mTOR Regulates Lysosomal ATP-Sensitive Two-Pore Na⁺ Channels to Adapt to Metabolic State

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http://dx.doi.org/10.1016/j.cell.2013.01.023

SUMMARY

Survival in the wild requires organismal adaptations to the availability of nutrients. Endosomes and lysosomes are key intracellular organelles that couple nutrition and metabolic status to cellular responses, but how they detect cytosolic ATP levels is not well understood. Here, we identify an endolysosomal ATP-sensitive Na⁺ channel (lysoNaATP). The channel is a complex formed by two-pore channels (TPC1 and TPC2), ion channels previously thought to be gated by nicotinic acid adenine dinucleotide phosphate (NAADP), and the mammalian target of rapamycin (mTOR). The channel complex detects nutrient status, becomes constitutively open upon nutrient removal and mTOR translocation off the lysosomal membrane, and controls the lysosome’s membrane potential, pH stability, and amino acid homeostasis. Mutant mice lacking lysoNaATP have much reduced exercise endurance after fasting. Thus, TPCs make up an ion channel family that couples the cell’s metabolic state to endolysosomal function and are crucial for physical endurance during food restriction.

INTRODUCTION

Cells use adenosine triphosphate (ATP) as an energy carrier. Sensing intracellular concentrations of ATP ([ATP]) is a crucial step in the regulation and coupling of [ATP] to cellular metabolism. One of the most extensively characterized cellular ATP sensors is the plasma membrane ATP-sensitive K⁺ channel (KATP) (McTaggart et al., 2010; Nichols, 2006, for review). In contrast to channels on the plasma membrane that regulate such functions as secretion, intracellular ion channels are more difficult to study and thus are less well understood. For example, ATP-sensitive K⁺ channels (mitoKATP) have been recorded from the mitochondrial inner membrane (Inoue et al., 1991), but their molecular identities are not established (Foster et al., 2008). Although it is clear that endosomes and lysosomes play key roles in cellular metabolism, we do not understand how metabolic signals are received by these organelles. Here, we report an ATP-sensitive Na⁺ channel (lysoNaATP) on endolysosomal membranes that is responsive to physiological [ATP]. The channel is formed by TPC1 and TPC2, two well-conserved proteins whose functions are largely unknown. The channel associates with the mammalian target of rapamycin (mTOR) complex and detects cellular nutrient status, becoming constitutively open when nutrients are depleted and when mTOR translocates away from the complex at the lysosomal membrane. LysoNaATP determines the sensitivity of endolysosome’s resting membrane potential to Na⁺ and cytosolic ATP, controls lysosomal pH stability, and regulates whole-body amino acid homeostasis. Strikingly, mutant mice lacking tpc1 and tpc2 have severely reduced endurance after fasting.

RESULTS

Endolysosomes Have an ATP-Sensitive Na⁺-Permeable Channel lysoNaATP

To determine whether endolysosomes have ionic mechanisms that sense intracellular physiological [ATP], we recorded whole-endolysosomal currents from endosomes/lysosomes mechanically released from the cytosol with a glass pipette (Saito et al., 2007). Endolysosomes were enlarged by treatment of cells with vacuolin-1, as previously described (Cerny et al., 2004; Dong et al., 2008). The optimal activities of many plasma membrane channels require phosphatidyllysinol 4,5-bisphosphate (PI(4,5)P₂) as a cofactor to prevent channel “run down”
GFP. ITRPML, though potentiated by exogenously applied HEK293T cells transfected with candidate proteins tagged with et al., 2006). We recorded endolysosomal currents from ATP-sensitive Na+-permeable channel (lysoNa ATP) that is sensitive to physiological [ATP]. Similar lysoNa ATP currents were also detected in endolysosomes from other cell types that we tested, including excitable cells such as beating cardiac myocytes and nonexcitable cells such as fibroblasts and liver hepatocytes (Figures 1F–1K).

**TPCs Form lysoNa ATP Channels in HEK293T Cells**

We used a candidate approach to identify proteins that reconstitute lysoNa ATP when transfected into HEK293T cells (these cells have little endogenous lysoNa ATP; Figure 2A). Among the hundreds of known ion channels, TRPML1 (a 6-transmembrane-spanning endolysosomal Na+-permeable cation channel in the TRP family) and TPC1/TPC2 proteins (endolysosomal channels of 12-transmembrane spanning proteins with similarity to that of voltage-gated Na+ and Ca2+ channels) are known to localize on endosomes and lysosomes (Braillou et al., 2009; Calcraft et al., 2009; Grimm et al., 2012; Ishibashi et al., 2000; Pryor et al., 2006). We recorded endolysosomal currents from HEK293T cells transfected with candidate proteins tagged with GFP. 

Strikingly, overexpression of human TPC1 increased endolysosomal ATP-sensitive currents by 13-fold (Figure 2C). Similarly, transfection with TPC2 resulted in a large lysoNa ATP current (Figures 2D and 2E) sensitive to [ATP] (IC50 = 0.92 ± 0.31 mM; Figure 2D), comparable to that recorded from macrophages. Because TPC2 has more prominent lysosomal localization than TPC1 (Calcraft et al., 2009) and is more readily identified in patch-clamp recordings, we focused on TPC2. In controls, lysoNa ATP recorded from macrophages, where lysosomes are well studied. Little current was detected when the pipette solution contained 150 mM K+ but no Na+ (−5.6 ± 2.3 pA with no PI(3,5)P2 in the bath and −7.7 ± 2.1 pA with 1 μM PI(3,5)P2; n = 7; −100mV). With a pipette solution containing Na+ (a major cation in the endolysosomal lumen [Steinberg et al., 2010]) and K+ in the cytosolic solution containing PI(3,5)P2, we recorded large inward currents (Na+ moving out of the endolysosome into the cytosol; Figure 1A) when no ATP was present in the bath (Figures 1B and 1C). Upon addition of ATP-Mg, however, the amplitude of the Na+ current was reduced in a dose-dependent manner with an IC50 of 0.32 ± 0.05 mM and a Hill coefficient of 1.25 ± 0.20 (Figures 1B–1E). Thus, peritoneal macrophage endolysosomes have an ATP-sensitive Na+-permeable channel (lysoNa ATP) that is sensitive to physiological [ATP]. Similar lysoNa ATP currents were also detected in endolysosomes from other cell types that we tested, including excitable cells such as beating cardiac myocytes and nonexcitable cells such as fibroblasts and liver hepatocytes (Figures 1F–1K).

**lysoNa ATP’s ATP Sensitivity Requires mTOR**

Unlike the plasma membrane KATP channel, which is inhibited by both ATP and the nonhydrolyzable analog ATPγS by direct binding (Nichols, 2006), lysoNa ATP is not inhibited by ATPγS (Figure S1H). Interestingly, the inhibition of lysoNa ATP by ATP is slow (T1/2: 43.3 ± 8.8 s, n = 3; Figure S1I) in contrast to the fast inhibition of KATP (ms). These slow kinetics were not due to ATP diffusion from the cytosol (bath) to the lumen, as dialyzing the endolysosomal lumen with 10 mM ATP in the pipette solution did not block lysoNa ATP, and the channel was still inhibited by ATP applied in the bath (Figure 2G). We conclude that ATP acts on the channel from the cytosolic surface of the endolysosome. The slow kinetics and the requirement of ATP hydrolysis suggest that ATP does not directly bind TPC to inactivate it. We next examined whether a slower process, such as activation of protein kinases, is responsible for lysoNa ATP’s ATP sensitivity.

A well-established cytosolic ATP-sensing kinase is the AMP-activated protein kinase (AMPK) (Hardie et al., 2012; Oakhill et al., 2012). In the presence of an AMPK inhibitor, dorsomorphin, however, lysoNa ATP was still ATP sensitive (Figure S2A). In addition, lysoNa ATP recorded from TPC2-transfected MEF cells lacking both AMPK catalytic subunit isoforms (AMPK 1/2 double-knockout [dKO]) was readily inhibited by ATP (Figure S2B). Thus, lysoNa ATP’s ATP sensitivity does not require AMPK.

Under our recording configurations in which soluble proteins are easily washed away (Figure 1A), a putative functional kinase should be associated with the endolysosomal membrane and TPC. One of the few kinases known to be tethered to the lysosome membrane is the mammalian target of rapamycin (mTOR) (Korolchuk et al., 2011; Sancak et al., 2010; Zoncu
Consistent with the role of mTOR in lysoNa\textsubscript{ATP}'s ATP sensitivity, the mTOR inhibitors rapamycin and Torin 1 profoundly reduced the ATP sensitivity of reconstituted lysoNa\textsubscript{ATP} in TPC2-transfected HEK293T cells (Figures 3A–3C) and that of the native channel in macrophages (Figures 3D–3F).

To test whether the ubiquitously expressed mTOR protein is required for lysoNa\textsubscript{ATP}’s ATP sensitivity, we knocked down the
endogenous mTOR protein in HEK293T cells using lentivirus encoding an shRNA against human mTOR (Figure 3G). The ATP sensitivity of lysoNaATP generated by TPC2 in the mTOR shRNA virus-infected cells was dramatically lowered to 0.24 ± 0.05 mM (Figures 3I and 3J). A control scrambled shRNA had no significant effect (Figures 3H and 3J).

Two mTOR complexes are active in mammalian cells (Laplante and Sabatini, 2012a for review): the Raptor-containing mTORC1 and Rictor-containing mTORC2. Knockdown of Raptor (Figure 3L), but not Rictor (Figure 3K), reduced the ATP inhibition of the channel (Figure 3M), suggesting that lysoNaATP’s ATP sensitivity is conferred by mTORC1.

**TPC Complexes with mTOR**
mTOR was coimmunoprecipitated with TPC1 or TPC2 from transfected HEK293T cells (Figure 4A), suggesting that the channel proteins and mTOR are in the same signaling complex. We did not detect any association between TPC2 and the lysosome-localized proteins RagB (Figure S3A), V-ATPase (Figure S3B), or lamptors (Figures S3C and S3D). These proteins
interact with mTOR under certain conditions (Kim et al., 2008; Sancak et al., 2010; Zoncu et al., 2011). In contrast to TPCs, the transfected ATP-insensitive endolysosomal channel TRPMLs (Figures 2B and S3E and Figure 4A, lane 2) had little or no detectable association with mTOR.

LysoNaATP’s ATP sensitivity was increased by ~5 fold when mTOR protein was increased above endogenous levels by mTOR cDNA transfection (Figures 4B–4E). When a rapamycin-resistant mTOR (residue S2035 mutated to T [S2035T] [Vilella-Bach et al., 1999]) was cotransfected with TPC2, the resulting lysoNaATP became insensitive to rapamycin (Figure 4F). Thus, mTOR determines lysoNaATP’s rapamycin sensitivity.

To determine whether lysoNaATP requires mTOR’s kinase activity, we inhibited the endogenous mTOR with rapamycin and then reintroduced mTOR activity by transfection of a rapamycin-resistant mTOR. Unlike wild-type, the mutant with an additional mutation in mTOR’s catalytic domain (D2357E, kinase dead mutant [Vilella-Bach et al., 1999]) was unable to support lysoNaATP’s ATP sensitivity (Figures 4G and 4H), suggesting that mTOR’s kinase activity is required. Through mutation of potential mTOR phosphosites of TPC2’s putative cytosolic domains, we have not located the sites of phosphorylation that are important for the channel’s ATP inhibition. The functionally important mTOR residues in lysoNaATP could be on cryptic sites.
of these TPCs or on TPC-complex-associated subunits yet to be identified.

**LysoNaATP Detects Nutrient Depletion**

In addition to sensing mM [ATP] (Dennis et al., 2001), mTOR is also a major sensor for cellular nutrients (Laplante and Sabatini, 2012b). Nutrient depletion is known to cause mTOR translocation away from lysosomal membranes (Korolchuk et al., 2011; Sancak et al., 2010), where TPC2 is localized (Callcraft et al., 2009). When cells were starved by depleting glucose and amino acids for 60 min, resulting in a drop of cellular ATP content from 3.9 ± 0.5 (n = 8) to 2.1 ± 0.3 (n = 10) nmole/10⁶ cells, ITPC2 was no longer inhibited by ATP (Figures 5A, 5B, and 5E), presumably as a result of loss of mTORC1 on the lysosomal membrane.

In nutrient-replete cells, mTORC1 is recruited to the lysosomal surface by the Rag GTPases (Kim et al., 2008; Sancak et al., 2008). When RagB(GTP, a GTP-bound mutant that retains mTOR on lysosomes (Sancak et al., 2008), was transfected with TPC2, the resulting lysoNaATP was inhibited by ATP even after cell starvation (Figures 5C and 5E). In contrast, transfection of a GDP-bound Rag mutant (RagB(GDP) that prevents mTOR from being localized on lysosomal surface rendered the channel insensitive to ATP even in replete cells (Figures 5D and 5E).
Amino acid depletion alone is sufficient to cause mTORC1’s translocation away from lysosomes (Sancak et al., 2008, 2010). Similarly, removal of amino acids (Figure 5G), but not glucose alone (Figure 5F), was also sufficient to render lysoNa_ATP largely ATP insensitive. Refeeding nutrient-deprived cells with amino acids for 10 min, a condition sufficient to relocate mTOR back onto the lysosomal membrane after starvation (Sancak et al., 2008), quickly restored the channel’s ATP sensitivity.
TPC1 and TPC2 Constitute Native lysoNaATP

To test the in vivo function of TPC1 and TPC2, we generated mice with tpc1, tpc2, or both tpc1 and tpc2 disrupted. In peritoneal macrophage endolysosomes isolated from the tpc1/tpc2 dKO, 1 μM PI(3,5)P2 elicited no inward current (Figures 6A and 6B). The small residual basal currents, presumably from other channels such as TRPML1, did not contribute to the ATP sensitivity (Figures 6B and 6C). The absence of lysoNaATP in the dKO is not a result of potential developmental defect, as transient transfection of a human (Figures 6D and 6E) or mouse (Figure 6E) TPC cDNA into the mutant macrophages restored the ATP-sensitive currents. Cells from mice lacking TPC1 or TPC2 alone had measurable lysoNaATP, although at reduced levels (current density: tpc1 KO, 71 ± 19 pA/pF, n = 8; tpc2 KO, 66 ± 31 pA/pF, n = 5; WT, 130 ± 16 pA/pF, n = 12). These data suggest that lysoNaATP is formed by TPC1 and/or TPC2 in the native cells and there are no other major ATP-sensitive cation channels in these organelles.

Because mTOR is a key component for the ATP sensitivity of lysoNaATP, we tested whether mTOR’s kinase activity also required lysoNaATP. In cultured hepatocytes, insulin stimulated mTOR-dependent (rapamycin-sensitive) phosphorylation of a major target p70S6K in both WT and dKO cells (Figure S4A). Similarly, amino acid feeding stimulated mTOR translocation to the lysosome in both WT and TPC1/2 dKO (Figure S4B). These data suggest that mTOR does not require TPCs for its function and place lysoNaATP downstream of mTOR in the signaling cascade.

lysoNaATP regulates lysosomal pH stability

Membrane potentials regulate a wide spectrum of physiological processes on the plasma membrane through proteins such as voltage-gated ion channels and voltage-sensitive enzymes (Hille, 2001). It is not clear whether endolysosomes have similar voltage-sensitive proteins. One potential consequence of an increase of ΔΨ (lumen more negative) during a decrease of available ATP is that the V-ATPase is able to maintain lysosomal pH at the pump’s set point. Consistent with this idea, WT lysosomes maintained a relatively stable pH upon starvation (4.73 ± 0.01 before and 4.74 ± 0.01 after starvation). In contrast, there was a significant shift of pH toward alkalization in the mutant (4.82 ± 0.01 before and 5.36 ± 0.02 after starvation; Figures 7D–7F).

lysoNaATP controls endolysosomal Na+ permeability, membrane potential, and sensitivity to ATP

The membrane potentials of endolysosomes have been only indirectly monitored using voltage-sensitive dyes. We directly measured the membrane potentials (ΔΨ, Vm, defined as Vcytosol – Vlumen; Bertl et al., 1992) using current-clamp recording. Similar to plasma membranes, endolysosomal Vm is sensitive to K+, Na+, H+, and Cl− (C.C. and D.R., unpublished data). In the absence of ATP, the membrane potential of the wild-type macrophage endolysosomes was +38.0 ± 1.4 mV (lumen more negative compared to cytosol). The endolysosome-expressing mutant lysoNaATP was ~20 mV more hyperpolarized than WT (+20.5 ± 1.1 mV) (Figures 7A–7C). In the WT, removal of cytosolic Na+ led to a change in ΔΨ of 9.7 mV (Figures 7A and 7C). The mutant endolysosomes’ membrane potential, however, was insensitive to changes in cytosolic Na+ (Figures 7C and 7D). These data suggest that lysoNaATP is a major determinant of the endolysosomal membrane’s resting Na+ permeability, similar to NALCN’s (a 24 transmembrane-spanning channel with sequence similarity to that of TPCs) effect on the plasma membrane of neurons (Ren, 2011).

ATP inhibition of TPCs led to a change of ΔΨ to +19.6 ± 1.8 mV in WT but had no effect on that of the mutant (Figures 7A–7C). These data suggest that lysoNaATP also controls the membrane potential’s ATP sensitivity: a drop of ATP leads to a depolarization of endolysosomes (lumen more negative) due to the opening of TPCs and an efflux of Na+ from endolysosomal lumen into cytosol.
lysoNaATP Is Required for Normal Amino Acid Homeostasis during Starvation Stress

A potential function for lysoNaATP’s control of lysosomal Vm and pH is to regulate the fusion between autophagosomes and lysosomes, one of the last steps of macroautophagy during which nutrients such as amino acids can be generated in response to starvation (Mizushima and Komatsu, 2011). The mutant mice, however, do not appear to have gross defects in autophagy (Figure S5A–S5C). During food deprivation stress, one of the homeostatic responses is an increase in the levels of certain amino acids in the circulation, partly due to protein digestion in the lysosomes and the subsequent export of amino acids from these organelles (Brady et al., 1978; Cahill, 2006). The transport of amino acids, particularly cations (lysine and arginine), by the lysosomal system c transportation system is likely influenced by lysosomal membrane potential and pH (Pisoni and Thoene, 1991). We loaded liver lysosomes with 14C-labeled lysine and monitored the efflux at various time points. With 2 mM ATP in the efflux buffer, sufficient to inhibit lysoNaATP, there was no significant efflux rate difference between WT and the dKO. With [ATP] reduced to 0.1 mM, a condition under which lysoNaATP is open, the amino acid efflux was significantly faster in the WT lysosomes than in the dKO (Figure S5D). We also measured the levels of circulating amino acids 6 hr and 3 days after the onset of food deprivation in the WT and dKO mice. In WT mice, the levels of plasma lysine and arginine increased by...
26% and 67%, respectively, upon prolonged fasting (Figure 7G). In the mutant, such increases were largely absent. Changes in the levels of total amino acids were less compromised in the mutant (WT, 38%; dKO, 7%; p = 0.06; Figure 7G).

**lysoNaATP is Required for Normal Fasting Endurance**

Under normal in-house conditions, tpc1/tpc2 dKO adult mice are viable, fertile, and have no obvious morphological abnormalities or obvious behavioral defects (observation and as measured with rotorod and treadmill tests; Figures 7H and 7I). Because lysoNaATP opens primarily when [ATP] is low or when nutrients are restricted, we subjected the mice to food deprivation. After fasting for 3 days, WT mice remained alert and active in food search activities. Endurance performance in the treadmill test was comparable to or better (five out of ten mice) than that before fasting, consistent with the findings of fasting-induced hyperactivity found in animal behavioral tests (Challet et al., 1997; Yamanaka et al., 2003). In contrast, dKO mice were less active after fasting, and endurance performance was reduced by 8.3-fold; none performed better than before fasting (Figures 7H and 7I). Two days after reintroduction of food, dKO mice regained endurance and became as active as before fasting (Figure 7I). In the less strength-demanding rotorod test, the mutants’ performances were less affected by fasting (Figure S5E).

**DISCUSSION**

We have shown that mTOR associates with the channel pore of lysoNaATP formed by TPC proteins and controls channel activation. Nutrient replete cells have high [ATP], which presumably enables mTOR to phosphorylate TPC and/or its associated proteins and maintain the channel in the closed state. During cell starvation, ATP levels fall, mTOR delocalizes from TPC, and lysoNaATP opens, allowing Na+ and other ions to leave the lysosome. Thus, TPCs link lysosomal control of nutrient recycling to the extensive signaling mTOR network that monitors and responds to nutrient depletion, hypoxia, and cell growth (Laplante and Sabatini, 2012b). Intracellular ATP concentrations are normally a few mM at rest but vary among cell types, during different metabolic conditions, and during circadian cycles. These levels can drop to <0.1 mM during starvation, hyperosmotic stress, hypoxia, and ischemia (Berg et al., 2009; Gribble et al., 2000; Imamura et al., 2009; Beis and Newsholme, 1975). With its ATP sensitivity tuned to the range of physiological concentrations and regulated by nutrient availability, lysoNaATP couples the cell’s energy status to endolysosomal function. In a natural environment, an animal’s survival depends on its ability to deal with stress, to maintain the ability to search for food, and to fend off attacks from intruders even during frequent starvation. TPC’s evolutionarily conserved endolysosomal function in fasting endurance presumably serves this purpose.

Currently, we do not know how the channel affects lysosomal regulation of such functions as protein degradation, energy generation, plasma membrane repair, and exocytosis to maintain cell viability (Laplante and Sabatini, 2012b; Luzio et al., 2007; Settembre et al., 2011). Plasma membrane potentials control many physiological responses ranging from fertilization, muscle contraction, neuronal excitability, synaptic transmission, and hormone release to immune responses. Opening of lysoNaATP depolarizes endolysosomal membranes, but how voltage changes across endolysosomal membranes are detected is poorly understood. The lysosomal membrane potential controls basic endolysosomal properties such as pH and amino acid transportation (Figure 5; Pisoni and Thoene, 1991), and this will require extensive new experiments. TPCs and mTORC1 are widely expressed in many organs including brain, heart, intestine, liver, and pancreas. As such, the cell types and circuitry in which lysoNaATP functions to regulate fasting endurance need to be determined.

Originally proposed to be activated by NAADP (Calcraft et al., 2009), recent data suggest that NAADP binds to proteins other than TPC1/2 (Lin-Moshier et al., 2012; Walseth et al., 2012) and does not activate TPCs (Figures S1F and S1G; see also Wang et al. [2012]). TPC has also recently been shown to be potentiated by PI(3,5)P2 under isolated endolysosome recording conditions (Wang et al., 2012). If not known whether PI(3,5)P2 is a basal requirement for channel activity under physiological conditions, similar to the requirement of PI(4,5)P2 for many plasma membrane ion channels and transporters, or whether there is significant change of PI(3,5)P2 levels during signaling to potentiate TPCs. Nevertheless, the ATP sensitivity of lysoNaATP is independent of PI(3,5)P2, as basal TPC current in the absence of added PI(3,5)P2 is similarly regulated by ATP.

The mTOR complexes are master integrators linking cellular metabolism to many adaptive cellular responses such as global gene expression (Laplante and Sabatini, 2012b). The targets of mTOR are primarily cytosolic, and mTOR is predominantly localized on endolysosomal membranes under physiological conditions (Korolchuk et al., 2011; Sancak et al., 2010; Zoncu et al., 2011). One functional consequence of the recruitment of mTOR to the lysosomal membrane is to detect amino acid content inside the lysosomes for the subsequent action on transcriptional cascades that are important for cellular responses such as lysosomal biogenesis (Han et al., 2012; Sancak et al., 2010; Settembre et al., 2011; Zoncu et al., 2011). Our data suggest that mTOR not only directly receives information from lysosomes, but also acutely controls endosomal and lysosomal functions through lysoNaATP in response to changes in the cell’s energy and nutrient status. Dysregulation of mTOR and the components in its signaling network is linked to many diseases such as diabetes, cancer, neurodegeneration, seizure, and autism. Because both mTOR and lysoNaATP are widely expressed, lysoNaATP channels may enable more precise downstream targeting in mTOR-associated disorders.

**EXPERIMENTAL PROCEDURES**

**Animals**

Animal use followed NIH guidelines and were approved by the IACUC at the University of Pennsylvania. To generate tpc1/tpc2 double-knockout mice, the exons containing the translational start site (ATG) were deleted. The mutant TPC1 and TPC2 proteins are predicted to lack the first 69 and 49 amino acids, respectively, and do not generate detectable current, as tested in HEK293T cells (Wang et al., 2012) and in the tpc1/tpc2 double-knockout cells (Figure 6). We cannot rule out the possibility that small, undetectable residual channels formed by the mutant proteins can support functions such as animal viability.
Individual knockouts were backcrossed to C57BL/6J for four (tpc1) or six (tpc2) generations before being used to generate the dKO.

cDNA Constructs, Transfection, and Cell Culture

Unless otherwise stated, all of the channel clones were GFP tagged for the identification of channel-protein-expressing endolysosomes used for patch-clamp recordings. Cell culture, transfection, and the ion channel and mTOR cDNA clones are described in Extended Experimental Procedures.

Knockdown of mTOR, Raptor, and Rictor

Infection with lentivirus-encoding shRNA of mTOR, Raptor, and Rictor was used for long-term knockdown of the target proteins (Sarbassov et al., 2005). To generate lentivirus, HEK293T cells were transfected with shRNA plasmids (Addgene plasmid 1885 [mTOR], 1857 [Raptor], and 1853 [Rictor] in pLKO.1 vector), lentivirus-packing plasmid psPAX2, and envelope plasmid pMD2.G (gifts from gifts Dr. Didier Trono) using FuGene 6 reagent (Roche Applied Bioscience). Two days after transfection, medium containing lentivirus particles was collected and added into a new dish of HEK293T cells cultured in antibiotic-free medium. Polybrene (8 μg/ml, Sigma-Aldrich) was added to increase the efficiency of viral infection. Infected cells were selected with puromycin (3 μg/ml, Sigma-Aldrich) starting the day after infection. Protein expression was tested using western blot 4 days after infection. For patch-clamp recordings, TPC2 plasmid was transfected into the cells at least 2 days after infection and recorded 2 days later. Control lentivirus-encoding scrambled shRNA (generated from Addgene plasmid #1864) was used as a negative control.

Protein Chemistry and Immunohistochemistry

Details of western blotting and immunohistochemistry are described in Extended Experimental Procedures.

ATP Measurement

ATP levels in HEK293T cells were measured using a luminescence ATP detection kit (ATP lite, PerkinElmer). Luminescence signals were detected using an Analyst HT plate reader (Molecular Device Corporation). ATP levels were calculated from standard curves.

Amino Acid Analysis

Plasma samples were obtained from heparinized blood by centrifugation. The concentration of amino acids in the plasma was determined with an Agilent 1100 Infinity LC system utilizing precolumn derivatization with o-phthalaldehyde-hyde, as previously described (Jones and Gilligan, 1983).

Lysosome pH Imaging

Ratiometric lysosome pH measurements were carried out as previously described (Steinberg et al., 2010). In brief, peritoneal macrophages were placed on glass coverslips and loaded overnight with Oregon-Green 488, Dextran pH-sensitive dye (250 mM) (at pH 4.6 with NaOH) (Dong et al., 2010; Wang et al., 2012). For voltage-clamp recordings, bath solution contained (in mM) 145 K-gluconate, 5 KCl, 2 CaCl2, 10 HEPES, 10 MES, and 10 glucose (pH adjusted to 7.2 with KOH). Pipette solution (luminal) contained (in mM) 145 NaCl, 5 KCl, 2 MgCl2, 10 HEPES, 10 MES, and 10 glucose (pH adjusted to 4.6 with NaOH) (Dong et al., 2010; Wang et al., 2012). For current-clamp recordings, bath solution contained (in mM) 10 Na-gluconate, 130 K-gluconate, 4 KCl, 2 MgCl2, 10 HEPES, 10 MES, and 10 glucose (pH adjusted to 7.2 with KOH), and pipette solution consisted of (in mM) 70 NaCl, 70 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, 10 MES, and 10 glucose (pH adjusted to 4.6 with NaOH). In 0-Na bath, Na-gluconate was substituted with K-gluconate. Liquid junction potential was corrected. Unless otherwise stated, recordings were obtained in the presence of 1 μM Pi(3,5)P2 (water-soluble diC8 form, from Echelon Biosciences) in the bath. ATP-Mg was used for the ATP inhibition experiment. Nucleotide solutions used for bath application were pH adjusted. ATP inhibition curves were fitted with the equation: 

\[ y = y_a + \frac{(y_b - y_a) \cdot [ATP]}{[ATP] + IC_{50}} \]

where \( y_a \) and \( y_b \) are the currents obtained in the presence and absence of ATP, respectively, \( I_{IC_{50}} \) is the ATP concentration required for half-maximal inhibition, and \( h \) is the Hill coefficient. Only the endolysosomes with currents >200 pA at +100 mV, without ATP were selected for the curve fitting.

Behavior Tests

Rotarod and treadmill tests were performed as described in the Extended Experimental Procedures.

Data Analysis

Data were analyzed using Clampfit (Molecular Device), Origin (Origin Lab), and Excel (Microsoft). Values of data were shown as mean ± SEM. Statistical analysis was performed using Student’s t test. Significant difference was considered when p < 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.01.023.

ACKNOWLEDGMENTS

We thank members of the Ren lab for discussion, Amita Tyaboochali for starting our interest in macrophages, Drs. Morris Birbaum and Russell Miller for...
AMPK1/2 dKO MEF cells, Dr. Sara Cherry for the use of plate reader, Dr. Jie Chen for mTOR and S6K1 clones, and Dr. Denia Ramirez-Montalegre for advice on amino acid efflux assay. This work was supported, in part, by funding from American Heart Association, NIH, and the University of Pennsylvania Research Foundation. Embryonic stem cell injections were performed in the Gene Manipulation Facility of the Children's Hospital Boston (tpc1) and the Transgenic and the Chimeric Mouse Facility at University of Pennsylvanian (tpc2). Amino acid measurement was performed by Ilana Nissim in the Metabolic Core Facility, Children's Hospital of Philadelphia Research Institute (supported, in part, by NIH grant number DK-053761). C.C. designed experiments and contributed all of the patch-clamp recordings. Y.Z. did protein chemistry experiments, immunocytochemistry, and lysosomal pH imaging. Y.-J.S. performed protein chemistry and ATP measurements. Y.S. and I.N. did amino acid analysis. C.C. and Y.Z. performed amino acid efflux assay. D.R. initiated the project, designed experiments, developed cDNA constructs and the mouse models, and performed the behavior studies. B.N. and S.B.-H. performed pilot studies. K.A. and L.S. developed reagents. D.R. and D.E.C. supervised the projects. C.C., D.E.C., and D.R. wrote the manuscript.

Received: May 11, 2012
Revised: August 31, 2012
Accepted: January 14, 2013
Published: February 7, 2013

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http://dx.doi.org/10.1016/j.cell.2013.01.023

SUMMARY

Survival in the wild requires organismal adaptations to the availability of nutrients. Endosomes and lysosomes are key intracellular organelles that couple nutrition and metabolic status to cellular responses, but how they detect cytosolic ATP levels is not well understood. Here, we identify an endolysosomal ATP-sensitive Na⁺ channel (lysoNaATP). The channel is a complex formed by two-pore channels (TPC1 and TPC2), ion channels previously thought to be gated by nicotinic acid adenine dinucleotide phosphate (NAADP), and the mammalian target of rapamycin (mTOR). The channel complex detects nutrient status, becomes constitutively open upon nutrient removal and mTOR translocation away from the lysosomal membrane, and controls the lysosome’s membrane potential, pH stability, and amino acid homeostasis. Mutant mice lacking lysoNaATP have much reduced exercise endurance after fasting. Thus, TPCs make up an ion channel family that couples the cell’s metabolic state to endolysosomal function and are crucial for physical endurance during food restriction.

INTRODUCTION

Cells use adenosine triphosphate (ATP) as an energy carrier. Sensing intracellular concentrations of ATP ([ATP]) is a crucial step in the regulation and coupling of [ATP] to cellular metabolism. One of the most extensively characterized cellular ATP sensors is the plasma membrane ATP-sensitive K⁺ channel (KATP) (McTaggart et al., 2010; Nichols, 2006, for review). In contrast to channels on the plasma membrane that regulate such functions as secretion, intracellular ion channels are more difficult to study and thus are less well understood. For example, ATP-sensitive K⁺ channels (mitoKATP) have been recorded from the mitochondrial inner membrane (Inoue et al., 1991), but their molecular identities are not established (Foster et al., 2008). Although it is clear that endosomes and lysosomes play key roles in cellular metabolism, we do not understand how metabolic signals are received by these organelles. Here, we report an ATP-sensitive Na⁺ channel (lysoNaATP) on endolysosomal membranes that is responsive to physiological [ATP]. The channel is formed by TPC1 and TPC2, two well-conserved proteins whose functions are largely unknown. The channel associates with the mammalian target of rapamycin (mTOR) complex and detects cellular nutrient status, becoming constitutively open when nutrients are depleted and when mTOR translocates away from the complex at the lysosomal membrane. LysoNaATP determines the sensitivity of endolysosome’s resting membrane potential to Na⁺ and cytosolic ATP, controls lysosomal pH stability, and regulates whole-body amino acid homeostasis. Strikingly, mutant mice lacking tpc1 and tpc2 have severely reduced endurance after fasting.

RESULTS

Endolysosomes Have an ATP-Sensitive Na⁺-Permeable Channel lysoNaATP

To determine whether endolysosomes have ionic mechanisms that sense intracellular physiological [ATP], we recorded whole-endolysosomal currents from endosomes/lysosomes mechanically released from the cytosol with a glass pipette (Saito et al., 2007). Endolysosomes were enlarged by treatment of cells with vacuolin-1, as previously described (Cerny et al., 2004; Dong et al., 2008). The optimal activities of many plasma membrane channels require phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) as a cofactor to prevent channel “run down” to neuronal excitability (McTaggart et al., 2010; Nichols, 2006, for review). In contrast to channels on the plasma membrane that regulate such functions as secretion, intracellular ion channels are more difficult to study and thus are less well understood. For example, ATP-sensitive K⁺ channels (mitoKATP) have been recorded from the mitochondrial inner membrane (Inoue et al., 1991), but their molecular identities are not established (Foster et al., 2008). Although it is clear that endosomes and lysosomes play key roles in cellular metabolism, we do not understand how metabolic signals are received by these organelles. Here, we report an ATP-sensitive Na⁺ channel (lysoNaATP) on endolysosomal membranes that is responsive to physiological [ATP]. The channel is formed by TPC1 and TPC2, two well-conserved proteins whose functions are largely unknown. The channel associates with the mammalian target of rapamycin (mTOR) complex and detects cellular nutrient status, becoming constitutively open when nutrients are depleted and when mTOR translocates away from the complex at the lysosomal membrane. LysoNaATP determines the sensitivity of endolysosome’s resting membrane potential to Na⁺ and cytosolic ATP, controls lysosomal pH stability, and regulates whole-body amino acid homeostasis. Strikingly, mutant mice lacking tpc1 and tpc2 have severely reduced endurance after fasting.
GFP. ITRPML, though potentiated by exogenously applied HEK293T cells transfected with candidate proteins tagged with et al., 2006). We recorded endolysosomal currents from 1B–1E). Thus, peritoneal macrophage endolysosomes have an tute lysoNaATP when transfected into HEK293T cells (these cells have little endogenous lysoNa ATP; Figure 2 A). Among the candidate approach to identify proteins that reconsti- mately found in intracellular organelle membranes, increases channel currents in endolysosomes (Dong et al., 2010). We first recorded endolysosomal currents from mouse peritoneal macrophages, where lysosomes are well studied. Little current was detected when the pipette solution contained 150 mM K− but no Na+ (−5.6 ± 2.3 pA with no PI(3,5)P2 in the bath and −7.7 ± 2.1 pA with 1 μM PI(3,5)P2; n = 7, −100mV). With a pipette solution containing Na+ (a major cation in the endolysosomal lumen [Steinberg et al., 2010]) and K+ in the cytosolic solution containing PI(3,5)P2, we recorded large inward currents (Na+ moving out of the endolysosome into the cytosol; Figure 1A) when no ATP was present in the bath (Figures 1B and 1C). Upon addition of ATP-Mg, however, the amplitude of the Na+ current was reduced in a dose-dependent manner with an EC50 of 0.32 ± 0.05 mM and a Hill coefficient of 1.25 ± 0.20 (Figures 1B–1E). Thus, peritoneal macrophage endolysosomes have an ATP-sensitive Na+-permeable channel (lysoNaATP) that is sensitive to physiological [ATP]. Similar lysoNaATP Currents were also detected in endolysosomes from other cell lines that we tested, including excitable cells such as beating cardiac myocytes and nonexcitable cells such as fibroblasts and liver hepatocytes (Figures 1F–1K).

**TPCs Form lysoNaATP Channels in HEK293T Cells**

We used a candidate approach to identify proteins that reconstitute lysoNaATP when transfected into HEK293T cells (these cells have little endogenous lysoNaATP; Figure 2A). Among the hundreds of known ion channels, TRPML1 (a 6-transmembrane-spanning endolysosomal Na+-permeable cation channel in the TRP family) and TPC1/TPC2 proteins (endolysosomal channels of 12-transmembrane spanning proteins with similarity to that of voltage-gated Na+ and Ca2+ channels) are known to localize on endosomes and lysosomes (Braliou et al., 2009; Calcraft et al., 2009; Grimm et al., 2012; Ishibashi et al., 2000; Pryor et al., 2006). We recorded endolysosomal currents from HEK293T cells transfected with candidate proteins tagged with GFP. TPC2, though potentiated by exogenously applied PI(3,5)P2 (Dong et al., 2010), was not sensitive to ATP, either at a saturating concentration (1 μM) (Figure 2B and Figure S1A available online) or a concentration close to its EC50 (0.1 μM) (Figure S1B).

Strikingly, overexpression of human TPC1 increased endolysosomal ATP-sensitive currents by 13-fold (Figure 2C). Similarly, transfection with TPC2 resulted in a large lysoNaATP current (Figures 2D and 2E) sensitive to [ATP] (IC50 = 0.92 ± 0.31 mM; Figure 2D), comparable to that recorded from macrophages. Because TPC2 has more prominent lysosomal localization than TPC1 (Calcraft et al., 2009) and is more readily identified in patch-clamp recordings, we focused on TPC2. In controls, GFP-tagged and -untagged TPC2 generated similar lysoNaATP currents (Figure S1C). ATP’s inhibition of lysoNaATP was not mediated by changes in free calcium ([Ca2+]i = 117 nM without ATP and 118 nM with 1 mM ATP). Application of ATP-Mg also increased the free Mg2+ concentration (1.94, 1.99, and 2.18 mM in the presence of 0, 1, and 5 mM ATP-Mg, respectively), but lysoNaATP was not sensitive to Mg2+, as addition of Mg2+ alone did not affect current amplitudes (Figure S1D). Unlike KATP (Nichols, 2006), lysoNaATP is insensitive to ADP (Figure 2E). Similarly, GTP did not inhibit lysoNaATP (Figure 2F).

The ATP sensitivity of plasma membrane KATP can be drastically shifted from several μM toward physiological mM concentrations by the plasma membrane species of PIP2, P(4,5)P2, as a result of interaction between the phospholipid and the channel gating machinery (Baukrowitz et al., 1998; Shyng and Nichols, 1998). Organellar PIP2 (P(3,5)P2), however, had no major effect on lysoNaATP’s ATP sensitivity (Figure 2D). In addition, basal ITPC2 was readily recorded from endolysosomes without the addition of P(3,5)P2 in TPC2-transfected cells, and the currents were also inhibited by ATP (Figure S1E). Therefore, the ATP sensitivity of the lysoNaATP channels is independent of P(3,5)P2.

Although TPC channels had been proposed to be activated by nicotinic acid adenine dinucleotide phosphate (NAADP) (Calcraft et al., 2009), recent data suggest that TPC proteins are not the direct targets of NAADP (Lin-Moshier et al., 2012; Wang et al., 2012). One potential mechanism for NAADP activation could be the release of ATP inhibition of TPC channels. However, NAADP had no effect on the ATP inhibition of lysoNaATP recorded from macrophages (Figure S1F) or TPC2-transfected HEK293T cells (Figure S1G), suggesting that NAADP is not an activator of TPCs in the lysoNaATP complex.

**lysoNaATP’s ATP Sensitivity Requires mTOR**

Unlike the plasma membrane KATP channel, which is inhibited by both ATP and the nonhydrolyzable analog ATPS by direct binding (Nichols, 2006), lysoNaATP is not inhibited by ATPS (Figure S1H). Interestingly, the inhibition of lysoNaATP by ATP is slow (T1/2: 43.3 ± 8.8 s, n = 3; Figure S1I) in contrast to the fast inhibition of KATP (ms). These slow kinetics were not due to ATP diffusion from the cytosol (bath) to the lumen, as dialyzing the endolysosomal lumen with 10 mM ATP in the pipette solution did not block lysoNaATP, and the channel was still inhibited by ATP applied in the bath (Figure 2G). We conclude that ATP acts on the channel from the cytosolic surface of the endolysosome. The slow kinetics and the requirement of ATP hydrolysis suggest that ATP does not directly bind TPC to inactivate it. We next examined whether a slower process, such as activation of protein kinases, is responsible for lysoNaATP’s ATP sensitivity.

A well-established cytosolic ATP-sensing kinase is the AMP-activated protein kinase (AMPK) (Hardie et al., 2012; Oakhill et al., 2012). In the presence of an AMPK inhibitor, dorsomorphin, however, lysoNaATP was still ATP sensitive (Figure S2A). In addition, lysoNaATP recorded from TPC2-transfected MEF cells lacking both AMPK catalytic subunit isoforms (AMPK 1/2 double-knockout [dkKO]) was readily inhibited by ATP (Figure S2B). Thus, lysoNaATP’s ATP sensitivity does not require AMPK.

Under our recording configurations in which soluble proteins are easily washed away (Figure 1A), a putative functional kinase should be associated with the endolysosomal membrane and TPC. One of the few kinases known to be tethered to the lysosome membrane is the mammalian target of rapamycin (mTOR) (Koroichuk et al., 2011; Sancak et al., 2010; Zoncu
et al., 2011). Consistent with the role of mTOR in lysoNaATP’s ATP sensitivity, the mTOR inhibitors rapamycin and Torin 1 profoundly reduced the ATP sensitivity of reconstituted lysoNaATP in TPC2-transfected HEK293T cells (Figures 3A–3C) and that of the native channel in macrophages (Figures 3D–3F).

To test whether the ubiquitously expressed mTOR protein is required for lysoNaATP’s ATP sensitivity, we knocked down the
endogenous mTOR protein in HEK293T cells using lentivirus encoding an shRNA against human mTOR (Figure 3G). The ATP sensitivity of lysoNa ATP generated by TPC2 in the mTOR shRNA virus-infected cells was dramatically lowered to 0.24 mM (Figures 3I and 3J). A control scrambled shRNA had no significant effect (Figures 3H and 3J).

Two mTOR complexes are active in mammalian cells (Laplante and Sabatini, 2012a for review): the Raptor-containing mTORC1 and Rictor-containing mTORC2. Knockdown of Raptor (Figure 3L), but not Rictor (Figure 3K), reduced the ATP inhibition of the channel (Figure 3M), suggesting that lysoNa ATP's ATP sensitivity is conferred by mTORC1.

**TPC Complexes with mTOR**

mTOR was coimmunoprecipitated with TPC1 or TPC2 from transfected HEK293T cells (Figure 4A), suggesting that the channel proteins and mTOR are in the same signaling complex. We did not detect any association between TPC2 and the lysosome-localized proteins RagB (Figure S3A), V-ATPase (Figure S3B), or lamptors (Figure S3C and S3D). These proteins

Figure 2. TPC1 and TPC2 Form lysoNa ATP in HEK293T Cells

(A–D) ATP sensitivity of endolysosomal currents was tested in HEK293T cells transfected with GFP (A) or GFP-tagged TRPML1 (B), TPC1 (C) and TPC2 (D). PI(3,5)P2 (1 μM) was included in the bath during recordings.

(A) Mock-transfected HEK293T cells had little lysoNa ATP current (24.9 ± 8.7 pA without ATP, 16.2 ± 6.2 pA with 1 mM ATP-Mg, at −100 mV).

(B) I_{TPRML1} is insensitive to ATP, as shown in the representative recordings (left) and the statistics of the averaged current amplitudes (right, at −100 mV).

(C and D) ATP-sensitive currents recorded from TPC1-transfected (C) and TPC2-transfected cells (D). The IC50 of ATP on I_{TPC2} was 0.55 ± 0.09 mM and 0.92 ± 0.31 mM in the presence of 0.1 μM and 1 μM PI(3,5)P2, respectively (n ≥ 4).

(E and F) I_{TPC2} is insensitive to ADP (E) and GTP (F).

(G) Similar to (D) but with a pipette solution containing 10 mM ATP-Mg.

Data are shown as mean ± SEM. See also Figure S1.
interact with mTOR under certain conditions (Kim et al., 2008; Sancak et al., 2010; Zoncu et al., 2011). In contrast to TPCs, the transfected ATP-insensitive endolysosomal channel TRPMLs (Figures 2B and S3E and Figure 4A, lane 2) had little or no detectable association with mTOR.

LysoNaATP’s ATP sensitivity was increased by ~5 fold when mTOR protein was increased above endogenous levels by mTOR cDNA transfection (Figures 4B–4E). When a rapamycin-resistant mTOR (residue S2035 mutated to T [S2035T] [Vilella-Bach et al., 1999]) was cotransfected with TPC2, the resulting lysoNaATP became insensitive to rapamycin (Figure 4F). Thus, mTOR determines lysoNaATP’s rapamycin sensitivity.

To determine whether lysoNaATP requires mTOR’s kinase activity, we inhibited the endogenous mTOR with rapamycin and then reintroduced mTOR activity by transfection of a rapamycin-resistant mTOR. Unlike wild-type, the mutant with an additional mutation in mTOR’s catalytic domain (D2357E, kinase dead mutant [Vilella-Bach et al., 1999]) was unable to support lysoNaATP’s ATP sensitivity (Figures 4G and 4H), suggesting that mTOR’s kinase activity is required. Through mutation of potential mTOR phosphosites of TPC2’s putative cytosolic domains, we have not located the sites of phosphorylation that are important for the channel’s ATP inhibition. The functionally important mTOR residues in lysoNaATP could be on cryptic sites.
of these TPCs or on TPC-complex-associated subunits yet to be identified.

**LysoNaATP Detects Nutrient Depletion**

In addition to sensing mM \([\text{ATP}]\) [Dennis et al., 2001], mTOR is also a major sensor for cellular nutrients (Laplante and Sabatini, 2012b). Nutrient depletion is known to cause mTOR translocation away from lysosomal membranes (Korolchuk et al., 2011; Sancak et al., 2010), where TPC2 is localized (Calcraft et al., 2009). When cells were starved by depleting glucose and amino acids for 60 min, resulting in a drop of cellular ATP content from 3.9 ± 0.5 (n = 8) to 2.1 ± 0.3 (n = 10) nmole/10^6 cells, \(I_{\text{TPC2}}\) was no longer inhibited by ATP (Figures 5A, 5B, and 5E), presumably as a result of loss of mTORC1 on the lysosomal membrane.

In nutrient-replete cells, mTORC1 is recruited to the lysosomal surface by the Rag GTPases (Kim et al., 2008; Sancak et al., 2008). When RagB\(_{\text{GTP}}\), a GTP-bound mutant that retains mTOR on lysosomes (Sancak et al., 2008), was transfected with TPC2, the resulting lysoNaATP was inhibited by ATP even after cell starvation (Figures 5C and 5E). In contrast, transfection of a GDP-bound Rag mutant (RagB\(_{\text{GDP}}\)) that prevents mTOR from being localized on lysosomal surface rendered the channel insensitive to ATP even in replete cells (Figures 5D and 5E).
Amino acid depletion alone is sufficient to cause mTORC1’s translocation away from lysosomes (Sancak et al., 2008, 2010). Similarly, removal of amino acids (Figure 5G), but not glucose alone (Figure 5F), was also sufficient to render lysoNaATP largely ATP insensitive. Refeeding nutrient-deprived cells with amino acids for 10 min, a condition sufficient to relocate mTOR back onto the lysosomal membrane after starvation (Sancak et al., 2008), quickly restored the channel’s ATP sensitivity.
TPC1 and TPC2 Constitute Native lysoNaATP
To test the in vivo function of TPC1 and TPC2, we generated mice with tpc1, tpc2, or both tpc1 and tpc2 disrupted. In peritoneal macrophage endolysosomes isolated from the tpc1/tpc2 dKO, 1 μM Pi(3,5)P2 elicited no inward current (Figures 6A and 6B). The small residual basal currents, presumably from other channels such as TRPML1, did not contribute to the ATP sensitivity (Figures 6B and 6C). The absence of lysoNaATP in the dKO is not a result of potential developmental defect, as transient transfection of a human (Figures 6D and 6E) or mouse (Figure 6E) TPC cDNA into the mutant macrophages restored the ATP-sensitive currents. Cells from mice lacking TPC1 or TPC2 alone had measureable lysoNaATP, although at reduced levels (current density: tpc1 KO, 71 ± 19 pA/pF, n = 8; tpc2 KO, 66 ± 31 pA/pF, n = 5; WT, 130 ± 16 pA/pF, n = 12). These data suggest that lysoNaATP is formed by TPC1 and/or TPC2 in the native cells and there are no other major ATP-sensitive cation channels in these organelles.

Because mTOR is a key component for the ATP sensitivity of lysoNaATP, we tested whether mTOR's kinase activity also required lysoNaATP. In cultured hepatocytes, insulin stimulated mTOR-dependent (rapamycin-sensitive) phosphorylation of a major target p70S6K in both WT and dKO cells (Figure S4A). Similarly, amino acid feeding stimulated mTOR translocation to the lysosome in both WT and TPC1/2 dKO (Figure S4B). These data suggest that mTOR does not require TPCs for its function and place lysoNaATP downstream of mTOR in the signaling cascade.

lysNaATP Controls Endolysosomal Na+ Permeability, Membrane Potential, and Sensitivity to ATP
The membrane potentials of endolysosomes have been only indirectly monitored using voltage-sensitive dyes. We directly measured the membrane potentials (ΔΨ, Vm, defined as Vcytosol − Vlumen, Bertl et al., 1992) using current-clamp recording. Similar to plasma membranes, endolysosomal Vm is sensitive to K+, Na+, H+, and Cl− (C.C. and D.R., unpublished data). In the absence of ATP, the membrane potential of the wild-type macrophage endolysosomes was +38.0 mV ± 1.4 mV (lumen more negative compared to cytosol). The endolysosome-expressing mutant lysNaATP was ~20 mV more hyperpolarized than WT (+20.5 mV ± 1.1 mV) (Figures 7A–7C). In the WT, removal of cytosolic Na+ led to a change in ΔΨ of 9.7 mV (Figures 7A and 7C). The mutant endolysosomes’ membrane potential, however, was insensitive to changes in cytosolic [Na+] (Figures 7B and 7C). These data suggest that lysNaATP is a major determinant of the endolysosomal membrane’s resting Na+ permeability, similar to NALCN’s (a 24 transmembrane-spanning channel with sequence similarity to that of TPCs) effect on the plasma membrane of neurons (Ren, 2011).

ATP inhibition of TPCs led to a change of ΔΨ to +19.6 mV ± 1.8 mV in WT but had no effect on that of the mutant (Figures 7A–7C). These data suggest that lysNaATP also controls the membrane potential ATP sensitivity: a drop of ATP leads to a depolarization of endolysosomes (lumen more negative) due to the opening of TPCs and an efflux of Na+ from endolysosomal lumen into cytosol.

lysNaATP Regulates Lysosomal pH Stability
Membrane potentials regulate a wide spectrum of physiological processes on the plasma membrane through proteins such as voltage-gated ion channels and voltage-sensitive enzymes (Hille, 2001). It is not clear whether endolysosomes have similar voltage-sensitive proteins. One potential consequence of an increase of ΔΨ (lumen more negative) during a decrease of available ATP is that the V-ATPase is able to maintain lysosomal pH at the pump’s set point. Consistent with this idea, WT lysosomes maintained a relatively stable pH upon starvation (4.73 ± 0.01 before and 4.74 ± 0.01 after starvation). In contrast, there was a significant shift of pH toward alkalization in the mutant (4.82 ± 0.01 before and 5.36 ± 0.02 after starvation; Figures 7D–7F).
lysoNaATP Is Required for Normal Amino Acid Homeostasis during Starvation Stress

A potential function for lysoNaATP’s control of lysosomal \( V_m \) and pH is to regulate the fusion between autophagosomes and lysosomes, one of the last steps of macroautophagy during which nutrients such as amino acids can be generated in response to starvation (Mizushima and Komatsu, 2011). The mutant mice, however, do not appear to have gross defects in autophagy (Figure S5A–S5C). During food deprivation stress, one of the homeostatic responses is an increase in the levels of certain amino acids in the circulation, partly due to protein digestion in the lysosomes and the subsequent export of amino acids from these organelles (Brady et al., 1978; Cahill, 2006). The transport of amino acids, particularly cations (lysine and arginine), by the lysosomal system c transportation system is likely influenced by lysosomal membrane potential and pH (Pisoni and Thoene, 1991). We loaded liver lysosomes with \(^{14}\text{C}-\text{labeled lysine and monitored the efflux at various time points. With 2 mM ATP in the efflux buffer, sufficient to inhibit lysoNaATP, there was no significant efflux rate difference between WT and the dKO. With [ATP] reduced to 0.1 mM, a condition under which lysoNaATP is open, the amino acid efflux was significantly faster in the WT lysosomes than in the dKO (Figure S5 D). We also measured the levels of circulating amino acids 6 hr and 3 days after the onset of food deprivation in the WT and dKO mice. In WT mice, the levels of plasma lysine and arginine increased by

**Figure 7. lysoNaATPs Control Lysosomal Membrane Potentials and Lysosomal pH and Are Required for Normal Fasting Endurance**

(A–C) Membrane potentials of macrophage lysosomes from wild-type (A) and dKO (B) were monitored with current-clamp recordings while ATP was added to the bath containing 0 or 10 mM Na\(^{+}\), as indicated by the bars above the recordings.

(D–F) Lysosomal pH measured with ratio-metric imaging from WT (D and F) and dKO macrophage lysosomes (E and F) before and after starvation. Distribution histograms of pH values (fitted to Gaussian distributions) are in (D) and (E), and averaged values are in (F).

(G) Amino acid analysis. Levels of 15 plasma amino acids (R, K, T, M, F, V, L, I, D, S, G, A, Y, and W) of each animal (5–6 in each group) were measured after 3 days of fasting and normalized to those at the beginning (6 hr) of fasting.

(H and I) Mice were tested before and after fasting for 3 days and 2 days after re-feeding. (H) Distance traveled at exhaustion. (I) Distance traveled of each mouse (represented by each point) after fasting and 2 days after refeeding, as normalized to that before fasting. Black and blue circles indicate behavioral tests after fasting and refeeding conditions, respectively. Red triangles indicate mean values. Numbers of animals tested are in (H). Several points overlap and are not distinguished.

*\( p < 0.05; **p < 0.001. \) NS, not significant. Data are presented as mean ± SEM. See also Figure S5.
lysoNaATP is Required for Normal Fasting Endurance  
Under normal in-house conditions, tpc1/tpc2 dKO adult mice are viable, fertile, and have no obvious morphological abnormalities or obvious behavioral defects (observation and as measured with rotarod and treadmill tests; Figures 7H and 7I). Because lysoNaATP opens primarily when [ATP] is low or when nutrients are restricted, we subjected the mice to food deprivation. After fasting for 3 days, WT mice remained alert and active in food search activities. Endurance performance in the treadmill test was comparable to or better (five out of ten mice) than that before fasting, consistent with the findings of fasting-induced hyperactivity found in animal behavioral tests (Challet et al., 1997; Yamanaka et al., 2003). In contrast, dKO mice were less active after fasting, and endurance performance was reduced by 8.3-fold; none performed better than before fasting (Figures 7H and 7I). Two days after reintroduction of food, dKO mice regained endurance and became as active as before fasting (Figure 7I). In the less strength-demanding rotarod test, the mutants’ performances were less affected by fasting (Figure S5E).

DISCUSSION

We have shown that mTOR associates with the channel pore of lysoNaATP formed by TPC proteins and controls channel activation. Nutrient replete cells have high [ATP], which presumably enables mTOR to phosphorylate TPC and/or its associated proteins and maintain the channel in the closed state. During cell starvation, ATP levels fall, mTOR delocalizes from TPC, and lysoNaATP opens, allowing Na+ and other ions to leave the lysosome. Thus, TPCs link lysosomal control of nutrient recycling to the extensive signaling mTOR network that monitors and responds to nutrient depletion, hypoxia, and cell growth (Laplante and Sabatini, 2012b). Intracellular ATP concentrations are normally a few mM at rest but vary among cell types, during different metabolic conditions, and during circadian cycles. These levels can drop to <0.1 mM during starvation, hyperosmotic stress, hypoxia, and ischemia (Berg et al., 2009; Gribble et al., 2000; Imamura et al., 2009; Beis and Newsholme, 1975). With its ATP sensitivity tuned to the range of physiological concentrations and regulated by nutrient availability, lysoNaATP couples the cell’s energy status to endolysosomal function. In a natural environment, an animal’s survival depends on its ability to deal with stress, to maintain the ability to search for food, and to fend off attacks from intruders even during frequent starvation. TPC’s evolutionarily conserved endolysosomal function in fasting endurance presumably serves this purpose.

Currently, we do not know how the channel affects lysosomal regulation of such functions as protein degradation, energy generation, plasma membrane repair, and exocytosis to maintain cell viability (Laplante and Sabatini, 2012b; Luzio et al., 2007; Settembre et al., 2011). Plasma membrane potentials control many physiological responses ranging from fertilization, muscle contraction, neuronal excitability, synaptic transmission, and hormone release to immune responses. Opening of lysoNaATP depolarizes endolysosomal membranes, but how voltage changes across endolysosomal membranes are detected is poorly understood. The lysosomal membrane potential controls basic endolysosomal properties such as pH and amino acid transport (Figure 5; Pisoni and Thoene, 1991), and this will require extensive new experiments. TPCs and mTORC1 are widely expressed in many organs including brain, heart, intestine, liver, and pancreas. As such, the cell types and circuitry in which lysoNaATP functions to regulate fasting endurance need to be determined.

Originally proposed to be activated by NAADP (Calcraft et al., 2009), recent data suggest that NAADP binds to proteins other than TPC1/2 (Lin-Moshier et al., 2012; Walseth et al., 2012) and does not activate TPCs (Figures S1F and S1G; see also Wang et al. [2012]). TPC has also recently been shown to be potentiated by PI(3,5)P2 under isolated endolysosome recording conditions (Wang et al., 2012). It is not known whether PI(3,5)P2 is a basal requirement for channel activity under physiological conditions, similar to the requirement of PI(4,5)P2 for many plasma membrane ion channels and transporters, or whether there is significant change of PI(3,5)P2 levels during signaling to potentiate TPCs. Nevertheless, the ATP sensitivity of lysoNaATP is independent of PI(3,5)P2, as basal TPC current in the absence of added PI(3,5)P2 is similarly regulated by ATP.

The mTOR complexes are master integrators linking cellular metabolism to many adaptive cellular responses such as global gene expression (Laplante and Sabatini, 2012b). The targets of mTOR are primarily cytosolic, and mTOR is predominantly localized on endolysosomal membranes under physiological conditions (Korolchuk et al., 2011; Sancak et al., 2010; Zoncu et al., 2011). One functional consequence of the recruitment of mTOR to the lysosomal membrane is to detect amino acid content inside the lysosomes for the subsequent action on transcriptional cascades that are important for cellular responses such as lysosomal biogenesis (Han et al., 2012; Sancak et al., 2010; Settembre et al., 2011; Zoncu et al., 2011). Our data suggest that mTOR not only directly receives information from lysosomes, but also acutely controls endosomal and lysosomal functions through lysoNaATP in response to changes in the cell’s energy and nutrient status. Dysregulation of mTOR and the components in its signaling network is linked to many diseases such as diabetes, cancer, neurodegeneration, seizure, and autism. Because both mTOR and lysoNaATP are widely expressed, lysoNaATP channels may enable more precise downstream targeting in mTOR-associated disorders.

EXPERIMENTAL PROCEDURES

Animals  
Animal use followed NIH guidelines and were approved by the IACUC at the University of Pennsylvania. To generate tpc1 and tpc2 knockouts, the exons containing the translational start site (ATG) were deleted. The mutant TPC1 and TPC2 proteins are predicted to lack the first 69 and 49 amino acids, respectively, and do not generate detectable current, as tested in HEK293T cells (Wang et al., 2012) and in the tpc1/tpc2 double-knockout cells (Figure 6). We cannot rule out the possibility that small, undetectable residual channels formed by the mutant proteins can support functions such as animal viability.
Individual knockouts were backcrossed to C57BL6/J for four (tpc1) or six (tpc2) generations before being used to generate the dKO.

cDNA Constructs, Transfection, and Cell Culture

Unless otherwise stated, all of the channel clones were GFP tagged for the identification of channel-protein-expressing endolysosomes used for patch-clamp recordings. Cell culture, transfection, and the ion channel and mTOR cDNA clones are described in Extended Experimental Procedures.

Knockdown of mTOR, Raptor, and Rictor

Infection with lentivirus-encoding shRNA of mTOR, Raptor, and Rictor was used for long-term knockdown of the target proteins (Sarbassov et al., 2005). To generate lentivirus, HEK293T cells were transfected with shRNA plasmids (Addgene plasmid 1855 [mTOR], 1857 [Raptor], and 1853 [Rictor] in pLKO.1 vector), lentivirus-packing plasmid psPAX2, and envelope plasmid pMD2.G (gifts from Dr. Didier Trono) using Fugene 6 reagent (Roche Applied Bioscience). Two days after transfection, medium containing lentivirus particles was collected and added into a new dish of HEK293T cells cultured in antibiotic-free medium. Polybrene (8 µg/mL, Sigma-Aldrich) was added to increase the efficiency of viral infection. Infected cells were selected with puromycin (3 µg/mL, Sigma-Aldrich) starting the day after infection. Protein expression was tested using western blot 4 days after infection. For patch-clamp recordings, TPC2 plasmid was transfected into the cells at least 2 days after infection and recorded 2 days later. Control lentivirus-encoding scrambled shRNA (generated from Addgene plasmid #1864) was used as a negative control.

Protein Chemistry and Immunohistochemistry

Details of western blotting and immunohistochemistry are described in Extended Experimental Procedures.

ATP Measurement

ATP levels in HEK293T cells were measured using a luminescence ATP detection kit (ATPlite, PerkinElmer). Luminescence signals were detected using an Analyt HT plate reader (Molecular Device Corporation). ATP levels were calculated from standard curves.

Amino Acid Analysis

Plasma samples were obtained from heparinized blood by centrifugation. The concentration of amino acids in the plasma was determined with an Agilent 1260 Infinity LC system utilizing precolumn derivatization with o-phthalaldehyde (Jones and Gilligan, 1983).

Lysosome pH Imaging

Ratiometric lysosome pH measurements were carried out as previously described (Steinberg et al., 2010). In brief, peritoneal macrophages were placed on glass coverslips and loaded overnight with Oregon-Green 488, Dextran pH-sensitive dye (250 µM of nigericin and monensin [pH 4.0 to 7.0]). Following 5 min of incubation for the equilibration of pH across lysosome membranes, pH images were acquired for each standard solution. The resulting fluorescence intensity ratios (F207/F440) as a function of pH were fitted to a Boltzmann sigmoid curve. Lysosome pH values were obtained by fitting the intensity ratios obtained during the experiment to the standard curves.

Lysosomal Amino Acid Efflux Measurement

Amino acid efflux was measured using liver lysosomes loaded with 14C-labeled amino acid methyl ester, as described in the Extended Experimental Procedures.

Electrophysiology

Whole-endolysosome recordings followed the previously described method (Saito et al., 2007) except that endolysosomes were enlarged with the treatment of vacuolin−1 (1 µM; Dong et al., 2008) for 1–6 hr (macrophyages and cardiac myocytes) or overnight (hepatocytes, fibroblasts, and HEK293T cells). In brief, the cell membrane was cut with a glass pipette at a position close to the endolysosome to be patched. Endolysosomes were pushed out through the cut position with the pipette tip. In experiments for which rapamycin or Torin 1 was used (Figures 3B, 3E, 4F–4H, and S2C), the drug was added to the bath solution 10 min before the lysosome was released. In cells transfected with mTOR-tagged TPR or TRPML, only the GFP-positive endolysosomes were selected for recording. Only one endolysosome was recorded from each coverslip. Patch recordings were performed with a Multiclamp 700B amplifier (Molecular Device) and a Digidata 1440A data acquisition system (Molecular Device). PClamp and Clampfit software were used to record and analyze data. Recording pipettes of borosilicate glass had resistances of 3–6 MΩ. For voltage-clamp recordings, bath solution (cystolic) contained (in mM) 140 K-glucolate, 4 NaCl, 2 MgCl2, 0.39 CaCl2, 1 EGTA, and 10 HEPES (pH adjusted to 7.2 with KOH). Pipette solution (luminal) contained (in mM) 145 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, 10 MES, and 10 glucose (pH adjusted to 4.6 with NaOH) (Dong et al., 2010; Wang et al., 2012). For current-clamp recordings, bath solution contained (in mM) 10 Na-glucolate, 130 K-glucolate, 4 KCl, 2 MgCl2, 0.39 CaCl2, 1 EGTA, 0.0001 PI(3,5)P2, and 10 HEPES (pH adjusted to 7.2 with KOH), and pipette solution consisted of (in mM) 70 NaCl, 70 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, 10 MES, and 10 glucose (pH adjusted to 4.6 with NaOH). In 0-Na bath, Na-glucolate was substituted with K-glucolate. Liquid junction potential was corrected. Unless otherwise stated, recordings were obtained in the presence of 1 µM PI(3,5)P2 (water-soluble diC8 form, from Echelon Biosciences) in the bath. ATP-Mg was used for the ATP inhibition experiment. Nucleotide solutions used for bath application were pH adjusted. ATP inhibition curves were fitted with the equation: \( [\text{IC50}] = [\text{IC50}]_0 + [\text{IC50}]_t / (1 + [\text{ATP}] / [\text{IC50}]_t) \), in which \([\text{IC50}]_0\) and \([\text{IC50}]_t\) are the currents obtained in the presence and absence of ATP, respectively. \( [\text{IC50}]_t \) is the ATP concentration required for half-maximal inhibition, and \( h \) is the Hill coefficient. Only the endolysosomes with currents >200 pA at −100mV, without ATP) were selected for the curve fitting.

Behavior Tests

Rotarod and treadmill tests were performed as described in the Extended Experimental Procedures.

Data Analysis

Data were analyzed using Clampfit (Molecular Device), Origin (Origin Lab), and Excel (Microsoft). Values of data were shown as mean ± SEM. Statistical analysis was performed using Student’s t test. Significant difference was considered when p < 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.01.023.

ACKNOWLEDGMENTS

We thank members of the Ren lab for discussion, Amita Tiyaboonchai for starting our interest in macrophages, Drs. Morris Birnbaum and Russell Miller for
AMPK1/2 dKO MEF cells, Dr. Sara Cherry for the use of plate reader, Dr. Jie Chen for mTOR and S6K1 clones, and Dr. Denia Ramirez-Montalegre for advice on amino acid efflux assay. This work was supported, in part, by funding from American Heart Association, NIH, and the University of Pennsylvania Research Foundation. Embryonic stem cell injections were performed in the Gene Manipulation Facility of the Children’s Hospital Boston (tpc2) and the Transgenic and the Chimeric Mouse Facility at University of Pennsylvania (tpc2). Amino acid measurement was performed by Ilana Nissim in the Metabolomic Core Facility, Children’s Hospital of Philadelphia Research Institute (supported, in part, by NIH grant number DK-053761). C.C. designed experiments and contributed all of the patch-clamp recordings. Y.Z. did protein chemistry experiments, immunocytochemistry, and lysosomal pH imaging. Y.-J.S. performed protein chemistry and ATP measurements. Y.S. and I.N. did amino acid analysis. C.C. and Y.Z. performed amino acid efflux assay. D.R. initiated the project, designed experiments, developed cDNA constructs and the mouse models, and performed the behavior studies. B.N. and S.B.-H. performed pilot studies. K.A. and L.S. developed reagents. D.R. and D.E.C. supervised the projects. C.C., D.E.C., and D.R. wrote the manuscript.

Received: May 11, 2012
Revised: August 31, 2012
Accepted: January 14, 2013
Published: February 7, 2013

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Cell 152, 778–790, February 14, 2013 ©2013 Elsevier Inc. 789


**Supplemental Information**

**EXTENDED EXPERIMENTAL PROCEDURES**

**cDNA Constructs Used in Transfection**

Unless otherwise stated, all the channel clones were GFP-tagged for the identification of channel protein-expressing endolysosomes used for patch-clamp recordings. Human isozymes were used in all the experiments except Figure 5H where the mouse TPC2 (mTPC2) was also used. TPC1, TPC2, TRPML1, TRPML2 and TRPML3 were cloned into the HindIII/EcoRI (TPC2), HindIII/BamHI (TPC1), or KpnI/SmaI (mTPC2, TRPML1, TRPML2, TRPML3) sites of pcEGFP-C1 (TRPML1, TRPML2, TRPML3), pEGFP-N1 (TPC1), or pEGFP-C3 (TPC2, mTPC2) vector. Lamtor1-3 were amplified from human liver cDNA with primers containing HA or Flag tag sequences and were subcloned into the EcoRI/XbaI sites of pcDNA3. All the clones were confirmed with sequencing. The human wt mTOR, mTOR-S2035T, mTOR-S2035T/D2357E, wt RagB, RagBGDP (T54L) and RagB^GTP (Q99L) clones were from Addgene (plasmid # 26603, 26604, 26605, 19313, 19314, 19315, respectively) (Sancak et al., 2010; Sancak et al., 2008; Vilella-Bach et al., 1999). Transfection was done with Lipofectamine LTX or Lipofectamine 2000 (Invitrogen).

**Cell Culture**

HEK293T cells were cultured in DMEM (GIBCO) medium supplemented with 10% FBS (Lonza) and 1x Glutamax (Invitrogen) at 37 °C in a humidified CO2 incubator. Human isozymes were used in all the experiments except Figure 5H where the mouse TPC2 (mTPC2) was also used. TPC1, TPC2, TRPML1, TRPML2 and TRPML3 were cloned into the HindIII/EcoRI (TPC2), HindIII/BamHI (TPC1), or KpnI/SmaI (mTPC2, TRPML1, TRPML2, TRPML3) sites of pcEGFP-C1 (TRPML1, TRPML2, TRPML3), pEGFP-N1 (TPC1), or pEGFP-C3 (TPC2, mTPC2) vector. Lamtor1-3 were amplified from human liver cDNA with primers containing HA or Flag tag sequences and were subcloned into the EcoRI/XbaI sites of pcDNA3. All the clones were confirmed with sequencing. The human wt mTOR, mTOR-S2035T, mTOR-S2035T/D2357E, wt RagB, RagBGDP (T54L) and RagB^GTP (Q99L) clones were from Addgene (plasmid # 26603, 26604, 26605, 19313, 19314, 19315, respectively) (Sancak et al., 2010; Sancak et al., 2008; Vilella-Bach et al., 1999). Transfection was done with Lipofectamine LTX or Lipofectamine 2000 (Invitrogen).

**Protein Chemistry**

For the experiments testing interaction between TPCs and mTOR (Figures 4, 5 and S3E), transfected HEK293T cells were lysed in ice-cold lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1% NP-40, and one tablet of complete protease inhibitors (Roche) per 25 ml. Lysates were cleared by spinning at 250 rpm for 5 min at 37 °C in D-Hank’s medium containing 0.08% (W/V) collagenase (type II, Worthington) and 1% BSA (Sigma-Aldrich). The supernatant was mixed with 10% FBS (V/V) to stop the digestion, and centrifuged at 800 rpm (68 xg) for 5 min at 4 °C. Cells were plated onto coverslips and cultured in DMEM/F-12 (GIBCO) supplemented with 10% FBS (Lonza) and 1x Pen/strep (Invitrogen) at 37 °C in a humidified CO2 incubator. Beating myocytes were used for recordings were visually identified. Fibroblasts were cultured for 4 passages before experiments. Peritoneal macrophages were extracted from sacrificed adult mice with 10 ml ice-cold PBS injected into the peritoneal cavity. Pelleted cells were plated onto coverslips and cultured in DMEM supplemented with 20% FBS and 1x Pen/strep at 37 °C in a humidified CO2 incubator. Liver hepatocytes used for electrophysiological recording were cultured from P0 pups. The liver was quickly dissected out and cut into small pieces in ice-cold Ca2+-free D-Hank’s medium. The pieces were digested by shaking at 250 rpm for 5 min at 37 °C in D-Hank’s medium containing 0.08% (W/V) collagenase (type II, Worthington) and 1% BSA (Sigma-Aldrich). The supernatant was mixed with 10% FBS (V/V) to stop the digestion, and centrifuged at 800 rpm (68 xg) for 5 min at 4 °C. Cells were plated onto coverslips and cultured in DMEM/F-12 (GIBCO) supplemented with 10% FBS (Lonza) and 1x Pen/strep (Invitrogen) at 37 °C in a humidified CO2 incubator. Beating myocytes were used for recordings were visually identified. Fibroblasts were cultured for 4 passages before experiments. Peritoneal macrophages were extracted from sacrificed adult mice with 10 ml ice-cold PBS injected into the peritoneal cavity. Pelleted cells were plated onto coverslips and cultured in DMEM supplemented with 20% FBS and 1x Pen/strep at 37 °C in a humidified CO2 incubator. Liver hepatocytes used for electrophysiological recording were cultured from P0 pups. The liver was quickly dissected out and cut into small pieces in ice-cold HBSS (GIBCO) containing 0.2 mM EDTA. Tissue was shaken at 80 rpm at 37 °C for 10 min, transferred into digestion solution containing 1 mM CaCl2 and 100 unit/ml collagenase (type 4, Worthington) in HBSS, and shaken for another 10 min. Digestion was stopped by adding 10% FBS. Cells were dissociated by pipette, plated in DMEM/F12 supplemented with 10% FBS and 1x Pen/strep, and cultured at 37 °C in a humidified CO2 incubator for more than 24 hr before recording. Hepatocytes used for biochemical studies were isolated from adults following previously described protocols (Glick et al., 2012) and cultured in serum-free William’s E medium supplemented with 1x Pen/strep.

For the experiments testing interaction between TPC2 and mTOR (Figures 4, 5 and S3E), transfected HEK293T cells were lysed in ice-cold lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1% NP-40, and one tablet of complete protease inhibitors (Roche) per 25 ml. Lysates were cleared by spinning at 13,200 rpm (18,506 xg) for 10 min. For Western blot, cleared lysates were resolved with 4%–12% NuPAGE Bis-Tris gels (Invitrogen), and analyzed with anti-mTOR (1:1000, Cell signaling, #2983) or anti-GFP (1 μg/ml, Santa Cruz, sc-9996) antibody. For immunoprecipitation, cell lysates were incubated with protein A agarose (Invitrogen) at 4 °C for 10 min. The cell lysates were divided into two groups, one used as total cell lysate input, and the other for immunoprecipitation with anti-GFP (2 μg/ml, Invitrogen, #A11210) at 4 °C for 2 hr, followed by an additional 2 hr incubation with protein A agarose. Precipitates were washed 3 times with cold lysis buffer and eluted with 1xLDS sample buffer (Invitrogen, NP0007) supplemented with 100 mM DTT. Samples were heated at 70 °C for 10 min, resolved in 4%–12% NuPAGE Bis-Tris gels, and analyzed with immunoblotting using anti-mTOR (1:1000), -GFP (1 μg/ml, Santa Cruz, sc-9996) or -GAPDH (2 μg/ml, Millipore, MAB374) antibody. HRP-conjugated secondary antibody from the Clean-Blot kit (Pierce) was used in ECL detection to minimize contaminating signals from the primary antibodies used in immunoprecipitation.

For the experiments testing interaction between TPC2 and V-ATPase, transfected cells were lysed in ice-cold buffer containing 150 mM NaCl, 40 mM HEPES (pH 7.4), 2 mM EGTA, 2.5 mM MgCl2, 1% Triton X-100, and one tablet of mini EDTA-free protease inhibitors (Roche) per 10 ml (Zoncu et al., 2011). Cell lysates were immunoprecipitated with anti-GFP (4 μg/ml, Invitrogen #A11210) or anti-Flag (4 μg/ml, Sigma F3165) at 4 °C for 2 hr. Immunoblots were analyzed using anti-V-ATPase D1 (1 μg/ml Santa Cruz, sc-166218). HRP-conjugated sheep anti mouse secondary antibody (GE Healthcare, NA931V) was used in ECL detection. To probe the interaction between TPC2 and RagB or lamtor 1/2/3, transfected HEK293T cells were lysed in ice-cold buffer containing 40 mM HEPES (pH 7.4), 10 mM pyrophosphate, 10 mM glycerophosphate, 1% Triton X-100 and one tablet of complete protease inhibitors (Roche) per 25 ml, as described before (Sancak et al., 2010). Cell lysates were immunoprecipitated at 4 °C for 2 hr with anti-GFP (4 μg/ml, Invitrogen #A11210), anti-Flag (4 μg/ml, Sigma F3165) or anti-HA antibody (4 μg/ml, Santa Cruz sc-805). Immunoblots were analyzed using anti-Flag (5 μg/ml), -GFP (1 μg/ml, Santa Cruz, sc-9996) or -HA (1 μg/ml) antibody. HRP-conjugated secondary antibody from the Clean-Blot kit (Pierce) was used in ECL detection to minimize contaminating signals from the primary antibodies used in immunoprecipitations.
Immunohistochemistry

Mouse embryonic fibroblast (MEF) cells were cultured from WT and dKO embryos as described before (Xu, 2005), and were plated on glass coverslips (12 mm diameter) one day before the assay. Cells were incubated in starvation buffer (in mM, 110 NaCl, 45 NaHCO₃, 5 KCl, 2 CaCl₂, 1 MgCl₂, pH 7.4) for 50 min, and were divided into two groups. One was directly used for immunostaining and the other was stimulated with 10X amino acid in culture medium for 10 min before immunostaining. Cells were washed once with PBS, fixed in 4% PFA at room temperature for 15 min, and washed twice with PBS. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 15 min, and blocked for 1 hr with 5% dry milk in PBST containing 0.1% Tween-20 in PBS. The coverslips were incubated with rabbit mTOR antibody (1:200, Cell Signaling) in 3% dry milk/PBST at 4°C overnight. After 3 times wash (10 min each) with PBST, the coverslips were incubated with TRITC-conjugated anti rabbit secondary antibody (1:25, Pierce, #31670) for 1 hr at room temperature in the dark and washed x3 with PBST. Washed coverslips were mounted on slides with Fluoromount (Sigma, F4680) and imaged with an Olympus IX71 microscope equipped with a VT-Hawk confocal unit (VisiTech International Ltd, United Kingdom) using a 60X water immersion objective.

Lysosomal Amino Acid Efflux Measurement

Lysosome Preparation

Lysosomes were prepared from mouse liver using differential centrifugation following previously described protocols (Reeves, 1979). Briefly, liver from each adult mouse (12-13 week old male) was homogenized with a Dounce homogenizer in 8 ml homogenization buffer (HB) containing 250 mM sucrose, 1 mM Na₂EDTA, 10 mM HEPES (pH 7.0). The homogenate was centrifuged at 1,000 x g for 10 min at 4°C. Supernatant was kept on ice; the pellet was homogenized again in 7 ml HB and spun again to obtain the supernatant. The two supernatants were combined and centrifuged at 20,000 x g for 20 min at 4°C. The pellet was resuspended in buffer containing 250 mM KCl and 1 mM MOPS (pH 7.4). After spinning at 1,600 x g for 10 min, the supernatant was diluted by 2.5x volume with buffer containing 450 mM sucrose, 0.5 mM Na₂EDTA and 5 mM HEPES (pH 7.2), and centrifuged again at 25,000 x g for 10 min. The pellet containing crude lysosomes was resuspended in 200 μl uptake buffer (250 mM sucrose, 5 mM MgCl₂, 20 mM HEPES, pH 7.0) and centrifuged at 600 x g for 2 min. The supernatant was kept on ice; the pellet was resuspended in (100 μl) uptake buffer by passing through a 30G needle for 30 times, and then combined with the supernatant. An aliquot of the crude lysosome was used for total protein determination with protein assay and the rest was kept on ice and used for amino acid efflux assays within 12 hr.

Preparation of Amino Acid Methyl Esters

14C-labeled lysine- and arginine- methyl esters were prepared from 14C-labeled lysine and arginine following previously described methods (Reeves, 1979; Steinherz et al., 1982). Briefly, 12.5 μCi 14C-labeled amino acid (from Perkin Elmer) was transferred into a 25-ml glass conical flask, dried under stream of nitrogen, dissolved in 3 N methanolic HCl (Sigma) and incubated for 24 hr at room temperature. The resulting amino acid-methyl ester was dried under stream of nitrogen, washed with methanol and dried again before being dissolved in 500 μl methanol, followed by volume reduction to 250 μl through evaporation. Shortly before efflux assay, appropriate amount was transferred into a 1.5 ml tube, dried under stream of nitrogen, and dissolved in uptake buffer (final concentration 0.005 μCi/μl).

Amino Acid Efflux Assay

The crude lysosomes were dispensed in 50 μl aliquots, each vial containing 100 μg of protein-equivalent amount of lysosomes. To initiate amino acid ester loading, 50 μl of amino acid methyl ester (0.25 μCi) in uptake buffer was added to each lysosome-containing vial and incubated at 37°C for 30 min (experimentally determined to be optimal for loading). At the end of loading, each vial was diluted into 5 ml pre-warmed efflux buffer (50 x dilution) containing 250 mM sucrose, 5 mM MgCl₂, 20 mM HEPES (pH 7.0), 0.1 μM Pi(3,5)P₂, and, in some assays, ATP (0.1 or 2 mM as indicated in the figures), mixed well and incubated at 37°C. At each given time point, a 5 ml efflux assay reaction was combined into 5 ml ice-cold PBS, filtered immediately through a Whatman filter (GF/F) under vacuum, and washed twice with 5 ml ice-cold PBS. For background count, lysosomes and amino acid methyl ester incubated separately were mixed and filtered immediately (without loading). The filter was soaked in scintillation cocktail overnight and counted using a Beckman LS6500 scintillation counter. Background-subtracted isotope counts were normalized to that obtained at efflux time point 0.

Behavior Tests

Male mice (9-11 weeks) were used for the tests. For fasting, animals had access to water but not food. Rotarod tests were performed using a 4-lane rotarod (Rotamex-5, Columbus Instrument). One day before the start of test sessions, each mouse was placed onto the rod for acclimation until it could remain on the rotarod for 30 s at 4 rpm. For test sessions, the rod rotation speed started at 4 rpm and increased 1 rpm every 8 s until the animal fell off. Test data from 3 daily sessions (1 hr apart) were averaged for each animal. Treadmill tests were performed using a 6-lane treadmill (Exer-3/6, Columbus Instrument) set at a 10 degree incline. Before the test sessions, each mouse was allowed to acclimate to the treadmill by running for 5 min at 10 m/min each day for 3 days. For test sessions, the belt speed was first increased to 10 m/min over 1 min. The animals were then stimulated (electrical shock of 0.47 mA at 1 Hz delivered in the resting grid in the rear end of the treadmill) to run at 10 m/min for 5 min. The speed was then increased by 1 m/min over each min. Electrical stimuli were turned off immediately after the animal was exhausted, defined by a lack of an active attempt to reengage the moving belt after 10 s of stimulation.
SUPPLEMENTAL REFERENCES


Figure S1. Electrophysiological Characterization of lysoNaATP, Related to Figure 2

Representative recordings are in left and averaged sizes (at –100mV) are in right in each panel. All the recordings had 1 μM PI(3, 5)P2 in the bath unless otherwise stated. Recordings were obtained from HEK293T cells transfected with GFP-tagged constructs except panel C (TPC2 without tag used) and panel F (from macrophage).

(A and B) TRPMLs do not form ATP-sensitive channels. Currents were recorded from TRPML3- (A) or TPRML1- (B) transfected endolysosomes in the presence of 1 μM (A) or 0.1 μM PI(3,5)P2 (B).

(C) LysoNaATP recorded from HEK293T cells transfected with a full-length TPC2 without any tag.

(D) Mg2+ does not inhibit TPC current. Currents were recorded with standard bath (containing 2 mM Mg2+, free [Mg2+] = 1.94 mM) or bath with additional 1 mM Mg2+ (total of 3 mM, free [Mg2+] = 2.91 mM).

(E) Basal TPC2 current is also inhibited by ATP in the absence of added PI(3,5)P2. Only currents > 50 pA (at –100mV without ATP) were included for the average analysis (349 ± 137 pA at –100mV, n = 5).

(F and G) NAADP does not activate TPC channels, as shown by the lack of effect of NAADP on the inhibition of lysoNaATP from mouse peritoneal macrophages (F) or TPC2- transfected HEK293T cells (G). (H) ATP-S does not inhibit lysoNaATP. Currents were recorded from TPC2-transfected HEK293T cells.

(I) The inhibition of lysoNaATP by ATP is slow. Left, current (at –100mV) was continuously recorded from a TPC2-transfected HEK293T cell during solution changes. Time 0 indicates break-in. Right, current amplitudes normalized to that before application of ATP indicating the time courses of ATP inhibition (n = 3 or 4).

Error bars represent SEM.
Figure S2. mTOR, but Not AMPK, Is Required for the ATP Sensitivity of lysoNa$_{ATP}$, Related to Figure 3

(A–C) Currents were recorded from macrophage endolysosomes in the presence of the AMPK inhibitor Dorsomorphin (A), mTOR inhibitor Torin 1 (C), or from TPC2- transfected AMPK1/2 double knockout embryonic fibroblasts (B). Error bars represent SEM.
Figure S3. Specificity of the Association between TPCs and mTOR, Related to Figure 4

(A–D) TPC2 does not associate with RagB, V-ATPase or lamptors. Cells were transfected with combination of plasmids as indicated in the table above each panel. Total proteins were immunoprecipitated (IP) with antibodies indicated below each lane and blotted with antibodies indicated on the left of each panel. (A–D) show that TPC2 does not associate with RagB (A, lamtor 1 used as a positive control), V-ATPase (B, endogenous V-ATPase detected with anti-V1 Subunit D; RagB as a positive control), lamtor 1 (C, RagB as positive control), or lamtor 2 (D, RagB as positive control).

(E) TRPML1–3 do not associate with mTOR. Immunoprecipitates with anti-GFP from cells transfected with plasmids as indicated were blotted with anti-mTOR (upper) or anti-GFP (lower).
Figure S4. LysoNaATPs Are Not Required for mTOR Activity, Related to Figure 6

(A) Total cell lysates from cultured hepatocytes treated with or without insulin (100 nM, 1 hr), amino acid (1x, 1 hr) and rapamycin (50 nM, 1 hr) as indicated were blotted with anti-pP70S6K (upper) or β-actin (lower).

(B) Immunostaining using anti-mTOR antibody with WT (upper) and TPC1/2 dKO (lower) MEF cells. Cells in the left panels were starved in amino acid-depleted medium for 50 min. Cells in the right panels were re-fed with amino acids for 10 min after starvation. Scale bars, 5 μm.
Figure S5. Phenotype Analysis of TPC1/2 Double-Knockout Mutant Mice, Related to Figure 7

(A–C) Autophagy analysis. (A) Total liver proteins from fed and fasted WT and TPC1/2 dKO mice were blotted with anti-LC3 (upper panel) and anti-GAPDH (lower panel, loading control). (B) Similar to (A), but from isolated hearts with or without oxygen-glucose deprivation (OGD) perfusion. Two pairs of WT and dKO are shown. OGD perfusion was done with Langendorff perfusion for 45 min (Matsui et al., 2007). (C) The mCherry- and GFP-tagged LC3 (tandem tag, illustrated; Kimura et al., 2007; Pankiv et al., 2007) was transfected into WT (upper panels) and TPC1/2 dKO (lower panels) macrophages. Cells were starved for 1 hr and imaged for GFP (left panels) and RFP (middle). Right panels are the merged signals between GFP and RFP. GFP- and RFP- positive puncta indicate autophagosomes before fusing with lysosomes. RFP- positive but GFP-negative puncta (see right panels) indicate fusion between autophagosomes and lysosomes. Scale bars, 5 μm.

(D) Lysosomal amino acid efflux assay with 14C-labeled lysine and liver lysosomes. *p < 0.05; **p < 0.01.

(E) Rotarod tests performed before (days 1–3) and during (days 7 and 8) fasting. The time at which each animal fell off the rod was recorded and averaged. Ten WT and 11 dKO mice were tested. Error bars represent SEM.