Two-pore Channels (TPC2s) and Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP) at Lysosomal-Sarcoplasmic Reticular Junctions Contribute to Acute and Chronic β-Adrenoceptor Signaling in the Heart*

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Background: The Ca2+-releasing messenger nicotinic acid adenine dinucleotide phosphate (NAADP) acts via lysosomal two-pore channels (TPC2).

Results: Tpcn2−/− cardiac myocytes showed reduced acute responses to β-adrenoceptor stimulation and chronically reduced cardiac hypertrophy and arrhythmogenesis.

Conclusion: Acute and chronic effects of cardiac β-adrenoceptor stimulation depend on NAADP acting via TPC2 in lysosomes.

Significance: NAADP/TPC2 signaling pathways offer new strategies for cardiac therapeutics.

Ca2+-permeable type 2 two-pore channels (TPC2) are lysosomal proteins required for nicotinic acid adenine dinucleotide phosphate (NAADP)-evoked Ca2+ release in many diverse cell types. Here, we investigate the importance of TPC2 proteins for the physiology and pathophysiology of the heart. NAADP-AM failed to enhance Ca2+ responses in cardiac myocytes from Tpcn2−/− mice, unlike myocytes from wild-type (WT) mice. Ca2+/calmodulin-dependent protein kinase II inhibitors suppressed actions of NAADP in myocytes. Ca2+ transients and contractions accompanying action potentials were increased by isoproterenol in myocytes from WT mice, but these effects of β-adrenoceptor stimulation were reduced in myocytes from Tpcn2−/− mice. Increases in amplitude of L-type Ca2+ currents evoked by isoproterenol remained unchanged in myocytes from Tpcn2−/− mice showing no loss of β-adrenoceptors or coupling mechanisms. Whole hearts from Tpcn2−/− mice also showed reduced inotropic effects of isoproterenol and a reduced tendency for arrhythmias following acute β-adrenoceptor stimulation. Hearts from Tpcn2−/− mice chronically exposed to isoproterenol showed less cardiac hypertrophy and increased threshold for arrhythmogenesis compared with WT controls. Electron microscopy showed that lysosomes form close contacts with the sarcoplasmic reticulum (separation ~25 nm). We propose that Ca2+-signaling nanodomains between lysosomes and sarcoplasmic reticulum dependent on NAADP and TPC2 comprise an important element in β-adrenoceptor signal transduction in cardiac myocytes. In summary, our observations define a role for NAADP and TPC2 at lysosomal/sarcoplasmic reticulum junctions as unexpected but major contributors in the acute actions of β-adrenergic signaling in the heart and also in stress pathways linking chronic stimulation of β-adrenoceptors to hypertrophy and associated arrhythmias.

NAADP11 is the most potent Ca2+-mobilizing messenger yet described, and it plays important roles in a wide variety of animal and plant cells (1, 2). NAADP uniquely evokes Ca2+ release from lysosomes (3–8), an action demonstrated to require two-pore channels (TPCs) (9–12). TPCs are evolutionarily ancient members of the superfamily of voltage-gated ion channels found in both plant and animal cells and localize to the endo-

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The abbreviations used are: NAADP, nicotinic acid adenine dinucleotide phosphate; SR, sarcoplasmic reticulum; TPC, two-pore channel; CaMKII, Ca2+/calmodulin-dependent protein kinase II; ER, endoplasmic reticulum; SERCA, sarco/endoplasmic reticulum Ca2+ -ATPase; RyR, ryanodine receptor; PSS, physiological salt solution; ISO, isoproterenol; PES, programmed electrical stimulation; NPE, 1(2-nitrophenyl)diazooethane; AIP, autocamide-2-related inhibitory peptide.
lyosomal system. TPC2 proteins are exclusively expressed on vacuolar or lysosomal membranes (13). Consisting of 12 transmembrane domains, they have so far been shown to be regulated by NAADP, inositol lipids, and voltage (13). The formation of functional channels seems likely to require dimerization and allows for physiological expression of homo- or heterodimers (14, 15), whereas responses to NAADP likely depend on the association of an unidentified NAADP-binding protein (16, 17). In this complex manner, TPCs are able to act as a signaling hub to regulate endo-lysosomal ion homeostasis with NAADP responsible for lysosomal contributions to Ca$$^{2+}$$-signaling events.

The diversity of cellular mechanisms that involve NAADP signaling is remarkably broad, including egg fertilization, insulin secretion, and neuronal differentiation (18). Recent work on the Ebola virus has added another example of the potential importance of this pathway, showing that inhibition of NAADP-mediated Ca$$^{2+}$$ signaling drastically reduces infectivity (19). The use of Tpcn knock-out mouse lines demonstrates the roles for TPC proteins in physiological processes, both at the cellular and whole-organism levels (9, 11, 19–30).

We have previously observed that β-adrenoceptor stimulation can increase NAADP levels in cardiac muscle (31, 32), and functional evidence supports a link between β-adrenoceptor stimulation and the effects of NAADP (31, 33). Recent work is also suggestive of a role for NAADP in arrhythmias induced by acute β-adrenoceptor stimulation (34). However, the role of TPCs in cardiac tissue and the contribution of this pathway to the pathology surrounding chronic β-adrenergic stimulation have yet to be described.

There has been some discussion over which cations pass through TPCs in lysosomes and in particular whether these channels are permeable to Ca$$^{2+}$$ (28, 35–37). A recent study shows that TPCs are a requirement for NAADP-mediated lysosomal Ca$$^{2+}$$ release (11) and that the actions of NAADP on lysosomes involve permeation of TPC channels by Ca$$^{2+}$$ (11, 38).

Two isoforms of TPCs are expressed in human and mouse cells, and we have investigated NAADP-evoked Ca$$^{2+}$$ release and β-adrenoceptor signaling in murine cells and mice that have been genetically modified to lack TPC2 protein (H9252 mreceptor signaling in murine cells and mice that have been genetically modified to lack TPC2 protein (H9252). This isoform was targeted because it has been shown to be specifically expressed in lysosomal and late endosomal membranes (9).

Our observations are the first to demonstrate the importance of TPC2 proteins for both acute and chronic consequences of stimulation of β-adrenoceptors in heart muscle, and they provide yet another major role for these ion channels, the NAADP pathway and acidic organelles, in the (patho-) physiology of Ca$$^{2+}$$ signaling.

**Experimental Procedures**

**Cell Isolation, Guinea Pig**—Male guinea pigs (300–500 g) were killed in accordance with Schedule 1 of The Animals (Scientific Procedures) Act 1986 (HMSO). The heart was rapidly excised, tied to a cannula via the aorta and perfused through a syringe containing a predissolved with 95% O$_2$, 5% CO$_2$ solution containing (mM) NaCl 125, NaHCO$_3$ 25, KCl 5.4, NaH$_2$PO$_4$ 1.2, MgCl$_2$ 1, CaCl$_2$ 1.0, glucose 5.5, pH 7.4, together with heparin (100 units/ml) and streptokinase (100 units/ml). The cannula was then removed from the syringe and mounted onto a Langendorff perfusion system (35). Once mounted, the hearts were perfused with a solution (36 °C) containing (mM) NaCl 130, KCl 5.4, MgCl$_2$ 3.5, glucose 10, HEPES 5, NaH$_2$PO$_4$ 0.4; pH 7.4, and gassed with 95% O$_2$, 5% CO$_2$. After 3 min, this isolation solution was replaced with a further 50 ml of a similar solution that also contained 3 mg/ml collagenase (type II, Worthington) and 0.1 mg/ml streptokinase (100 units/ml). The cannula was then removed from the syringe and mounted onto a Langendorff perfusion system. Once mounted, the hearts were perfused with a solution (36 °C) containing (mM) NaCl 130, KCl 5.4, MgCl$_2$ 3.5, glucose 10, HEPES 5, NaH$_2$PO$_4$ 0.4; pH 7.4, and gassed with 95% O$_2$, 5% CO$_2$. After 3 min, this isolation solution was replaced with a further 50 ml of a similar solution that also contained 0.3 mg/ml collagenase (type II, Worthington) and 0.1 mM CaCl$_2$. Following enzymatic digestion (for a maximum time of 7 min), the heart was removed from the cannula. The ventricles were cut into pieces, and the myocytes were released during several gentle suspensions, then filtered through 250-μm mesh, and stored at room temperature in medium containing (in mM) NaCl 130, KCl 5.4, MgCl$_2$ 3.5, CaCl$_2$ 0.1, glucose 10, HEPES 5, NaH$_2$PO$_4$ 4.0, taurine 20; 0.1% bovine serum albumin; pH 7.4, and gassed with 95% O$_2$, 5% CO$_2$.

**Electrophysiology**—Cardiac myocytes were mounted in a perfusion bath placed at the stage of an inverted microscope and perfused with a physiological salt solution (PSS) containing (in mM) NaCl 125, NaHCO$_3$ 25, KCl 5.4, NaH$_2$PO$_4$ 1.2, MgCl$_2$ 1, glucose 5.5, CaCl$_2$ 1.8 and oxygenated with 95% O$_2$, 5% CO$_2$ to maintain a pH of 7.4. The PSS used for mouse experiments was identical except that CaCl$_2$ concentration was reduced to 1 mM. All electrophysiology was performed at 36 °C. Glass microelectrodes were manufactured from thin-walled, filamented borosilicate glass capillary tubing (GC150TF, Harvard Apparatus Ltd., Kent, UK). Electrode resistances of 3.0 ± 1.5 meegohms were used for whole-cell experiments. During whole-cell recordings, a whole-cell patch solution was used containing (in mM) K$^+$ aspartate 110, KCl 10, NaCl 5, MgCl$_2$ 5.2, HEPES 5, K$_2$ATP 5, pH 7.2, with KOH, to which Ca$^{2+}$ indicator dye,
caged NAADP, and inhibitors were added at the stated concentrations. Perforated patch was carried out using whole-cell patch solution plus amphotericin B at 250 μg/ml. Current clamp recordings were made using an AxoClamp 2A amplifier system (Axon Instruments). Cells were stimulated to fire action potentials using a 2-ms current pulse applied via the microelectrode at a rate of 1 Hz. Voltage clamp recordings were made using an AxoPatch 200B amplifier system (Axon Instruments). To stimulate the L-type Ca$^{2+}$ current ($I_{CaL}$), cells were maintained at a holding potential of −40 mV and stimulated by a 200-ms step depolarization to 0 mV at a rate of 0.2 Hz. $I_{CaL}$ amplitude was measured as maximum current minus current at the end of the voltage pulse.

**Ca$^{2+}$ Fluorescence, Dye Loading, and Cell Stimulation Protocol**—During whole-cell fluorescence experiments, Fluo-5F salt (100 μM) was added to the whole-cell patch solution. In mouse experiments, myocytes were incubated with Fluo-5F AM (20 μM) for 20 min. Ca$^{2+}$ transients were stimulated at 1 Hz either by a patch electrode (all guinea pig experiments) or by carbon fiber electrodes placed at the side of the superfusion bath (mouse experiments).

**Epifluorescence**—Guinea pig atrial myocytes were visualized using a Leica DMIRB inverted microscope. Excitation light was provided by 470 nm LED illumination, passed through a 475 ± 15 nm bandpass interference filter, and transmitted through the objective. Emitted light was passed through a 520-nm long pass filter and collected using a photomultiplier system (Cairn Research Ltd., Kent, UK). Photomultiplier signals were directed through an amplifier, followed by a low pass 100 Hz electronic filter, digitized (Axon Digidata 1200, Axon Instruments), and recorded with pClamp software at an acquisition rate of 10 kHz.

**Spinning Disk Confocal Microscopy**—Guinea pig and mouse ventricular myocytes were visualized using a Nikon Axiocam 200 inverted microscope with attached Nipkow spinning disk confocal unit (CSU-10, Andor Technology, UK). Excitation light was provided by a 488-nm diode laser (Cairn Research Ltd.) passed through the Nipkow unit and delivered to the sample through the objective. Emitted light passed back through the CSU-10 unit and was detected using an Andor iXON897 EM-CCD camera (Andor Technology) at a frame rate of 50 Hz. Images were recorded and analyzed using Andor iQ software (Andor Technology).

**Line Scan Confocal Microscopy**—Mouse ventricular myocytes were imaged with a confocal microscope system that consisted of a Leica TCS NT scanning head coupled to a Leica DMIRB inverted microscope with a 100× oil immersion objective lens (1.2 NA, Leica) in line scan mode (2.6 ms per line). Excitation light (488 nm) was provided by an air-cooled 488-nm argon ion laser system (Uniphase Ltd.), and emitted light was collected at wavelengths above 515 nm using a long-pass filter. Images were recorded using Leica TCS NT software and analyzed using ImageJ software.

**Analysis of Ca$^{2+}$ Transient Data**—For analysis, background fluorescence was subtracted, and multiple transients were averaged to collect data at a given time point. Data are presented as F/F₀ such that fluorescence data are presented relative to diastolic fluorescence. In the case of experiments in which NAADP was photoreleased in guinea pig ventricular myocytes, there was a small baseline change, and data are presented as F/F₀ where F₀ is equal to diastolic fluorescence before photo-release of NAADP.

**Electron Microscopy**—Rabbit ventricular tissue was fixed in Karnovsky fixative (39), resin-embedded, and sectioned at 80 nm (Reichert Ultracut). These were post-stained with 2% uranyl acetate and Reynolds lead citrate. Images were obtained using transmission electron microscopy (Jeol 1200EX II).

**Contraction Studies, Mouse Ventricular Myocytes**—Cells were stimulated to contract via a patch electrode (permeabilized configuration), and contractile properties were studied using the IonWizard system (IonOptix Corp.) to measure sarcomere length. Cells were visualized via a ×40 oil objective using an IonOptix MyoCam (IonOptix Corp.) that sampled images at a frequency of 240 Hz. Sinusoidal optical density traces arising from the alternating light and dark bands of the contractile machinery were then transformed into a signal of sarcomere length by application of a fast Fourier transform by the IonWizard sarcomere length acquisition software (IonOptix Corp.). Length measurements were calibrated using a stage micrometer with 2-μm graduations such that the number of pixels/μm recorded by the MyoCam could be entered into the software as a fixed value. Amplitude of sarcomere shortening was calculated by deduction of systolic from diastolic sarcomere length and expressed as a percentage of resting sarcomere length. Analysis was performed using IonWizard 5 software (IonOptix Corp.). All values represent an average of 10 contractions.

**Langendorff-perfused Whole Mouse Hearts**—Mice were killed in accordance with Schedule 1 of The Animals (Scientific Procedures) Act 1986 (HMSO). Hearts were excised, cannulated via the aorta under a microscope, and perfused for 30 s with PSS solution containing streptokinase (100 units/ml) via a syringe connected to the cannula. After initial perfusion, the syringe was removed and the cannula mounted on a Langendorff apparatus for retrograde perfusion with PSS containing 1.8 mM CaCl₂ (36 °C, gassed with 95% O₂, 5% CO₂) at a constant flow rate (3.5 ml/min). Hearts were allowed to beat spontaneously, and contractile force was measured by means of a hook placed through the ventricles near the apex, attached to a tension transducer. Resting tension was set between 0.5 and 1.0 g initially. Heart rate was calculated from the force signal as the reciprocal of the time interval between contractions. Inflow pressure was measured using a pressure transducer positioned close to the aorta so that changes in coronary artery resistance could be measured as pressure changes at a constant perfusion flow rate. Data were acquired and analyzed using Chart software (ADInstruments, UK), with a data acquisition sampling rate of 100 samples/s. The tension signal was low pass-filtered with a cutoff frequency of 50 Hz.

**Photorelease of Caged NAADP**—NPE-caged NAADP, synthesized in-house by a method described previously (40), was included in whole-cell patch solution for use in both atrial and ventricular myocytes at the concentrations stated. NPE-caged NAADP is inactive until “uncaged” by exposure to UV light. UV photolysis was provided by rapid (~1 ms) arc-lamp flash directed through a 355-nm filter and transmitted to the cell via the objective (atrial cells, Cairn Photolysis System, Cairn...
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Research Ltd.; ventricular cells, Rapp Photolysis System, Rapp OptoElectronic, Vedel, Germany).

Cell Superfusion with NAADP-AM—NAADP-AM was synthesized in-house by a method described previously (41). Loading of NAADP into the cytosol was achieved by rapid switching the extracellular solution to one containing the membrane-permeant acetoxymethyl ester of NAADP (allowing rapid access of NAADP-AM to the cytosol and subsequent liberation of NAADP following action of intracellular esterases).

Programmed Electrical Stimulation (PES)—To assess propensity to arrhythmias, Langendorff-perfused hearts from WT and Tpcn2−/− mice were subjected to PES. Monophasic action potentials were recorded from the left ventricular epicardial surface.

Hearts from mice not previously exposed to chronic β-adrenergic stimulation were subjected to PES in the presence of 50 nm isoproterenol. Ventricular pacing first occurred with a progressively increasing pacing current (from pacing capture current to 35 mA). At each current amplitude, three bursts of 50 stimuli were delivered. The cycle length between each stimulus was held at 20 ms, and the bursts were separated by a 2-s pacing-free interval. Data were expressed as a cumulative percentage of pacing-free time during which ventricular arrhythmias were observed. A second set of experiments was then performed in which the cycle length was progressively reduced (from 90 to 10 ms), and the pacing current was held at 10 mA. These data were analyzed using the same method.

Hearts from mice treated for 2 weeks with isoproterenol were subjected to a similar protocol in the absence of further β-adrenergic stimulation. A pacing train of eight stimuli (S1) was delivered with a cycle length of 100 ms, with a single (S2) premature extra stimulus introduced at progressively shorter intervals until arrhythmia was induced or the ventricular refractory period was reached. For the burst pacing protocol, ventricular pacing was carried out with a train of 50 S1 at a cycle length of 20 ms. Pacing current amplitude was progressively increased from the threshold of ventricular capture until ventricular tachycardia or fibrillation was induced or a current of 35 mA was reached. Ventricular tachycardia was defined as six or more consecutive premature ventricular waves (tachycardia with regular waves defined as ventricular tachycardia, whereas ventricular fibrillation was characterized by irregular fibrillating waves).

Hypertrophy Studies—Cardiac hypertrophy was induced by administration of isoproterenol (ISO, Sigma) at 10 mg/kg/day for 14 days via osmotic mini-pumps (Alzet) implanted subcutaneously in 8–10-week-old WT and Tpcn2−/− mice.

Echocardiography—Mice were anesthetized with Avertin (Sigma, 200 mg/kg) via intraperitoneal injection. Transthoracic M-mode echocardiographic recordings were performed using an Acuson Sequoia C256 system (Siemens) following a protocol described previously (42). Measurements taken at end-systole and end-diastole were averaged to calculate parameters of end-diastolic left ventricular posterior wall thickness, left ventricular mass, and fractional shortening.

Electrocardiography—To monitor cardiac rhythms, we carried out in vivo electrocardiographic analysis on mice anesthetized with isoflurane (2.5%). RR interval, P wave duration, PR interval, QRS, JT, and QT durations were recorded.

Statistics—Statistical comparisons were made using paired or unpaired Student’s t tests or one- or two-way analysis of variance (with repeated measures if appropriate) followed by either Tukey, Bonferroni, or Dunnett’s post hoc test. Where a data set could not be deemed normally distributed, a Mann-Whitney test was used instead. A statistically significant difference was concluded when p was < 0.05. All data are expressed as mean values ± S.E.

Results

We first demonstrated the absence of TPC2 expression at the mRNA level in cardiac tissue from Tpcn2−/− mice, as shown in the inset to Fig. 1C (compared with wild type (WT)). Effects of exogenous NAADP were investigated in cells from these Tpcn2−/− mice by superfusing myocytes with NAADP-AM, a membrane-permeant acetoxymethyl ester of NAADP (31, 33, 41). Myocytes were stimulated at 1 Hz to evoke Ca2+ transients accompanying action potentials. Application of NAADP-AM (240 nm) caused a significant increase in the amplitude of these Ca2+ transients (Fig. 1, A, panel i, and C, 16 ± 5%, n = 20) in myocytes from WT mice, but there was a striking failure of NAADP-AM to increase Ca2+ transient amplitude in ventricular myocytes from Tpcn2−/− mice (Fig. 1, A, panel ii, and C, −6 ± 4%, n = 20). No difference was seen in Ca2+ transient amplitude between the two genotypes under control conditions (Fig. 1B). Therefore, the expression of TPC2 proteins is required for NAADP-mediated enhancement of Ca2+ transients in cardiac myocytes.

We then tested for the involvement of TPC2 in the response to β-adrenoceptor stimulation. Fig. 1, D–F, shows effects of isoproterenol (3 nm) on contraction of myocytes electrically stimulated at 1 Hz. Increases in contraction amplitude caused by β-adrenoceptor stimulation were greatly reduced in myocytes from Tpcn2−/− mice (64 ± 14%; n = 7) as compared with the increases observed in WT myocytes (161 ± 32%; n = 13, p < 0.05). Action potential recordings made during the contraction study showed no difference between WT and Tpcn2−/− cells under control conditions but showed a smaller increase in the “late plateau” phase of Tpcn2−/− action potentials during exposure to ISO (an increase in amplitude of 8.3 ± 1.6 mV in myocytes from Tpcn2−/− mice, n = 7, as compared with 13.0 ± 1.5 mV in myocytes from WT mice, n = 13, p < 0.05).

In view of the evidence presented above, and given that the late plateau phase of the action potential is largely dependent on Na+/Ca2+ exchanger activity, the reduced effect of isoproterenol on contraction in myocytes lacking TPC2 proteins was hypothesized to arise from reduced effects on Ca2+ transients. This was investigated directly using the Ca2+ probe Fluo-5F. Fig. 1, G and H, shows representative Ca2+ transients in myocytes stimulated at 1 Hz. In line with the contraction data, the effects of isoproterenol (3 nm) on Ca2+ transients in myocytes from Tpcn2−/− mice were significantly reduced compared with WT (maximum increase from control amplitude of 123 ± 24% in WT and 57 ± 17% in Tpcn2−/− myocytes, n = 15 for both groups, p < 0.05, see Fig. 1F).
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FIGURE 1. NAADP actions were absent in cardiac ventricular myocytes from mice lacking TPC2 protein (Tpcn2−/−), and effects of β-adrenergic receptor stimulation on contractions and Ca2+ transients evoked by electrical stimulation were reduced in myocytes from Tpcn2−/− as compared with wild-type myocytes. A, panels i and ii show superimposed Ca2+ transients (Fluo-5F as probe, 1 Hz electrical stimulation) in myocytes before and after application of NAADP-AM (240 nM). B, shows that the mean amplitudes of Ca2+ transients in the absence of drugs were similar in myocytes from Tpcn2−/− and WT mice; ns, not significant. C shows mean data for effects of NAADP-AM (n = 20, both data sets). The inset to this panel shows that Tpcn2 mRNA is found in hearts from WT but not Tpcn2−/− mice. D shows mean increases in contraction amplitude during ISO (3 nM) application in myocytes from Tpcn2−/− (n = 7) or wild-type mice (WT, n = 13, 1 Hz stimulation). *, p < 0.05; **, p < 0.01; ***, p < 0.001. E and F show representative action potential and contraction traces before and after ISO application in WT and Tpcn2−/− cells. G and H show superimposed Ca2+ transients before and during ISO application in myocytes from Tpcn2−/− or WT mice. Mean data are summarized in (n = 15 for both data sets). J shows mean data from whole hearts perfused by the Langendorff technique. All observed effects of ISO were reduced in Tpcn2−/− (n = 7) as compared with WT (n = 5); * indicates p < 0.05. K shows bar graph representing mean effects of isoproterenol on the amplitudes of L-type Ca2+ currents in response to step depolarizations from −40 to 0 mV (red, WT; green, Tpcn2−/−; n = 7 for both groups). Superimposed representative traces in the presence and absence of isoproterenol are shown in L (WT, black before and red after isoproterenol) and M (Tpcn2−/−, blue before and green after isoproterenol). The effects of isoproterenol were similar in WT and Tpcn2−/−.

Previous work has demonstrated that NAADP has no effect on Ca2+ influx through L-type channels (31, 33). We therefore compared the change in L-type current recorded from Tpcn2−/− and WT cells during β-adrenergic stimulation. The effect of 3 nM isoproterenol to increase the amplitude of L-type Ca2+ currents remained unchanged in myocytes from Tpcn2−/− mice (Fig. 1, K–M, 78 ± 5.8% increase in WT and 81 ± 15% increase in Tpcn2−/−, p = 0.88, n = 7 for both groups) showing that the β-adrenoceptor signaling pathway per se remained intact, allowing PKA to phosphorylate L-type Ca2+ channels in the usual way.

The observations in myocytes from Tpcn2−/− mice were complemented by observations in whole hearts perfused by the Langendorff technique. In spontaneously beating hearts, isoproterenol (5 nM) increased the force of ventricular contraction by 92 ± 4% (n = 5) in age-matched WT controls compared with 63 ± 9% (n = 7) in hearts from Tpcn2−/− animals, a significant reduction in animals lacking TPC2 (p < 0.05, Fig. 1).

The reduced response to isoproterenol in Tpcn2−/− myocytes shows a striking parallel to additional observations made during pharmacological suppression of the NAADP pathway in guinea pig ventricular myocytes; exposure to bafilomycin A (1 μM, which abolishes acidic store Ca2+ loading (31, 33)) or Ned-19 (1 μM, an antagonist of NAADP (43)) both reduced the Ca2+ transient response to isoproterenol (166 ± 8% increase in control cells, n = 9, 92 ± 20% increase in the presence of bafilomycin, n = 5, and 105 ± 19% increase in the presence of Ned-19, n = 6, Fig. 2, A–D and H). Although suppression of the NAADP pathway reduced the ability of isoproterenol to increase Ca2+ transients, the effect of isoproterenol on L-type Ca2+ currents was unaffected by Ned-19 showing that other stages of the β-adrenoceptor pathway remained operational (Fig. 2, E–G and I).

Taken together, the observations reported above provide a compelling case for the contention that NAADP, most likely acting via the TPC2 protein, plays a major role in the acute effects of isoproterenol on the magnitude of stimulated Ca2+ transients and contractions in ventricular myocytes from mouse and guinea pig.

Previous work has shown that the effects of NAADP to increase the amplitude of Ca2+ transients triggered by action potentials are associated with an increased Ca2+ load of the SR (31, 33). This increased Ca2+ load provides a major mechanism for the increased amplitude of the Ca2+ transient accompanying Ca2+-induced Ca2+ release, although additional effects of NAADP to influence the “gain” of Ca2+-induced Ca2+ release cannot be excluded. Does the reported increase in SR Ca2+ load in response to NAADP result solely from the small amounts of Ca2+ released from lysosomes or might there be an amplification mechanism? One possibility is that Ca2+ released from the lysosome could activate CaMKII causing phosphorylation of phospholamban and disinhibition of SERCA activity resulting in additional SR Ca2+ load (44). This was tested in guinea pig myocytes by employing two CaMKII inhibitors (KN-93, 1 μM, in atrial myocytes and AIP peptide, 1 μM, in ventricular myocytes). Following photorelease of NAADP from a caged NPE derivative (40), there were increases in the amplitude of Ca2+ transients in guinea pig atria (37 ± 8% increase after 5 min, n = 10) similar to those described previously (Fig. 3, A, D, and E) (31, 33). These effects of photoreleased NAADP on Ca2+ transients were abolished by KN-93 (Fig. 3, B, D, and E). KN-92 is a struc-
naturally related analogue of KN-93 without effects on CaMKII, and this substance failed to suppress the effects of photoreleased NAADP in atrial myocytes (Fig. 3, C and E).

Effects of the active KN-93 were also tested in ventricular myocytes from WT mice exposed to NAADP-AM. Again, Ca\textsuperscript{2+} transients were increased following exposure to NAADP-AM in the absence (n = 10 control, n = 6 KN-93). Note suppression by KN-93 of effect of photoreleased NAADP on Ca\textsuperscript{2+} transients. E shows mean data comparing KN-92 and KN-93 over 10 min (n = 4 for each). Note lack of effect of KN-92 in comparison to KN-93. *p < 0.05.

FIGURE 3. CaMKII is required for atrial cardiomyocyte responses to NAADP from guinea pig. A and B show superimposed representative Ca\textsuperscript{2+} transients (Fluo-5F, 1 Hz) before and after photorelease of NAADP from a caged NPE derivative in the absence (A) or presence (B) of 1 μM KN-93 or C) 1 μM KN-92 (a structural analogue of KN-93 lacking effects on CaMKII) in guinea pig atrial myocytes; D shows mean data (n = 10 control, n = 6 KN-93). Note suppression by KN-93 of effect of photoreleased NAADP on Ca\textsuperscript{2+} transients. E shows mean data comparing KN-92 and KN-93 over 10 min (n = 4 for each). Note lack of effect of KN-92 in comparison to KN-93. *p < 0.05.

FIGURE 4. CaMKII is required for ventricular cardiomyocyte responses to NAADP from guinea pig or WT mice. A and B show traces from similar experiments in guinea pig ventricular myocytes in the absence (A) and presence (B) of inhibitor peptide (AIP, 1 μM in the patch solution, Fluo5F, 1 Hz stimulation); C shows mean data (n = 8 control, n = 7 AIP). D and E show Ca\textsuperscript{2+} transients (Fluo-5F, 1 Hz) in mouse ventricular myocytes before and after application of NAADP-AM in the absence (D) and presence (E) of 1 μM KN93. F shows mean data (n = 20 for NAADP-AM, n = 10 for KN93, and n = 5 for DMSO controls). * indicates significant difference from 0-min value p < 0.05.

AIP, n = 7). These observations are therefore consistent with a role for CaMKII in the pathway-mediating effects of NAADP in guinea pig and mouse myocytes, in part through phospholamban/
NC2, although additional effects, for example on ryanodine receptors, cannot be excluded.

For an amplification mechanism to work in the way we have hypothesized above, a close structural relationship would be required to exist between lysosomes and the SR. This was investigated using transmission electron microscopy of rabbit ventricular tissue, and sample images are shown in Fig. 5. It can be seen that lysosomes were observed close to the SR, both in regions that might be associated with Ca\(^{2+}\) uptake (Fig. 5A) and in locations thought to mediate Ca\(^{2+}\) release via RyRs (Fig. 5B). The mean distance of lysosomes from the SR was 25.4 ± 1.8 nm (n = 30). The significance of these observations is considered in more detail under the “Discussion.”

From these observations showing that NAADP and TPC2 appear to contribute to the effects of the β-adrenergic agonist isoproterenol to increase the amplitude of Ca\(^{2+}\) transients in electrically stimulated myocytes, and taking into account the finding that arrhythmias caused by isoproterenol could be suppressed by the NAADP antagonist BZ194 (34), we hypothesized that isoproterenol-mediated increases in NAADP acting via TPC2 proteins might also be involved in the susceptibility to cardiac arrhythmias in the intact heart. We first investigated acute exposure to the β-adrenergic agonist. In the presence of isoproterenol, hearts from Tpcn2\(^{-/-}\) mice were significantly (p < 0.001) less prone to arrhythmias when subjected to a ventricular burst pacing protocol compared with WT controls. The induction threshold for ventricular tachycardia or fibrillation using a burst pacing protocol in hearts exposed to isoproterenol was significantly higher in hearts from Tpcn2\(^{-/-}\) mice compared with hearts from WT mice (Fig. 6).

Building upon the above observations showing a role for NAADP and TPC2 proteins in the acute effects of β-adrenergic receptor stimulation and acute arrhythmogenesis, we investigated whether NAADP-mediated Ca\(^{2+}\) signaling may also play a role in the development of hypertrophy that is caused by chronic stimulation of β-adrenoceptors. To test this hypothesis, both Tpcn2\(^{-/-}\) and WT mice were stressed by chronic administration of isoproterenol at 10 mg/kg/day for 14 days via osmotic mini-pumps. The control groups were given isotonic saline following the same protocol. Notably, after 14 days of treatment with isoproterenol, Tpcn2\(^{-/-}\) mice displayed less hypertrophy and showed better preserved cardiac function than WT mice as demonstrated by echocardiography, morphometry, and histology (Fig. 7, A–C). In particular, the increase in heart weight after chronic exposure to isoproterenol (expressed either as left ventricular mass or the ratio of heart weight to tibia length) was significantly less in Tpcn2\(^{-/-}\) mice than in WT (heart weight/tibia length after 14 days of isoproterenol treatment 0.0127 ± 0.0004 g mm\(^{-1}\) in wild type against 0.0110 ± 0.0003 g mm\(^{-1}\) in Tpcn2\(^{-/-}\), p < 0.05). Additional ex vivo electrophysiological studies indicated that hearts from isoproterenol-treated Tpcn2\(^{-/-}\) mice showed less susceptibility to arrhythmia when subjected to a ventricular burst pacing protocol in the absence of further acute isoproterenol exposure (Fig. 8, A and B), with threshold currents required to elicit ventricular fibrillation increasing from 8.8 ± 4.2 to 21.7 ± 6.9 mA, p < 0.05.

**Discussion**

The observations described in this study demonstrate the requirement for TPC2 proteins in order for NAADP actions in mammalian cardiac tissue, and they highlight the contribution of these proteins during acute and chronic actions of the β-adrenergic signaling pathway. We have shown by using both genetic and pharmacological techniques that the NAADP pathway is physiologically important during acute β-adrenergic stimulation. Furthermore, pharmacological observations show that the actions of NAADP on cellular Ca\(^{2+}\) signaling require the presence of functional CaMKII. TPC2 proteins also appear to contribute to arrhythmogenic effects during excessive acute β-adrenergic stimulation. In addition, our work highlights the contribution of this pathway during chronic β-adrenergic stimulation leading to pathological hypertrophy and suggests that its inhibition is partially protective against the negative consequences of chronic activation of β-adrenergic signaling pathways.

The role of two-pore channels in the generation of Ca\(^{2+}\) signals is an emerging field. Ca\(^{2+}\) release from lysosomes dependent on TPC2 has been demonstrated to regulate a disparate number of cellular processes, and it is the principal way in which NAADP evokes intracellular Ca\(^{2+}\) signals. Roles for TPCs in pathophysiological processes to date include triggering of the acrosome reaction in mammalian fertilization (20), angiogenesis (23), cell differentiation (45–47), muscarinic receptor-mediated smooth muscle contraction (27), endocytic...
trafficking of cholesterol in the liver (25), insulin secretion from mouse pancreatic β-cells (21), T cell activation (48), exocrine pancreatic Ca\(^{2+}\) signaling (24), melanosome concentration in *Xenopus* oocytes (26), defective vesicular trafficking in mouse models of Parkinson disease (49), and Ebola infectivity (19). The overriding principle of NAADP-evoked Ca\(^{2+}\) signaling is that localized Ca\(^{2+}\) signals are evoked by the release of lysosomal Ca\(^{2+}\). This has three major roles. Localized Ca\(^{2+}\) release in the lysosomal system promotes vesicular trafficking and also exocytosis, whereas localized sub-plasma membrane release modulates membrane excitability (18). However, a major role for NAADP is to enhance the excitability of the ER by triggering Ca\(^{2+}\)-induced Ca\(^{2+}\) release leading to global Ca\(^{2+}\) signals at lysosomal-ER nanojunctions (18).

The slow time course of the increase in Ca\(^{2+}\) signals following photorelease of NAADP led us to hypothesize that there may be more to the observed effects than the simple two-pool model (3) in which Ca\(^{2+}\) rapidly released from lysosomes is taken up into the ER/SR and leads to increased ER/SR release during Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Whether such communication involves an increase in cytosolic peri-SR Ca\(^{2+}\) to activate inositol 1,4,5-trisphosphate receptors or RyRs or whether a local increase in luminal SR Ca\(^{2+}\) is required is not clear.

In this study we have shown that CaMKII is necessary to mediate the effects of NAADP. We suggest that CaMKII may amplify the effect of Ca\(^{2+}\) released from acidic lysosomes, leading to phosphorylation of proteins on the SR membrane. Our previous observations showing that NAADP actions in the
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A train of 50 S1 at a cycle length of 20 ms at a progressively increasing amplitude perfused hearts after exposure to chronic ISO stress (ventricular burst pacing with representative examples of monophasic action potential recordings in Langendorff-to ventricular arrhythmias than hearts from wild-type mice.

Future experiments will be needed to further test this hypothesis. In particular, it will be important to investigate whether there is a preferential location of lysosomes/late endosomes bearing TPC2 proteins. It has been acknowledged for many years that close associations between subcellular structures known as nanojunctions or microdomains play an important role in localized Ca^{2+} signaling. Membrane contact sites are vital to the formation of functional signaling microdomains and are regions where membranes typically come within 30 nm of each other. This small space allows the release of a small amount of Ca^{2+} to result in a high concentration and therefore more efficient and rapid signaling. Examples include the t-tubule membrane and the SR (allowing close proximity between L-type Ca^{2+} channels and ryanodine receptors) and also the mitochondrial membrane and the SR (allowing a similar relationship between inositol 1,4,5-trisphosphate receptors and mitochondrial Ca^{2+} transporters). These relationships are not simply a structural coincidence; they not only facilitate major physiological processes, such as excitation-contraction coupling and regulation of oxidative phosphorylation respectively, but recent work has suggested molecular tethering between specific proteins on these structures to maintain their close apposition (50, 51).

Such microdomains have been observed in extracardiac tissues between lysosomes and the SR/ER (52). In pulmonary artery smooth muscle cells NAADP-dependent Ca^{2+} release was observed principally in the perinuclear region in association with RyR3, which colocalized with lysosomal/late endosomal markers such as LAMP-1 (1).

Our results suggest that these lysosome-SR microdomains also exist in cardiac tissue. In the future, it will be important to investigate whether there is a preferential location of lysosomes that bear TPC2 proteins in the heart, as this would indicate where microdomains involved in NAADP-related Ca^{2+} signaling are likely to be located. Although our observations of lysosomes using transmission electron microscopy were not sufficient to provide information regarding the exact distribution of TPC2 proteins, they do provide good evidence that lysosomes are located in positions that are consistent with our hypothesis. Those lysosomes that are closely associated with t-tubules might be expected to facilitate effects of NAADP on Ca^{2+} release from the SR via RyR2. Similarly, those lysosomes observed to be more distant from the t-tubules could be associated with SERCA and phospholamban or with CaMKII and be involved with the facilitation of Ca^{2+} reuptake and increased SR Ca^{2+} load (although SERCA may be more widely distributed in SR membranes).

The proposed novel effects of CaMKII as part of the cardiac NAADP signaling pathway provide an additional mechanism for the extensive contributions of CaMKII to β-adrenoceptor signaling in the heart (53). A role for CaMKII has frequently been associated with cardiac hypertrophy (54–57) and arrhythmogenesis (58), and the pathway involving NAADP and TPC2 proposed here might provide insight into a novel additional mechanism by which such effects could arise.

Our observations can be readily incorporated in the accepted scheme that β-adrenoceptor agonists lead to stimulation of adenyl cyclase, formation of cAMP, and activation of PKA, followed by phosphorylation of proteins, including L-type Ca^{2+} channels (to promote Ca^{2+} entry) and phospholamban (speeding relaxation and increasing the amount of Ca^{2+} stored in the heart) without CaMKII-mediated phosphorylation are likely to occur from relief of phospholamban-mediated inhibition of SERCA (31, 33), but additional actions on, for example, the ryanodine receptor (that might also be prevented by CaMKII inhibition) cannot be excluded.

A scheme summarizing our proposals is shown in Fig. 9. Future experiments will be needed to further test this hypothesis.
SR. To this scheme, we would add that NAADP synthesis is also increased during β-adrenergic stimulation (31, 32). A family of multifunctional enzymes termed ADP-ribosyl cyclases has been suggested to synthesize both cADP-ribose (when the substrate is NAD) and NAADP (when the substrate is NADP with nicotinic acid). These enzymes may be located in the sarcotendium but have also been suggested to be associated with the SR (59, 60). Given that NAADP synthesis has been shown to be stimulated by activation of β-adrenergic receptors, it seems plausible that these enzymes might be an additional target of phosphorylation by PKA (59, 61, 62) or as a consequence of other coupling mechanisms that have yet to be elucidated. The resultant NAADP could act on TPC2 proteins in acidic lysosomes to bring about effects that supplement other well-established effects of β-adrenergoreceptor stimulation on L-type Ca²⁺ currents, RyR, and phospholamban. PKA-mediated phosphorylation of phospholamban is a major mechanism underlying the increased Ca²⁺ load of the SR following β-adrenergoreceptor stimulation (58, 63), although there may be an additional contribution arising from phosphorylation by CaMKII (44, 53, 54). The reported actions of NAADP to increase cardiac muscle Ca²⁺ transients and contraction are also associated with an increase in SR Ca²⁺ load in both atrial and ventricular myocytes (31, 33).

The mechanisms underlying cardiac hypertrophy following chronic β-adrenergoreceptor stimulation are likely to be complex, but the evidence reported here supports a role for TPC2 proteins.

In summary, our observations provide strong support for a role in NAADP and TPC2 proteins as major contributors in the actions of β-adrenergoreceptor agonists to increase contraction amplitude in cardiac ventricular myocytes and suggest that these effects require the presence of functional CaMKII. TPC2 proteins also appear to play a major role in the pathway linking the stress of chronic stimulation of β-adrenergoreceptors to hypertrophy and associated tendency to arrhythmias. These proteins are likely to provide a promising new target for future therapy, because inhibiting the NAADP/TPC2 pathway has minimal effect on normal excitation-contraction coupling but suppresses adrenergic signaling in the heart.

**Author Contributions**—R. A. C. carried out all guinea pig work; E. L. B. and W. K. L. carried out all Tpcn2⁻/⁻ physiology; cell isolations were carried out by E. L. B., R. A. C., and W. K. L. K. L. synthesized NAADP-AM; D. B.-Y. and G. C. C. synthesized caged NAADP. M. R., J. P., and A. G. were responsible for the development of the Tpcn2⁻/⁻ mouse line and subsequent breeding. K. T. S. carried out confirmation of Tpcn2⁻/⁻ mRNA. R. A. B. B. and D. A. performed electron microscopy studies. A. G., M. R., and D. A. T. were responsible for experimental design, with assistance from R. A. C., E. L. B., W. K. L., and X. W. W. L. carried out echocardiography. D. A. carried out experiments on arrhythmias during acute isoproterenol stimulation. Y. W. carried out osmotic minipump experiments and chronic isoproterenol stimulation work. All experiments were carried out in the laboratories of D. A. T. and M. R. All authors contributed to the writing and editing of the manuscript.
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