirmed direct action of Pyrs on TRPC3 through observation that Pyr3 inhibition of TRPC3 activity or incorporation of Pyr-PP does not require receptors or their activation. Electrophysiological recording (Fig. 3) locates the action site on the extracellular side of TRPC3 proteins. Amino acid residues unique for TRPC3 in functionally important domains such as E3 (39) may be responsible for the TRPC3 selectivity of Pyr3. Recently, we reported a reconstruction of a 3-dimentional (3D) structure of TRPC3 at 15 Å resolution by single particle analysis of images taken by a cryoelectron microscope (33). It would be interesting to locate Pyr-binding site in 3D reconstitution of TRPC3 proteins at this resolution.

Functions of native TRPC3 channels are yet to be established. Initial overexpression studies reported that TRPC3 forms SOCs (5). However, constitutive channel activity suppressed by strong intracellular Ca^{2+} buffering (40, 41) and DAG-induced activation via a membrane-delimited pathway have been reported for TRPC3 (6). Therefore, depending on the expression system and protein expression levels, TRPC3 can function in a store-dependent or -independent manner (42, 43). Physical interactions have been suggested for TRPC3 also with IP_3 receptors, PLCγ1, and PLCγ2 (12, 13, 44). Interestingly, BCR stimulation failed to induce PLCγ2 translocation to PM in DT40 B cell mutant lines engineered with disruption of TRPC3 localization at PM (unpublished results), in support of the importance of TRPC3 in the PLCγ2 translocation. We have also found that TRPC3 mediates Ca^{2+} influx responsible for translocation of PKCβ to the PM and anchors PKCβ at PM. This suggests that TRPC3 functions not only as an ion conducting channel but also as a protein scaffold. Pyr3 should be extremely useful in selectively dissecting multiple roles of native TRPC3.

$[Ca^{2+}]_i$ elevation by various hypertrophic stimuli plays a critical role in the development of cardiac hypertrophy (36). Continuous blockage of L-type voltage-dependent Ca^{2+} channels by Ca^{2+} antagonists effectively suppresses the development of cardiac hypertrophy (16). However, because L-type Ca^{2+} channels controls excitation-contraction coupling, these Ca^{2+} channel antagonists may exert serious influence on the myocardial contraction. Recently, TRPC3 and TRPC6 channels have been reported to regulate cardiac hypertrophy (15–19). Suppression of Ang II- or mechanical stretch-induced NFAT activation and hypertrophic growth of cardiomyoctes by Pyr3 strongly suggest a potential of Pyr3 in the treatment of cardiac hypertrophy. Compared with Pyr2, which was reported to inhibit ANP secretion and β-myosin heavy chain expression in rat neonatal ventricular myocytes stimulated with α-adrenergic agonist, phenylephrine (15), Pyr3 has improved potency in inhibiting hypertrophic responses. Moreover, attenuation of pressure over-

load-induced cardiac hypertrophy in vivo by Pyr3 will give efficacy for the pathological heart without serious influence of the normal myocardial contraction. This Pyr3 property may lead to a safer therapeutic approach to cardiac hypertrophy.

Methods

The detailed methods for cell culture and cDNA expression, $[Ca^{2+}]_i$ measurements, electrophysiology, photoaffinity labeling, coimmunoprecipitation, confocal image analysis, measurements of NFAT and ERK activation, and analysis of cardiac hypertrophy in vitro and in vivo are described in *SI Appendix*.

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