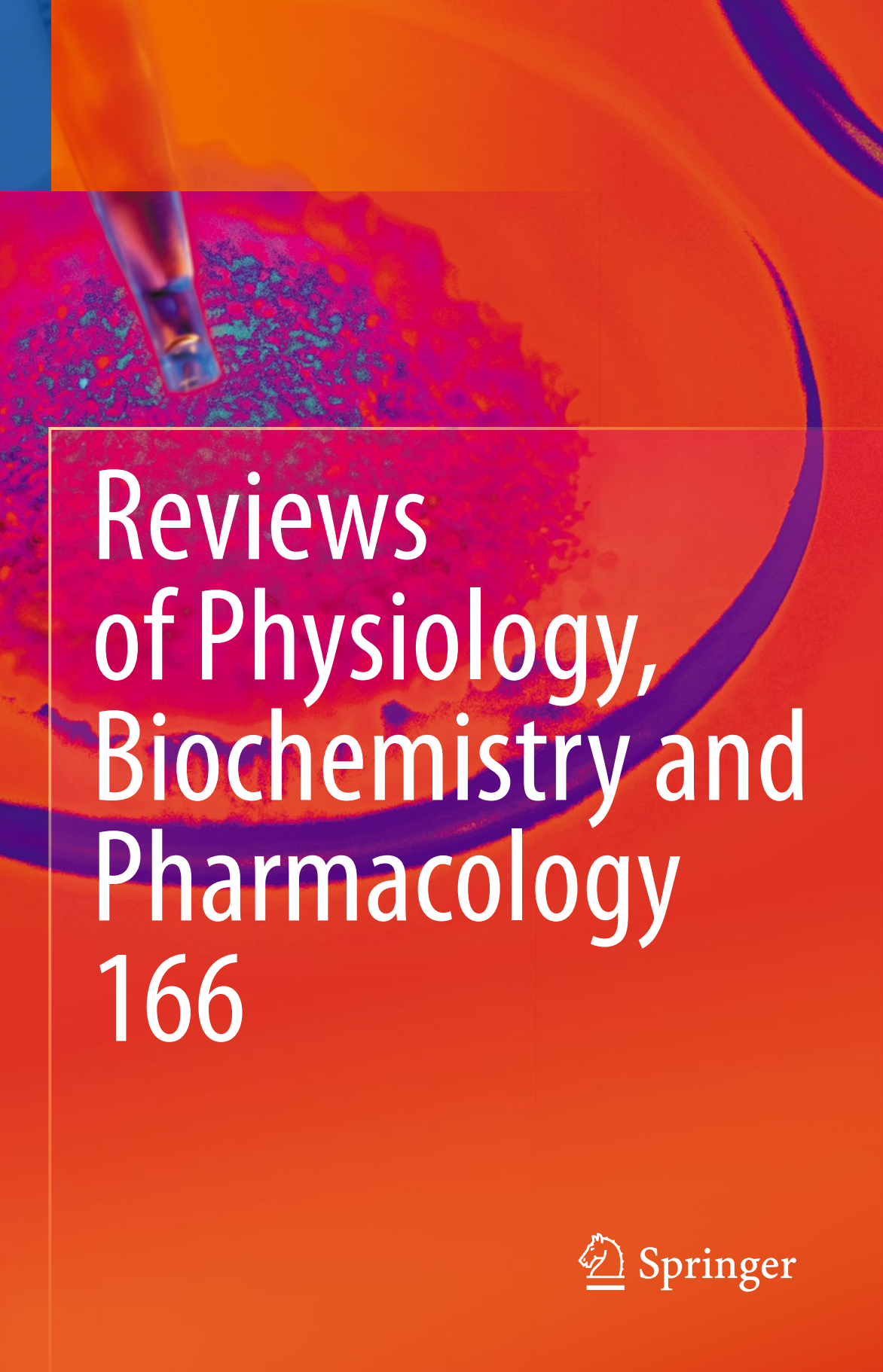
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Contents

TRP Channels Coordinate Ion Signalling in Astroglia

Alexei Verkhratsky, Reno C. Reyes, and Vladimir Parpura

Abstract Astroglial excitability is based on highly spatio-temporally coordinated fluctuations of intracellular ion concentrations, among which changes in Ca^{2+} and Na^{+} take the leading role. Intracellular signals mediated by Ca^{2+} and Na^{+} target numerous molecular cascades that control gene expression, energy production and numerous homeostatic functions of astrocytes. Initiation of Ca^{2+} and Na^{+} signals relies upon plasmalemmal and intracellular channels that allow fluxes of respective ions down their concentration gradients. Astrocytes express several types of TRP channels of which TRPA1 channels are linked to regulation of functional expression of GABA transporters, whereas TRPV4 channels are activated following osmotic challenges and are up-regulated in ischaemic conditions. Astrocytes also ubiquitously

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express several isoforms of TRPC channels of which heteromers assembled from TRPC1, 4 and/or 5 subunits that likely act as stretch-activated channels and are linked to store-operated Ca^{2+} entry. The TRPC channels mediate large Na^{+} fluxes that are associated with the endoplasmic reticulum Ca^{2+} signalling machinery and hence coordinate Na^{+} and Ca^{2+} signalling in astroglia.

Keywords Astrocyte · Ca^{2+} signalling · Na^{+} signalling · Metabotropic receptors, endoplasmic reticulum · TRPC channels · TRPCA1 · TRPV4 · Store-operated Ca^{2+} entry · Stretch-activated channels · Mechanosensitivity · Volume regulation · Plasticity · Brain homeostasis

Contents

1 Astrocytes: The Homeostatic Cells of the CNS

Evolution of the central nervous system (CNS) progressed through specialisation of the cellular elements composing neural networks. Functional dissociation between executive cellular branch represented by electrically excitable neurones and housekeeping branch represented by electrically non-excitable glial cells developed immediately after appearance of centralised masses of neural cells and attained highest degree of complexity in the mammalian CNS (Kettenmann and Ransom 2013; Verkhratsky and Butt 2013). Astroglia refers to a highly heterogeneous cell population present in the grey matter and in the white matter of the brain and of the spinal cord that are responsible for a remarkable array of homeostatic functions that control the CNS environment and provide for seemingly slick operation of neural cells (Nedergaard and Verkhratsky 2012; Parpura and Verkhratsky 2012). Astrocytes, in particular, are fundamentally important for rapid regulation of extracellular ions (Kofuji and Newman 2004; Olsen and Sontheimer 2008) and neurotransmitters (Conti et al. 2004; Danbolt 2001), that, to a large extent, shape neuronal excitability and synaptic transmission. Astrocytes are critical for providing glutamatergic and GABA-ergic neurones with glutamine, which is indispensable for

maintaining releasable pool of these transmitters (Hertz et al. 1999); similarly, astrocytes are mainly responsible for adenosine turnover (Boison et al. 2010). Astrocytes, unlike neurones, can synthesise glutamate de novo owing to the entry of pyruvate to the citric acid cycle via astrocyte-specific mitochondrial enzyme pyruvate carboxylase (Hertz and Zielke 2004). Astroglial cells are also capable of releasing various neurotransmitters and neuromodulators that provide for regulation of synaptic connectivity and plasticity (Henneberger et al. 2010; Parpura and Zorec 2010). Astrocytes support neuronal energetics with lactate (Magistretti 2011) and hold at bay extracellular accumulation of reactive oxygen species using nonenzymatic antioxidant defences, such as ascorbate and glutathione (Fernandez-Fernandez et al. 2012; Swanson et al. 2004). Astroglial cells also contribute to regulation of brain microcirculation by linking neuronal activity with functional hyperaemia (Carmignoto and Gomez-Gonzalo 2010; Iadecola and Nedergaard 2007). Finally astroglial cells are fundamental elements of brain defence through evolutionary conserved multistage programmes of reactive astrogliosis (Sofroniew 2009). To maintain all these functions, astroglial cells are in need of real-time monitoring of their immediate environment, including neuronal activity, with rapid activation of multiple intracellular signalling cascades regulating varieties of molecules responsible for homeostatic response.

2 Ion Signalling Defines Astroglial Excitability

Astrocytes are electrically non-excitable cells incapable of producing plasmalemmal regenerative responses based on coordinated activity of voltage-gated ion channels, that is, action potentials that underlie signalling in neuronal networks. There are however numerous types of voltage-gated channels expressed in astroglia (Verkhratsky and Steinhauser 2000), although densities of Na^+ and Ca^{2+} permeable channels (otherwise necessary proviso for generation of action potentials) are low and membrane depolarisation is prevented by large K^+ permeability and shunting through gap junctions. Nonetheless astrocytes are mounting active responses to external stimulation (with chemical and mechanical stimulation being physiologically relevant) by producing changes in intracellular ion concentration coordinated in spatio-temporal domains.

Intracellular Ca^{2+} signals were the first kind of ionic signalling recognised to be universally present in astroglia. Early experiments have found that stimulation of cultured astrocytes with neurotransmitters (such as glutamate) or with mechanical displacement of membrane produced transient changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) that were able to propagate through a glial monolayer in the form of Ca^{2+} waves (Charles et al. 1991; Cornell Bell et al. 1990; Finkbeiner 1993). Subsequently, experiments in vitro demonstrated that astrocytes are capable of expressing numerous receptors linked to Ca^{2+} signalling (Verkhratsky and Kettenmann 1996). These receptors, triggering Ca^{2+} signals, were further characterised in situ revealing

remarkable region-dependent variability, with receptors' patterns matching immediate neurochemical environment, that is, nearby neurotransmission (Verkhratsky et al. 1998). In recent years, astroglial Ca^{2+} signals and astroglial Ca^{2+} waves were also identified in vivo and linked to various forms of sensory stimulation (Kuga et al. 2011; Wang et al. 2006). Importantly, astroglial Ca^{2+} signals can induce neuronal responses (Nedergaard 1994; Parpura et al. 1994), although the detailed physiological consequences of such interactions remained to be clarified that warrant further investigations (Gourine et al. 2010; Poskanzer and Yuste 2011).

The second kind of intracellular ion signalling in astroglia is associated with transient changes in cytosolic concentration of Na^+ ($[Na^+]_i$). It appears that physiological (i.e. chemical and mechanical) stimulation triggers rapid and substantial transient increases in $[Na^+]_i$ in astrocytes in culture (Reyes et al. 2012; Rose and Ransom 1996) and in situ (Kirischuk et al. 1997; Langer and Rose 2009). These $[Na^+]_i$ transients also follow synaptic stimulation (Kirischuk et al. 2007; Langer and Rose 2009), and Na^+ can propagate through astroglial syncytia in the form of Na^+ waves ((Langer et al. 2012; Rose and Ransom 1997), for detailed description of glial Na^+ signalling, see (Kirischuk et al. 2012; Rose and Karus 2013) and references therein). Importantly, these $[Na^+]_i$ fluctuations are involved in regulation of multiple astroglial homeostatic cascades (Kirischuk et al. 2012). The sources of Na^+ signalling in astroglia are associated with Na^+ influx through ion channels and Na^+ transport through multiple Na^+ secondary transporters. Of these the Na^+-dependent glutamate and GABA transporters are of particular importance, because they are activated in the course of synaptic transmission (Kirischuk et al. 2007; Unichenko et al. 2012). The above-mentioned two forms of ion (Na^+ and Ca^{2+}) signalling are interlinked through, for example, the plasmalemmal sodium-calcium exchangers (NCXs) and transient receptor potential (TRP) channels, the role of which will be discussed in detail below.

The functional consequences of Ca^{2+} and Na^+ signalling in astrocytes are many and they are mediated through multiple molecular cascades sensitive to cytosolic ion concentrations (see Table 1 for selected targets). Ions regulate molecular function either through selective binding (which is common for Ca^{2+} sensors) or through changes in electrochemical driving force across cellular membranes (which is more common for Na^+ targets). Another important determinant for ion signalling is focalisation, that is, microdomains of high ion concentrations that are spatially confined to the functionally relevant areas. Spatial restriction of Ca^{2+} signals are mainly achieved through cytosolic Ca^{2+} buffers that limit Ca^{2+} diffusion; mechanisms of localisation of Na^+ signals remain uncharacterised. Of note, however, the sites of Na^+ entry are often co-localised with Na^+ pumps and transporters; these latter can act as dynamic Na^+ buffers and contribute to focalisation of $[Na^+]_i$ fluctuations.

Table 1 Selected functional and molecular targets of Ca^{2+} and Na^{+} signals in astroglia

Functional responses	Molecular targets	References
Ca^{2+} signals		
Gene expression	Transcriptional factors/regulators (e.g. CREB/DREAM)	(Cebolla et al. 2008; Zhao and Brinton 2004)
Exocytosis	Synaptotagmins (functionally uncomfirmed)	(Mittelsteadt et al. 2009; Zhang et al. 2004)
	Calcineurin/calmodulin-mediated modulation of secretory machinery	(Reyes et al. 2011)
Mitochondrial ATP production	Ca^{2+}-sensitive mitochondrial dehydrogenases; pyruvate dehydrogenase phosphatase; F_1–F_0 ATP synthase	(Tarasov et al. 2012)
Ca^{2+} transport	Plasmalemmal Ca^{2+} ATPase (PMCA); sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA)	(Burdakov et al. 2005; Reyes et al. 2012)
Na^{+} signals		
K^{+} buffering	Inward rectifying K^{+} channel ($K_{ir}4.1$)	(Kucheryavykh et al. 2012)
	Na^{+}/K^{+} ATPase	(Walz and Hertz 1984)
	$Na^{+}/K^{+}/Cl^{-}$ co-transporter (NKCC1/SLC12A2)	(MacVicar et al. 2002)
Glutamate-glutamine shuttle:		
Glutamate uptake	Excitatory amino acid transporters 1, 2 (EAAT1/SLCA2, EAAT2/SLCA3)	(Anderson and Swanson 2000)
Glutamine transport	Na^{+}/H^{+}-dependent sodium-coupled neutral amino acid transporters (SN1/SNAT3/SLC38A3 and SN2/SNAT5/SLC38A5)	(Broer and Brookes 2001; Hertz 1979; Uwechue et al. 2012)
Glutamine-GABA shuttle: GABA uptake	GABA transporter (GAT3/SLC6A11)	(Unichenko et al. 2012)
Glycine uptake	Glycine transporter 1 (GlyT1/SLC6A9)	(Gomeza et al. 2003)
Plasmalemmal Na^{+}/Ca^{2+} exchange	Sodium calcium exchangers (NCX1/SLC8A1, NCX2/SLC8A2 and NCX3/SLC8A3)	(Kirischuk et al. 2007, 2012; Reyes et al. 2012)
Mitochondrial Na^{+}/Ca^{2+} exchange	Mitochondrial sodium calcium exchanger (NCLX/SLC8B1)	(Parnis et al. 2013; Reyes and Parpura 2008)
pH homeostasis: H^{+} transport	Na^{+}/H^{+} exchanger (NHE1/SLC9A1)	(Kintner et al. 2005)
pH homeostasis: HCO_3^{-} transport	Sodium bicarbonate co-transporter (NBC/SLC4A5)	(Deitmer and Rose 2010; Lascola and Kraig 1997)
Lactate shuttle	Na^{+}/K^{+} ATPase	(Magistretti 2011; Pellerin and Magistretti 1996, 2012)

3 Astroglial Na^+/Ca^{2+} Channels

Astrocytes, in physiological conditions, express several sets of cationic channels permeable to both Na^+ and Ca^{2+}. There is no firm evidence for expression of highly selective Ca^{2+} channels in astroglial cells in situ. There are indications for expression of several types of voltage-dependent Ca^{2+} channels in astrocytes in culture, which, however have not been confirmed for mature astrocytes neither in brain slices nor in vivo (reviewed in Parpura et al. (2011)). Voltage-gated Ca^{2+} channels can be confined to immature astroglial precursors, to NG-2 cells (with which astrocytes can be often mistaken) and to reactive astroglia (Parpura et al. 2011; Verkhratsky et al. 2012). Similarly, highly selective Ca^{2+}-release activated Ca^{2+} channels (of I_{CRAC} variety) have not been hitherto recorded from mature astroglial cells in brain tissue, while Orai channels and their respective currents have been recently recorded in primary cultured astrocytes and astroglial cell lines (Moreno et al. 2012; Motiani et al. 2013). Likewise, evidence for expression of voltage-gated Na^+ channels in cultured astroglia (Black et al. 2010) have not been corroborated by direct electrophysiological recordings in situ. It appears that the majority of ion channels expressed in astroglial membrane is permeable to both Na^+ and Ca^{2+} supporting the idea of interwoven intracellular Na^+ and Ca^{2+} excitability of astroglia.

Astrocytes express several types of cationic ionotropic receptors, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and N-methyl-D-aspartate (NMDA) receptors and purinergic P2X receptors (Lalo et al. 2006, 2008; Steinhauser and Gallo 1996). These receptors have (in contrast to neurones) an intermediate to small Ca^{2+} permeability (Pankratov et al. 2009). In some types of astroglia, the AMPA receptors lack the GluA2 subunit which underlies their Ca^{2+} permeability ($P_{Ca}/P_{monovalent} \sim 1$ (Burnashev et al. 1992; Muller et al. 1992)). This, however, corresponds to ~4 % of fractional Ca^{2+} current, which together with rapid physiological AMPA receptor desensitisation very much limits Ca^{2+} entry. In Bergmann glial cells, Ca^{2+} permeable AMPA receptors have minimal, if any, contribution to Ca^{2+} signals (Kirischuk et al. 1999). Similarly, astroglial NMDA and $P2X_{1/5}$ receptors have relatively low Ca^{2+} permeability ($P_{Ca}/P_{monovalent} \sim 3$ and $P_{Ca}/P_{monovalent} \sim 2$, respectively (Palygin et al. 2010)). There is fragmentary evidence (Sharma and Vijayaraghavan 2001) for astroglial expression of α7 Ca^{2+} permeable nicotinic cholinoreceptors (α7nAChRs), although another study in hippocampal slices produced somewhat inconclusive results (Shen and Yakel 2012), and specific parameters and functional role of astroglial α7nAChRs similarly remain unknown. Another important pathway for membrane Na^+ entry in astroglia is represented by TRP channels.

4 TRP Channels as Multi-ion Carriers

The TRP channel family (for the somewhat controversial history of its discovery, see (Hardie 2011; Minke 2010; Montell 2011)) is widely present in many cell types of all multicellular organisms, from *Caenorhabditis elegans* to mammals, although the phylogenetic roots of this channel are found in yeasts (the TRPY channel family (Venkatachalam and Montell 2007)). There are 28 members of the superfamily in vertebrates, of which 27 are present in humans (Nilius et al. 2012; Owsianik et al. 2006; Pedersen et al. 2005) and classified into 6 subfamilies. The TRP channels are fundamental for all types of sensing including, thermal sensation, nociception, chemoception, equilibrioception and interoception (Nilius and Appendino 2013; Nilius and Owsianik 2011; Vennekens et al. 2012). The TRP channels are cationic channels permeable to multiple cations with great heterogeneity of permeation properties (Owsianik et al. 2006). They are found in the CNS, being expressed in cells from all regions of the brain and the spinal cord with particularly high expression of TRPV, TRPC and TRPM channels, and more restricted expression of TRPA1, TRPP1 and TRP-ML proteins (for many details and exhaustive reference list, see (Nilius 2012; Vennekens et al. 2012)).

5 TRP Channels in Astroglia

5.1 TRPA1 Channels

TRPA1 (where 'A' stands for ankyrin) is the only member of this subfamily identified in mammals (Nilius et al. 2011) with high single channel conductance (~110 pS) and relatively high Ca^{2+} permeability ($P_{Ca}/P_{monovalent}$ ~ 5.9). This Ca^{2+} permeability can be increased even further upon channel activation that is accompanied with pore dilation. In dilated state the $P_{Ca}/P_{monovalent}$ is ~7.9, corresponding to fractional Ca^{2+} current of ~23 % (Nilius et al. 2011). These TRPA1 channels can be activated by noxious cold (below 17 °C), by pungent substances derived from plants, by growth factors (via G-protein-coupled receptors) and by pro-inflammatory factors (Nilius et al. 2012).

Functional expression of TRPA1 channels was suggested for hippocampal astrocytes, although neither specific mRNA nor TRPA protein was detected in these cells (Shigetomi et al. 2012). Nonetheless a complex of Ca^{2+} imaging (with a genetically encoded Ca^{2+} probe Lck-GCaMP that monitors near-membrane $[Ca^{2+}]$), electrophysiology, silencing RNA and pharmacology provided reasonably convincing evidence for operation of these channels in sub-population of astroglia (Shigetomi et al. 2012). The fundamental observation was a detection (in cultured astrocytes) of near-membrane local spontaneous $[Ca^{2+}]_i$ transients (called by the authors 'spotty' Ca^{2+} signals) that were inhibited by Gd^{3+} and La^{3+} as well as by broad spectrum TRP channel antagonist HC 030031. Similarly these 'spotty' Ca^{2+}

signals were blocked by anti-TRP silencing RNA, whereas the TRPA1 agonist allyl isothiocyanate (AITC) increased frequency of these events; AITC also activated currents in voltage-clamped astrocytes. Further studies have found evidence for functional activity of TRPA1 channels in astroglial cells in situ in hippocampal slices. Activity of TRPA1 channels apparently contributed to setting the resting $[Ca^{2+}]_i$ in astrocytes (both in cultures and in situ) and inhibition of these channels resulted in a significant (from ~120 to ~50 nM) decrease in basal $[Ca^{2+}]_i$. This decrease in resting $[Ca^{2+}]_i$ in turn reduced functional expression of astroglial GABA plasmalemmal GAT-3 transporters, which, as authors suggested, resulted in an elevated extracellular concentration of GABA, desensitization of $GABA_A$ receptors in neighbouring hippocampal neurones and hence a decrease in the inhibitory synaptic transmission (Shigetomi et al. 2012).

5.2 TRPC Channels

Mammalian TRPC ('C' denotes canonical) channels are represented by seven members (TRPC1–7) which are all cationic channels with $P_{Ca}/P_{monovalent}$ varying between 1 and 9 (Owsianik et al. 2006). These channels can be activated by phospholipase C, by diacylglycerol (DAG) and by mechanical stimulation, and are responsible for store-operated Ca^{2+} entry in some types of cells. The TRPC channels can form both homo- and heteromeric channels, which underlie substantial heterogeneity in their biophysical properties (Nilius et al. 2007).

Embryonic cultured astrocytes (also often referred to as astrocytes type I) express mRNA for TRPC1 to TRPC6 (Grimaldi et al. 2003; Pizzo et al. 2001) and were reported to produce Ca^{2+} fluxes and $[Ca^{2+}]_i$ oscillations in response to oleyl-acetyl-glycerol (an analogue of DAG) and following stimulation of glutamate receptors and endoplasmic reticulum (ER) store depletion. In spinal astrocytes, the mRNAs for TRPC1, 2, 3, 4 and 6 were detected (Miyano et al. 2010). At the protein level relatively high expression of TRPC1 channel was detected in the embryonic astroglial cultures. It appeared that TRPC1 channels were located in the portions of plasmalemma closely associated with the ER (i.e. at plasmalemma-ER junctions) and, moreover, TRPC1 proteins were co-immunoprecipitated with inostiol 1,4,5 trisphospate ($InsP_3$) receptors and ER Ca^{2+}-ATPases of SERCA 2b subtype suggesting intimate functional relations between ER receptors, Ca^{2+} transporters and plasmalemmal TRPC1-containig channels (Golovina 2005). Likewise, co-immunoprecipitation of TRPC1 channels, $InsP_3$ receptors type II and Homer proteins was found in cortical astrocytes cultured from 3- to 5-day-old rats (Weerth et al. 2007). Similar co-localisation of TRPC4 channels with ZO-1 scaffolding proteins was detected in cultured foetal human astrocytes (Song et al. 2005). Besides TRPC1 expression, TRPC4, TRPC5 and TRPC6 proteins were also detected in cultured and freshly isolated embryonic astrocytes (Beskina et al. 2007).

In primary astrocytes cultured from visual cortices of newborn rats or freshly isolated from the same region of 1-, 8- and 55-day-old rats, expression of TRPC1,

TRPC4 and TRPC5 channels was detected in Western blots and their cellular localisation was mapped with immune labelling showing that TRPC1 channels were predominantly localised to the plasma membrane (Malarkey et al. 2008). The percentage of astrocytes expressing TRPC isoforms increased with age. At 1 day of age, percentage of astrocytes expressing TRPCs was 47 %, 7 % and 70 % for TRPC1, TRPC4 and TRPC5 proteins, respectively, whereas at 55 days of age all astrocytes expressed all three isoforms (Malarkey et al. 2008). Indeed, several TRPC isoforms are expressed in the brain, where the predominant types are represented by TRPC1, 4 and 5 subunits that are generally believed to form heteromers, in which TRPC1 acts as an obligatory, channel forming subunit and TRPC4/5 function as ancillary ones (Hofmann et al. 2002; Strubing et al. 2001).

Activation of TRPC1 channels in astroglia has been observed in various physiological and pathophysiological contexts. The TRPC1 channels contribute to $[Ca^{2+}]_i$ transients induced by stimulation of purinergic and glutamatergic metabotropic receptors (Malarkey et al. 2008), because treatment of astrocytes with anti-TRPC blocking antibody substantially reduced the plateau phase, as well as a component of the peak, of these Ca^{2+} responses (Fig. 1a). The TRPC1 channels are also instrumental for astroglial Ca^{2+} signalling following mechanical stimulation. The ability of TRPC1 to act as a stretch-activated polycationic channel (first identified as Na^+, K^+, Ca^{2+} and Mg^{2+} mechanosensitive cation channel MscCa) was initially demonstrated in frog oocytes, and the similarity between MscCa and TRPC1 was confirmed following heterologous expression studies (Maroto et al. 2005). Of note, the role of TRPC channels as mechanosensors remains controversial, while recent discoveries of Piezo1/2 channels open new avenues in understanding mechanisms of mechano-transduction (Nilius and Honore 2012). Be it all as it may, it was demonstrated that inhibition of TRPC1 channels substantially reduces $[Ca^{2+}]_i$ transients induced by mechanical stimulation of cultured astrocytes (Malarkey et al. 2008; Reyes et al. 2013) (Fig. 1b) as well as consequential Ca^{2+}-dependent glutamate release from these glial cells (Fig. 1c). The TRPC channels in cortical astrocytes are also activated by hypo-osmotic shock, and the resulting $[Ca^{2+}]_i$ elevation triggers translocation of aquaporin-1 water channels to the plasma membrane that increases water transport (Conner et al. 2012). The TRPC6 channels were claimed to contribute to Ca^{2+} entry following stimulation of interleukin-1β (IL-1β) receptors in embryonic astrocytes (Beskina et al. 2007).

5.3 *TRPC Channels as Molecular Substrate of Store-Operated Ca^{2+} Entry in Astroglia*

The store-operated (also known as 'capacitative') Ca^{2+} entry (SOCE) mechanism (Putney 1990, 2007) is expressed in virtually all types of non-excitable cells and in some excitable cells. This mechanism is operated by a dynamic molecular link between the ER and the PM. The molecular sensor that monitors the intra-ER

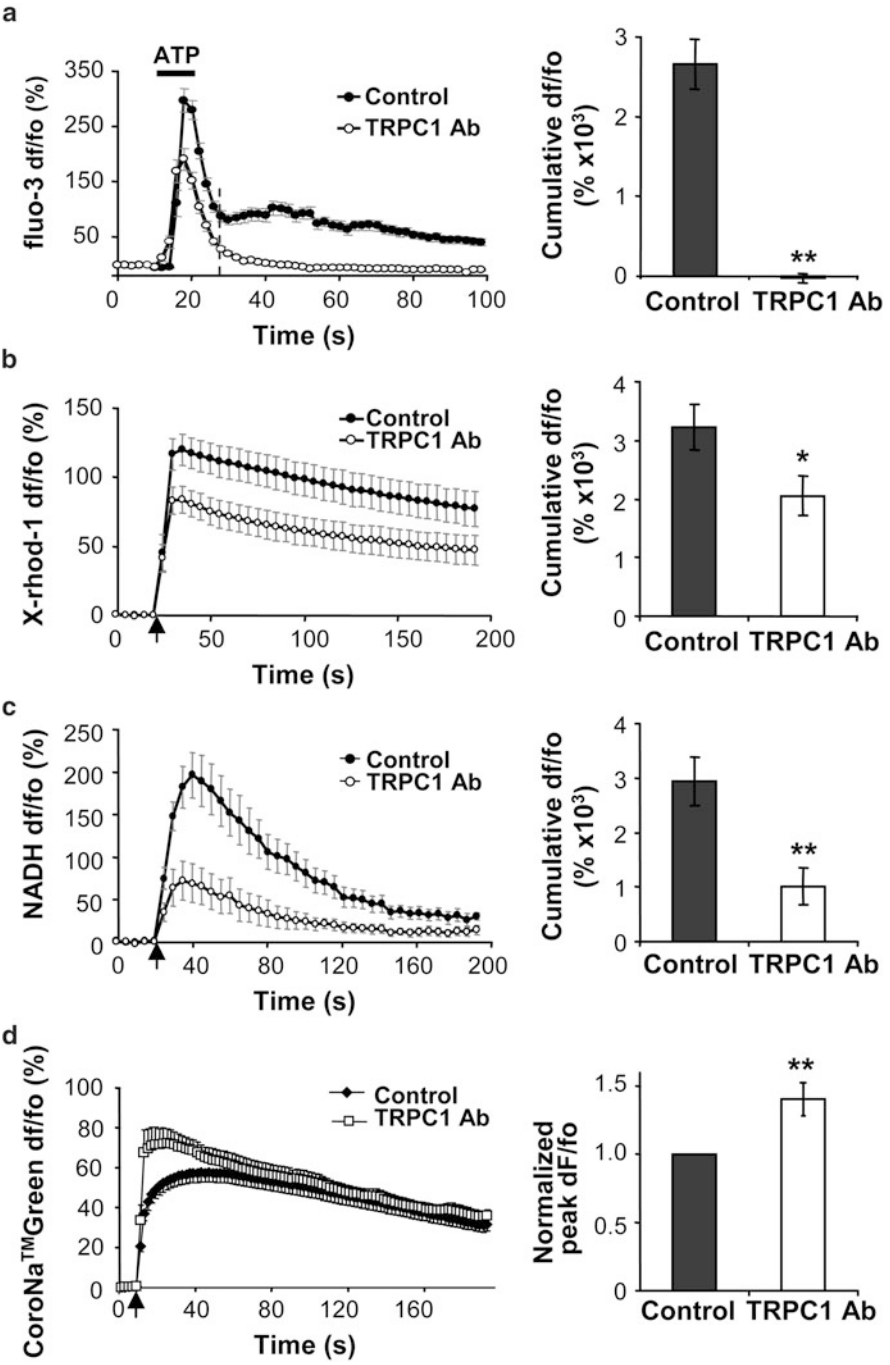

Fig. 1 The role of TRPC1 in intracellular Ca^{2+} and Na^{+} dynamics in cultured astroglia. (**a**) TRPC1 plays a role in receptor activation-elicited intracellular Ca^{2+} elevations in astrocytes. Application of ATP (100 μM) to astrocytes from rat visual cortex results in a biphasic intracellular Ca^{2+} response:

Ca^{2+} concentration is represented by the stromal interacting molecule proteins (STIM1 and STIM2). Upon ER Ca^{2+} depletion, STIM molecules oligomerise and drift towards the ER-PM junction where they interact with and activate plasmalemmal Ca^{2+} channels. These latter are (i) I_{CRAC} channels formed by Orai proteins and/or (ii) TRPC channels (for review of and references about molecular physiology of SOCE, see (Cahalan 2009; Carrasco and Meyer 2011; Feske et al. 2006; Owsianik et al. 2006; Parekh 2010; Parekh and Penner 1997; Soboloff et al. 2012; Zeng et al. 2008)). Channels formed by Orai and TRPC have distinct biophysical identity and their corresponding currents and functional responses can be easily distinguished.

This SOCE pathway is functioning in virtually all types of neuroglial cells (Hartmann and Verkhratsky 1998; Malarkey et al. 2008; Moller et al. 1997; Muller et al. 2013; Paez et al. 2009; Pivneva et al. 2008; Pizzo et al. 2001; Reyes and Parpura 2009; Toescu et al. 1998; Tuschick et al. 1997). To the best of our knowledge, characteristic I_{CRAC} channels have not been hitherto recorded from mature astrocytes and evidence about functional operation of Orai/STIM complex derives from neoplastic cell lines and astrocytes in vitro (Moreno et al. 2012; Motiani et al. 2013).

The role for TPRC1 channels in SOCE in astroglial cells is based on functional studies deploying immunological inhibition and down-regulation of TRPC1 channels' expression in combination with Ca^{2+} imaging. The antisense RNA knock-down of TRPC1 as well as inhibition of the channel with blocking antibody directed at an epitope in the pore forming region of the TRPC1 protein substantially reduced SOCE (activated either following metabotropic stimulation or following ER store depletion with SERCA blockers, namely, cyclopiazonic acid or thapsigargin) in cultured cortical astrocytes (Golovina 2005; Malarkey et al. 2008). As alluded to earlier, this TRPC1 inhibition underlies reduction of plateau phase of $[Ca^{2+}]_i$ transients induced by ATP in astrocytes in vitro (Golovina 2005; Malarkey et al. 2008). Similarly the SOCE-mediated plateau of Ca^{2+} responses

Fig. 1 (continued) the initial transient Ca^{2+} elevation and sustained (plateau) Ca^{2+} elevation. Intracellular Ca^{2+} measurements were obtained using the Ca^{2+} indicator fluo-3. If TRPC1 containing channels are blocked by incubating cells with an antibody against TRPC1, the sustained (plateau) Ca^{2+} elevation, reporting on SOCE, is abolished. *Vertical dashed line* indicates the initial point of a sustained plateau Ca^{2+} response, of which cumulative is shown in bar graph. (**b**, **c**) TRPC1 plays a role in mechanically elicited intracellular Ca^{2+} responses in astrocytes and resulting Ca^{2+}-dependent glutamate release from these glial cells. (**b**) Mechanical stimulation causes cytoplasmic Ca^{2+} elevations in astrocytes, as recorded using the Ca^{2+} indicator X-rhod-1. (**c**) Glutamate release from astrocytes, reported by an increase in extracellular NADH fluorescence, can be induced by mechanical stimulation. Both responses (Ca^{2+} and glutamate) are reduced when astrocytes were incubated with TRPC1 antibody. (**d**) TRPC1 plays a role in mechanically elicited intracellular Na^+ responses in astrocytes. Mechanical stimulation causes cytoplasmic Na^+ elevations in astrocytes, as recorded using the Na^+ indicator CoroNa^{TM}Green. The peak Na^+ responses are enhanced when astrocytes were incubated with TRPC1 antibody. *Point* and *bars* indicate means ± SEMs. *Asterisks* indicate a significant change of measurements compared with the control group ($^{*}p < 0.05$, $^{**}p < 0.01$). *Arrows* in b–d indicate the time of mechanical stimulation. (**a**–**c**: Modified from Malarkey et al. (2008); **d**: Modified from Reyes et al. (2013))

to glutamate, ATP and endothelin-1 were inhibited by Zn^{2+}, Gd^{3+} and La^{3+} in astrocytes in culture and in hippocampal slices (Kresse et al. 2005). This inhibition likely reflects upon the action of these metal ions on the TRPC1 channel. In-depth analysis of the SOCE induced by activation of bradykinin receptors in cultured astrocytes isolated from the cortex of newborn mice revealed the leading role of TRPC1 and to a lesser extent TRPC3 isoforms (Akita and Okada 2011). In contrast, in spinal astrocytes (stimulated by neurokinin-1 receptor agonists substance P and GR73632) the SOCE was predominantly mediated by TRPC3 channels being sensitive to specific inhibitor ethyl-1-(4-(2,3,3-trichloroacrylamide)phenyl)-5-(trifluoromethyl)-1H-pyrazole-4-carboxylate or Pyr3 (Miyano et al. 2010). Incidentally, chronic (3–21 days) treatment of cultured astrocytes with the serotonin $5\text{-}HT_{2B}$ receptor agonist fluoxetine substantially (by 50–90 %) reduced TRPC1-dependet SOCE (Li et al. 2011). The expression of TRPC1 and TRPC1-mediated SOCE in astrocytes also seems to be mediated by amyloid precursor protein (APP) and knocking out of APP substantially reduced both (Linde et al. 2011).

All in all, the wealth of data seems to indicate that TRPC1 (most likely heteromeric with TRPC3, 4 and 5) is, to a large extent, responsible for astroglial SOCE. This is, incidentally, contrasting to microglia, where SOCE is mostly (if not exclusively) mediated by Orai-composed I_{CRAC} channels (Verkhratsky and Parpura 2013).

5.4 TRPV Channels

TRPV ('V' for vanilloid) channels family covers six members which are activated by various chemical, thermal, and noxious stimuli; TRPV4 channels are also sensitive to osmotic pressure. All TRPV channels are Ca^{2+} permeable with $P_{Ca}/P_{monovalent}$ between 1 and 10 for TRPV1–4, and $P_{Ca}/P_{monovalent} > 100$ for TRPV5 and 6 (Owsianik et al. 2006).

Astrocytes in cortex and in hippocampus express TRPV4 channels (localised mainly in their processes) that are involved in osmotic sensing and together with aquaporins (AQP) contribute to cell volume regulation (Bai and Lipski 2010; Benfenati et al. 2007; Butenko et al. 2012; Liu et al. 2006). These TRPV4 channels were found to be activated by hypotonicity that triggered substantial Ca^{2+} influx resulting in $[Ca^{2+}]_i$ elevation. This could be blocked by the TRPV inhibitor ruthenium red (Benfenati et al. 2007). The TRPV4-mediated outwardly rectifying currents were also monitored in voltage-clamp configuration following stimulation with the selective TRPV4 agonist 4-α-phorbol 12,13-didecanoate (Benfenati et al. 2007). Similarly, TRPV-4-mediated currents and $[Ca^{2+}]_i$ transients were recorded from astrocytes in hippocampal slices. Both events were blocked by ruthenium red and the TRPV-4 selective inhibitor RN1734 (Butenko et al. 2012). In cortical astroglia, TRPV4 were shown to interact with AQP-4; the resulting TRPV4-AQP4 complexes were critical for regulatory volume decrease ensuing hypo-osmotic shock (Benfenati et al. 2011; Benfenati and Ferroni 2010).

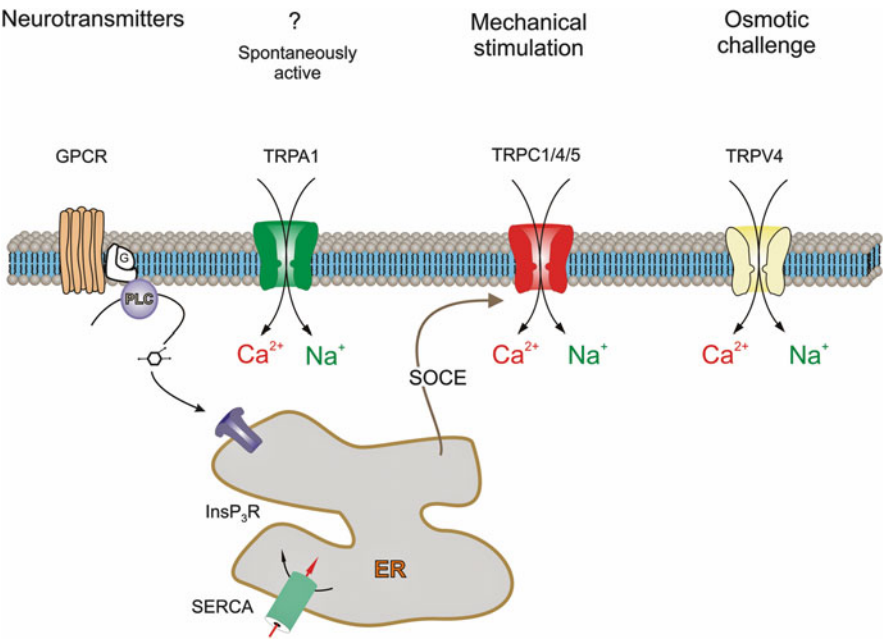

Fig. 2 Variety of astroglial TRP channels. Note the link between metabotropic stimulation and TRPC channels through the ER and store-operated Ca^{2+} entry

Recently (Mannari et al. 2013), TRPV1 channels were also detected (by PCR, Western blotting and immunohistochemistry) in astrocytes in circumventricular organs (more specifically within the organum vasculosum of the lamina terminalis, subfornical organ and area postrema) that contain chemosensitive area of the brain. These channels were found especially abundant in the thick processes of astrocytes that surround blood vessels, and hence could presumably be activated by a blood-borne stimulus. In particular, the blood infusion of the TRPV1 selective agonist, resiniferatoxin, triggered expression of immediate early gene *c-Fos* in astrocytes from circumventricular organs (Mannari et al. 2013).

6 TRPC Channels Coordinate Multi-ion Signalling in Astroglia

In astrocytes, out of all TRP proteins, the channels of TRPC family seem to be the most abundant, and are poised to have a specific functional importance in coordinating Ca^{2+} and Na^{+} signalling in response to widely heterogeneous stimuli (Fig. 2). First, TRPC channels are sensitive to mechanostimulation, which occurs quite frequently in astroglia that show a remarkable degree of morphological plasticity and are prone to rapid changes in their volume. These volume changes

can develop on a relatively rapid scale (seconds), accompanying, for example, synaptic transmission. Synaptic activity is directly associated with a transient local shrinkage of the extracellular space which is controlled by water transport across astroglial perisynaptic membranes mediated by AQP-4 with subsequent water redistribution through the glial syncytium (Haj-Yasein et al. 2012; Nagelhus et al. 2004). These local volume changes may activate TRPC channels with the subsequent initiation of local Na^+/Ca^{2+} signals; incidentally, activation of TRPC1 channels may regulate expression of AQP channels (Conner et al. 2012). Similarly, TRPC channels are activated during hypo-osmotic stress.

Second, TRPC1-containing channels (which predominantly mediate Na^+ fluxes) are under control of Ca^{2+} signalling machinery (being astroglial substrates for SOCE). As a result, it is plausible that metabotropic stimulation of astroglia that depletes ER Ca^{2+} stores would trigger opening of TRPC channels and induces substantial Na^+ fluxes (Fig. 2). This mechanism may translate activation of G-protein-coupled receptors into Na^+ signalling events developing in parallel with ER-mediated Ca^{2+} signals.

The role for TRPC channels in regulation of $[Na^+]_i$ and Na^+-dependent processes was first discovered in HEK cells in which TRPC3 protein appeared to be closely associated with NCX via the C-terminus of the channel. Interactions were reciprocal as the inhibition of NCX affected the Ca^{2+} flux through the TRPCs (Eder et al. 2005). In addition, a $[Na^+]_i$ elevation following the opening of TRPC channels led to a reversal of NCX with obvious consequences for Ca^{2+} signalling, that is, Ca^{2+} entry to the cytosol from the extracellular space. The role for TRPC-mediated Na^+ influx and resulting intracellular Na^+ signals in Ca^{2+}astrocytes could be even more important than that of Ca^{2+} dynamics, as astrocytes possess numerous molecular systems relevant for homeostatic responses that are controlled by the transmembrane Na^+ gradient (see (Kirischuk et al. 2012; Verkhratsky et al. 2013a) and Table 1).

TRPC channels seem to have a dual selectivity filter, as unveiled by site-directed mutagenesis and immunological approaches. Hence, substitution of seven acidic residues to basic amino acids in the channel region of TRPC1 subdued Ca^{2+}, but not Na^+ fluxes (Liu et al. 2003). Introduction of a single mutation (E630Q) to the selective filter of TRPC3 caused a reduction in Ca^{2+} current with a concomitant enhancement of Na^+ currents (Poteser et al. 2011). Having this in mind, Reyes et al. (2013) used a functional anti-TRPC1 antibody targeting the putative selective filter of the TRPC1 channel. As we already disclosed, mechanical stimulation of astrocytes triggers increases in both $[Ca^{2+}]_i$ and $[Na^+]_i$ (Fig. 1b and d, respectively, and (Malarkey et al. 2008; Reyes et al. 2013)). Inhibition of TRPC1 channels by the anti-TRPC1 antibody resulted in a decrease in the peak and cumulative $[Ca^{2+}]_i$ responses (Fig. 1b) and, in parallel, in an increase in the peak amplitude of $[Na^+]_i$ response (Fig. 1d) (Reyes et al. 2013). Taken together, Ca^{2+} and Na^+ fluxes of TRPC channels can thus be dissociated following molecular biology or immunological interventions. It is tempting to speculate that such mechanism of regulation of TRPC permeability could represent a physiological event, perhaps mediated by yet unknown enzymatic, protein-protein binding or post-translational modifications.

7 Pathological Potential of Astroglial TRP Channels

Astroglia, being the central homeostatic and defensive cellular elements of the CNS, are involved in the absolute majority of neurological diseases, and astroglial reactions to pathological insults to a great extent determine progression and outcome of neuropathology (Giaume et al. 2007; Verkhratsky et al. 2013b). Investigations of contribution and possible pathophysiological relevance of astroglial TRP channels are in statu nascendi with only several studies having been performed hitherto.

TRPA1 channels were detected in glial fibrillary acidic protein-positive astrocytes of the superficial laminae of the rat trigeminal caudal nucleus using electron microscopy in combination with immunohistochemistry and immuno-silver-gold labelling (Lee et al. 2012). Peripheral inflammation (induced by injection of complete Freund's adjuvant into the capsule of a temporomandibular joint) increased the number of labelled TRPA1 channels in astroglial processes contacting nociceptive primary afferent terminals of the joint. This was considered as an indication of a possible role for TRPA1 channels in the stimulation of astrogliotic reactions following activation of a nociceptive input.

The TRPC channels are implicated in Ca^{2+} signalling generated by thrombin that were demonstrated to be linked to initiation of astrogliotic remodelling (Nakao et al. 2008; Shirakawa 2012). On similar lines, astroglial Ca^{2+} signalling in response to acute administration of IL-1β results, in part, from activation of TRPC1 and TRPC6 channels, and chronic treatment with IL-1β increased TRPC6 expression that contributed to dysregulation of overall Ca^{2+} homeostasis (Beskina et al. 2007). TRPV4 channels also have been linked to astrogliotic response. Expression of TRPV4 channels in hippocampal astrocytes substantially increased following brief (15 min) episode of cerebral hypoxia/ischaemia produced by bilateral occlusion of the common carotid arteries together with systemic hypoxia (Butenko et al. 2012). This increased presence of TRPV4 channels resulted in an increase in respective ion currents and TRPV4-mediated Ca^{2+} signals. It has been also suggested that TRPV4 contributes to ischaemia-induced $[Ca^{2+}]_i$ elevations (Butenko et al. 2012). The TRPV4 channels were also implicated in astroglial cell death triggered by oxidative stress (Bai and Lipski 2010).

The TRP channels being multi-ion carriers can also be implicated in astroglial regulation of homeostasis of various metals and in metal-induced toxicity. The TRPC channels, for example, have been shown to participate in the buffering of iron, which ability increased in reactive cells (Pelizzoni et al. 2013). Astrocytes are also primary targets for the main forms of toxic encephalopathies induced by heavy metals. Accumulation of these metals in astroglia generally disrupts astroglial homeostatic abilities and often compromises astroglial glutamate uptake which in turn results in excitotoxic neuronal death. These astroglial impairments are central, for example, in poisoning by methylmercury or Minamata disease (Yin et al. 2007), lead toxic encephalopathy (De Keyser et al. 2008), manganese neurotoxicity (De Keyser et al. 2008) and aluminium toxic encephalopathy (Struys-Ponsar et al. 2000; Suarez-Fernandez et al. 1999). In part, accumulation of these metals into

astroglia is mediated by specific transporters. However, the role of TRP channels cannot be excluded, and this possible route for heavy metal entry has not been yet experimentally addressed.

8 Conclusions

Channels of TRP family are expressed in astroglia where they perform various, mainly yet undetermined functions in physiology and pathophysiology. The TRPC channels are uniquely placed to coordinate astroglial Ca^{2+} and Na^{+} signalling because of their Na^{+}/Ca^{2+} permeability and because of association of TRPC-mediated Na^{+}/Ca^{2+}influx with ER store depletion of releasable Ca^{2+}, which establishes a direct link between activation of metabotropic receptors and Na^{+} signalling.

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Conflict of Interest. The authors declare that they have no conflict of interest.

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Pharmacological Inhibition of Actin Assembly to Target Tumor Cell Motility

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Abstract Tumor metastasis remains an unsolved clinical problem. An initial and essential step in this process is active migration of tumor cells, which critically depends on reorganization of the actin cytoskeleton. Factors regulating actin assembly are just beginning to emerge as potential targets for preventing dissemination and invasion of tumor cells. Recent studies have shown that actin-dependent cellular processes, including tumor invasion, can be pharmacologically modulated by small-molecule inhibitors of actin assembly. In this chapter, we summarize reports on newly identified small-molecule inhibitors that target a growing number of actin nucleation and assembly factors relevant for human disease.

Contents

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Abbreviations

APC	Adenomatous polyposis coli
Arp	Actin-related protein
CDDO-Im	1-(2-cyano-3,12-dioxooleana-1,9-dien-28-oyl) imidazole
CDDO-Me	Methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate
EGF	Epidermal growth factor
FH2	Formin homology 2 domain
GBD	GTPase-binding domain
JMY	Junction-mediating and -regulatory protein
NPF	Nucleation promoting factor
PDGF	Platelet-derived growth factor
PIP_2	Phosphatidylinositol-(4,5)-biphosphate
SDF-1	Stromal cell-derived factor-1
SMIFH2	Small-molecule inhibitor of FH2 domains
WIP	WAS/WASL-interacting protein family

1 Introduction

Metastasis represents the turning point of tumor progression that marks the transition from a potentially curable disease into a terminal illness. The metastatic cascade is a complex process that begins with the detachment of tumor cells from the primary tumor and invasion into the surrounding environment. Through this step, tumor cells gain access to the systemic circulation, which allows them to spread and to colonize distant sites. Hence, the capability to move and invade is absolutely essential for metastatic spread. Indeed, cellular motility and invasiveness along with the ability for self-renewal belong to the fundamental traits of high-grade malignancy (Chaffer and Weinberg 2011).

Cellular motility is based on the reorganization of the actin cytoskeleton (Pollard and Cooper 2009). During cell migration, actin polymerization drives the formation of membrane protrusions, while movement of myosin motors along actin tracks provides the contractile force required to overcome physical resistance of the extracellular matrix and to translocate the cell body. The actin cytoskeleton is also involved in a variety of other cellular processes, such as vesicle trafficking, cell division, and regulation of gene expression (Olson and Nordheim 2010; Firat-Karalar and Welch 2011). Accordingly, highly invasive tumor cells exhibit an enhanced actin polymerization activity and aberrant expression of actin-regulating proteins (Bravo-Cordero et al. 2012).

De novo formation of an actin filament begins with the association of monomeric actin into a trimer, a so-called nucleus. This process relies upon the activity of specialized proteins termed actin nucleation factors or simply actin nucleators (Campellone and Welch 2010). Thus, in contrast to microtubules, the assembly of

actin networks does not require dedicated organizing centers. It is this spatial and temporal flexibility of actin filament formation that enables rapid reorganization of the actin cytoskeleton. Importantly, actin nucleators represent ultimate effectors of many signaling cascades involved in the regulation of actin turnover as well as of cellular motility as a whole.

These signaling pathways are orchestrated by members of the Rho GTPase family (Heasman and Ridley 2008), such as Cdc42, Rac, and Rho. Rho GTPases are of particular significance for the coordination of actin nucleation activities, with many actin nucleators being their direct effectors. Notably, Rho GTPases are often upregulated in tumors and play important—as yet only partially understood—roles in tumorigenesis and metastasis (Karlsson et al. 2009). Thus, there is mounting evidence for a critical link between tumor progression and signaling processes involved in the regulation of actin dynamics.

Specific treatment aimed at actin assembly factors emerges as a promising concept for the development of antitumor or anti-inflammatory therapies (Nürnberg et al. 2011). Recent reports have demonstrated that actin-dependent cellular processes can be specifically modulated by pharmacological inhibition of actin nucleation factors (Table 1). In this chapter, we focus on these newly identified small-molecule inhibitors of actin assembly, highlighting their potential use for future antimetastatic therapies.

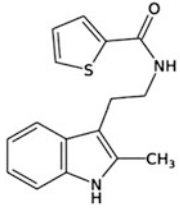
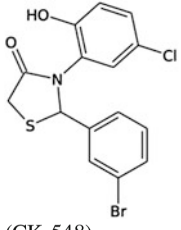
2 Targeting Modules of Actin Nucleation and Assembly

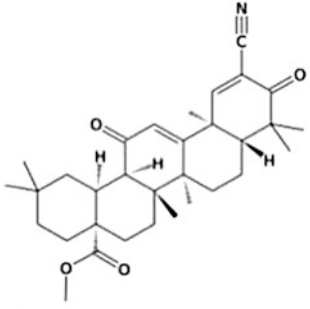
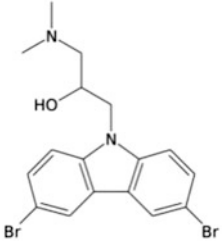
Actin nucleation factors facilitate the formation of an otherwise highly unstable actin nucleus, a process called actin nucleation (Fig. 1). Once an actin nucleus is formed, further incorporation of actin monomers occurs spontaneously, although at relatively slow pace. In the cellular context, proteins known as actin elongation factors support the association of actin monomers with the growing filament thereby strongly increasing actin polymerization rates. Besides these factors, a wide array of actin-binding proteins, such as actin-bundling proteins, contributes to the shaping of actin filament networks in vivo. However, actin nucleation factors remain the driving force for actin network assembly for they initiate the de novo formation of actin filaments.

Actin nucleators represent a heterogeneous set of proteins that includes actin-related protein 2 (Arp2) and 3 (Arp3) complex (known as the Arp2/3 complex), formin proteins (formins), and a recently discovered group of tandem actin monomer-binding proteins (Firat-Karalar and Welch 2011) (Fig. 1). Being functionally similar, these proteins differ substantially in the molecular mechanism of actin nucleation as well as in signaling processes that control their activity.

Tandem actin monomer-binding proteins are not covered in this review, as pharmacological inhibitors of their activity have not been described. However, it is worth noting that members of this group, junction-mediating and -regulatory protein (JMY) and adenomatous polyposis coli (APC), have been associated with tumor progression. APC is a prominent tumor suppressor implicated in the development of colon tumors (McCartney and Nathke 2008), whereas JMY is involved in the p53 response

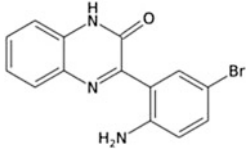
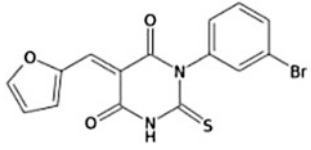
Table 1 Small-molecule inhibitors of actin assembly

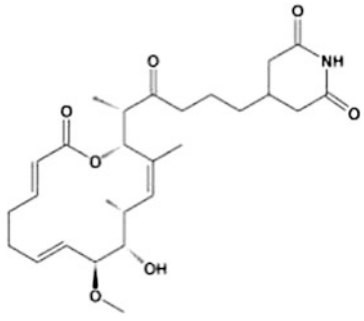
Compounds	Chemical structure	Molecular target	IC_{50}[a]	Inhibitory effects on actin-dependent structures and cell motility	References
CK-636 CK-666 CK-548 CK-869	 (CK-636)  (CK-548)	Arp2/3	4 μM	Listeria motility in living cells Podosome formation in THP-1 cell line Melanoblast migration in skin explants Chemotaxis in fibroblasts by reducing migration speed Haplotaxis in fibroblasts by disrupting directional sensing Fibroblast migration in wound healing assay	Nolen et al. (2009), To et al. (2010), Li et al. (2011), Wu et al. (2012)

CDDO-Im CDDO-Me	 (CDDO-Me)	Arp2/3 (and others)	50 μM	Fibroblast migration in wound healing assay	To et al. (2010)
Wiskostatin		N-WASP/ WASP	<10 μM	Dorsal ruffle formation in fibroblasts and MDCK cells Induces podosome disassembly in a macrophage cell line Formation of phagocytic caps and phagocytosis in macrophages Integrin-dependent migration of NK cells towards CXCL12/SDF-1 and CX3CL1/fractalkine	Peterson et al. (2004), Legg et al. (2007), Dovas et al. (2009), Park and Cox (2009), Abella et al. (2010), Stabile et al. (2010), King et al. (2011)
187-1	cyclo(*L*Lys-*D*Phe-*D*Pro-*D*Phe-*L*Phe-*D*Pro-*L*Gln)2	N-WASP/ WASP	<10 μM		Peterson et al. (2001)

(continued)

Table 1 (continued)

Compounds	Chemical structure	Molecular target	IC_{50}[a]	Inhibitory effects on actin-dependent structures and cell motility	References
3-(2-amino-5-bromophenyl)-1H-quinoxalin-2-one		mDia1, mDia2	2 μM		Gauvin et al. (2009)
SMIFH2		Formins (FH2)	15 μM	Induces disassembly of actin cables and contractile rings in fission yeast Migration rate of NIH 3T3 fibroblasts and induces non-apoptotic membrane blebbing Induces bleb-like structures in a carcinoma cell line A549 Induces disassembly of basal actin bundles in MDCK cells Diminishes radial stress fibers in U2OS cells Migration speed of highly metastatic MDA MB231 cells in Matrigel	Rizvi et al. (2009), Poincloux et al. (2011), Oakes et al. (2012), Tang and Brieher (2012)

Migrastatin		Fascin	–	Migration of EC17 esophageal cancer cells (IC_{50} ~ 10 μg/ml[b]), B16 melanoma cells (IC_{50} ~ 3 μg/ml[c]), 4T1 mouse breast cancer cells (IC_{50} ~ 29 μM), MDA MB231 human breast cancer cells (IC_{50} ~ 17 μM, IC_{50} ~ 0.35 μM[d]), lung cancer cell lines (IC_{50} ~ 1.5–8.2 μM[e]), human colon cancer cells (IC_{50} ~ 0.023 μM[d]), and human prostate cancer cells (IC_{50} ~ 0.17 μM[d]) Lamellipodia formation in 4T1 cancer cell line Metastasis in mice breast tumor model and a human small-cell lung carcinoma model[d]	Nakae et al. (2000), Takemoto et al. (2001), Gaul et al. (2004), Shan et al. (2005), Ju et al. (2009), Lecomte et al. (2011)

[a] Actin polymerization in vitro
[b] Purified migrastatin
[c] Migrastatin containing teleocidin-related compounds
[d] Macroketone
[e] Migrastatin core ether

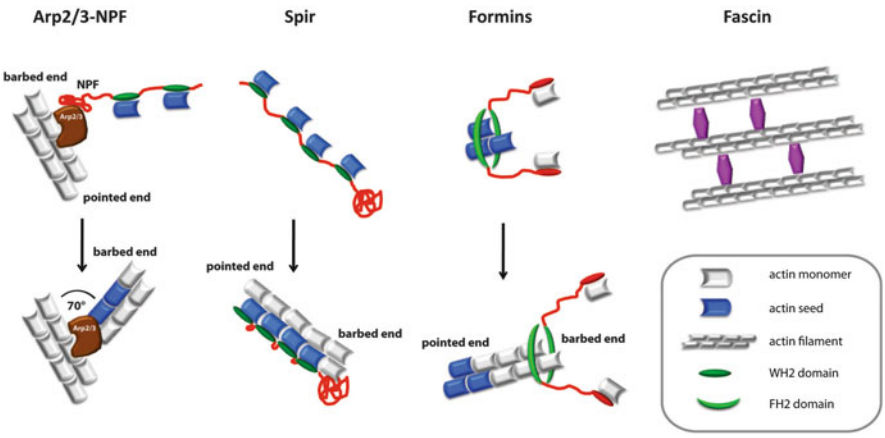


Fig. 1 *Mechanisms of actin nucleation and bundling*. Actin nucleation by the Arp2/3 complex: Arp2/3 is activated by a nucleation promoting factor (NPF), which also delivers two actin monomers (bound to WH2 domains) required for actin nucleus formation. Arp2/3 nucleates actin on the side of existing actin filaments, thereby initiating the formation of actin filament branches. Actin nucleation by tandem actin monomer-binding proteins (e.g., Spir/Spire): These proteins can bind four actin monomers, arranging them in a way that leads to the formation of an actin nucleus. Actin nucleation and elongation by formins: Formins act as homodimers with FH2 domains forming the actin processing core. FH1 domains bind actin monomers and deliver them to the FH2 core for incorporation into the growing filament. Actin bundling by fascin: Fascin binds to the sites of actin filaments via its two binding sites and thereby stabilizes high order actin structures

(Zuchero et al. 2009) and appears to positively influence tumor cell motility under stress or hypoxia conditions (Coutts et al. 2009). Moreover, overexpression of JMY in tumor stroma is correlated with poor prognosis of esophageal adenocarcinoma (Saadi et al. 2010), suggesting that actin nucleation factors also play a role in tumor—stroma interaction and in establishing tumor microenvironment.

2.1 Inhibition of the Arp2/3 Complex

The Arp2/3 complex is a stable heteroheptameric protein complex composed of Arp2 and Arp3 as well as five additional subunits named ARPC1 to ARPC5. All Arp2/3 subunits are highly conserved and expressed in virtually all eukaryotic cells. The nucleation activity of the Arp2/3 complex critically depends upon an association with proteins known as nucleation promoting factors (NPFs), which serve as an interface between upstream signaling cascades and Arp2/3 (Goley and Welch 2006). Acting in a multiprotein complex, Arp2/3-NPF can nucleate actin from the side of an existing filament, leading to the formation of actin filament branches (Mullins et al. 1998; Pantaloni et al. 2000; Goley et al. 2010) (Fig. 1). Further growth of these newly formed filament branches is aided by actin elongation factors from the enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family of proteins (Chesarone and Goode 2009) as well as by formins (Yang et al. 2007; Lee et al. 2010). Other regulators of Arp2/3-dependent actin assembly are cofilin,

whose severing activity increases actin turnover (DesMarais et al. 2004), and coronin, an Arp2/3 inhibitor (Chan et al. 2011).

Arp2/3-dependent assembly of branched actin filament networks drives protrusive structure, lamellipodia, at the leading edge of migrating cells (Sarmiento et al. 2008). Importantly, Arp2/3 is essential for the formation of specialized matrix-degrading protrusions in invasive cancer cells, named invadopodia (Yamaguchi et al. 2005). An increased Arp2/3 expression has been associated with highly invasive phenotypes of cancer cells (Wang et al. 2002, 2004, 2007; Otsubo et al. 2004) as well as metastatic disease (Iwaya et al. 2007).

Two chemically distinctive compounds named as CK-636 and CK-548 (Table 1) have been identified as inhibitors of the Arp2/3-NPF-induced actin polymerization in vitro and have been shown to suppress actin-dependent processes, such as *Listeria* motility in vivo (Nolen et al. 2009). The inhibitory activity of these compounds is based on direct interaction with Arp2/3 and is independent of NPFs. Structural analysis revealed that CK-636 binds at the interface between Arp2 and Arp3 subunits of the Arp2/3 complex, preventing Arp2/3 activation (Nolen et al. 2009; Baggett et al. 2012). In contrast, CK-548 binds to a hydrophobic region in the core of the Arp3 subunit probably leading to a conformational change that interferes with actin branch formation. Importantly, amino acid residues that interact with these inhibitors are highly conserved, implying that mutational changes in the binding pocket would be an unlikely mechanism for development of drug resistance.

These Arp2/3 inhibitors have already been used in studies on mechanisms of cell motility. In mouse embryonic skin explants, these compounds inhibit melanoblast migration without disrupting the keratinocyte actin network (Li et al. 2011). Pharmacological inhibition of the Arp2/3 complex also reduces the migration speed of Rat2 fibroblasts exposed to a gradient of platelet-derived growth factor (PDGF), but it does not affect directional sensing (Wu et al. 2012). In contrast, migration speed on a gradient of fibronectin is not affected by this treatment; however, treated cells fail to detect changes in extracellular matrix concentration. These findings support the notion that pharmacological interventions on actin dynamics may produce different effects depending on the context of the tumor microenvironment. In addition, these compounds interfere with cell contacts, as Arp2/3 inhibition was shown to reduce the F-actin content in apical cell—cell junctions (Kovacs et al. 2011; Tang and Brieher 2012), which could potentially lead to epithelial tissue damage.

Interestingly, derivatives of a synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), which belongs to a novel class of anti-inflammatory and cytoprotective agents with antitumor effects (Liby et al. 2007), were reported to interact with the Arp2/3 complex and inhibit Arp2/3-dependent actin polymerization in vitro (To et al. 2010). These compounds reduce cell migration in wound healing assays and prevent Arp2/3-driven actin network formation at the leading edge. However, given the multitude of their intracellular targets (Liby et al. 2007), synthetic triterpenoids will unlikely be used as specific pharmacological agents influencing actin nucleation activity.

2.2 Drugs Affecting Nucleation Promoting Factors

The hematopoietic-specific Wiskott—Aldrich syndrome protein (WASP) and its ubiquitously expressed homolog neural WASP (N-WASP) are prominent NPFs of the Arp2/3 complex. WASPs interact with Arp2/3 via the conserved WCA element (also referred to as VCA), which is located at their C-terminus and is sufficient to initiate Arp2/3-dependent actin nucleation in vitro (Rohatgi et al. 1999; Padrick et al. 2011) (Fig. 1). The N-terminus of WASPs is modularly organized and contains the WASP homology 1 domain (WH1), the GTPase-binding domain (GBD), and the Proline-rich domain. In vivo, WASPs are autoinhibited by an intramolecular interaction between the WCA and the GBD. This autoinhibition is released upon binding to Cdc42 and phosphatidylinositol-(4,5)-biphosphate (PIP_2), which synergistically activate WASPs (Prehoda et al. 2000). In addition, a variety of regulatory signaling proteins, such as Grb2, Nck, or IQGAP, as well as phosphorylation contribute to the modulation of their activity (Takenawa and Suetsugu 2007; Padrick and Rosen 2010).

WASPs regulate a plethora of cellular processes associated with directional motility. N-WASP is crucial for the formation of invadopodia (Lorenz et al. 2004; Yamaguchi et al. 2005; Oser et al. 2009; Gligorijevic et al. 2012; Yu et al. 2012) and is involved in the directional sensing at the leading edge (DesMarais et al. 2009). Although N-WASP plays a role in filopodia assembly (Miki et al. 1998; Lee et al. 2010), its activity is not essential for this process, as fibroblasts and cancer cells lacking N-WASP can still protrude filopodia (Snapper et al. 2001; Sarmiento et al. 2008). Apart from these processes involving Arp2/3-driven actin filament assembly, N-WASP was reported to act as an Arp2/3-independent actin nucleator, promoting myofibril formation in skeletal muscles (Takano et al. 2010).

N-WASP is also implicated in the regulation of proliferative and chemotactic signaling. Acting downstream of β1-integrin, N-WASP promotes chemotactic responses towards PDGF and facilitates PDGF receptor traffic in fibroblasts (King et al. 2011). In ovarian cancer cells, N-WASP associates with CD44 and this complex binds Her2 to promote phosphorylation and nuclear translocation of β-catenin (Bourguignon et al. 2007). In concordance with these findings, N-WASP-deficient mouse keratinocytes have decreased nuclear localization of β-catenin, which is accompanied by a reduction in Wnt-dependent transcription (Lyubimova et al. 2010). Moreover, N-WASP seems to facilitate epidermal growth factor (EGF) receptor internalization (Innocenti et al. 2005) and it may therefore decrease EGF receptor-Src signaling in EGF-sensitive tumor cells. Lack of N-WASP also leads to increased TGFβ signaling in vivo, but not in vitro (Lefever et al. 2010), indicating that the inhibition of such multifunctional molecules as N-WASP can produce different consequences depending on the extracellular and intracellular contexts.

The first identified inhibitor of N-WASP was a 14-amino acid cyclic peptide, 187–1 (Table 1), which was identified as an inhibitor ($IC_{50} \sim 2$ μM) of PIP_2-induced actin polymerization in cytoplasmic extracts of *Xenopus laevis* eggs. This peptide

inhibits N-WASP-Arp2/3-initiated actin polymerization in vitro ($IC_{50} < 10$ μM) through an interaction with N-WASP (Peterson et al. 2001). Based on this work, Peterson et al. identified a small-molecule inhibitor (EC50 ~ 4 μM) of WASPs, named wiskostatin (Table 1), and demonstrated that wiskostatin stabilizes the autoinhibited conformation of WASPs through binding to the GBD. In this way, wiskostatin reduces WASP activity and competitively antagonizes WASP activation by Cdc42 and PIP_2 (Peterson et al. 2004). Accordingly, wiskostatin inhibits the activation of a FRET-based WASP biosensor in COS-7 cells expressing a constitutive active Cdc42 mutant (Cammer et al. 2009). At much higher concentrations ($IC_{50} > 140$ μM), wiskostatin also suppresses spontaneous actin polymerization (Peterson et al. 2004). This effect may account for the reported failure of cytokinesis in HeLa upon wiskostatin treatment, which could not be mimicked by knockdown of N-WASP or Arp2/3 (Bompard et al. 2008).

The discovery of wiskostatin has provided researchers with a useful tool for studying the cellular roles of WASP proteins. Especially in immune cells, wiskostatin has been shown to interrupt many actin-dependent processes. It induces disassembly of podosomes in a murine monocyte cell line (Dovas et al. 2009). Podosomes—protrusive structures formed by monocyte-derived cells—are involved in proteolytic matrix degradation, thus closely resembling invadosomes of cancer cells (Linder et al. 2011). In murine bone marrow-derived macrophages, wiskostatin reduces actin assembly in response to IgG stimulation and attenuates phagocytic cap formation and subsequently phagocytosis (Park and Cox 2009). Consequently, HIV entry into macrophages is inhibited by pretreatment with wiskostatin (Carter et al. 2011).

Furthermore, wiskostatin inhibits integrin-dependent migration of human NK cells stimulated with CXCL12/stromal cell-derived factor-1 (SDF-1) or CX3CL1/fractalkine and prevents upregulation of the β2-integrin neoepitope in these cells (Stabile et al. 2010). In another study, wiskostatin induced the redistribution of the intercellular adhesion molecule ICAM-1 on bronchial epithelial cell surfaces thereby affecting transepithelial migration of T cells (Porter and Hall 2009). Wiskostatin was also shown to interfere with actin assembly at cell–cell junctions (Ivanov et al. 2005). These findings again indicate that effects of pharmacological intervention on actin dynamics likely depend upon the cellular context.

As N-WASP is involved in the regulation of signaling processes in the cells, wiskostatin has been shown to affect a number of signaling processes involved in cell migration. In MDCK cells, wiskostatin-induced disruption of actin structures at adherens junctions causes the release of Rac guanine exchange factor Tiam1 (Ten Klooster et al. 2006). Resulting increase of Rac1 activity could, in turn, affect actin dynamics via its downstream targets. In fibroblasts, PDGF receptor-induced formation of dorsal ruffles—actin-enriched structures involved in growth factor receptor endocytosis (Orth and McNiven 2006)—is inhibited by treatment with wiskostatin (Legg et al. 2007; King et al. 2011), which also leads to an increased PDGF receptor phosphorylation upon PDGF stimulation (King et al. 2011). Similarly, wiskostatin inhibits dorsal ruffle formation in MDCK cells after hepatocyte growth factor stimulation (Abella et al. 2010). Thus, apart from its direct effect on

actin assembly, wiskostatin can interfere with upstream signaling processes that affect actin dynamics.

2.3 Inhibitors of Formin Homology Proteins

In contrast to Arp2/3, formin proteins can be self-sufficient, being able to nucleate actin independently of existing filaments or NPFs (Pruyne et al. 2002; Sagot et al. 2002). Formin-mediated actin assembly is driven by the highly conserved formin homology 2 domain (FH2), which defines this group of actin nucleation factors (Pruyne et al. 2002; Sagot et al. 2002). Similar to N-WASP, the activity of formins is tightly regulated by autoinhibition, which may be relieved upon binding of activated Rho GTPases to the GBDs of formins. Activated formins associate into homodimers that can both nucleate actin and efficiently promote the growth of newly formed actin filaments (Goode and Eck 2007). The latter is supported by the FH1 domain, which precedes FH2 and can bind profilin-associated actin, providing monomers for processive elongation of an actin filament (Kovar 2006) (Fig. 1). The ability to support elongation of actin filaments clearly distinguishes formins from other actin nucleators, making them one of the most powerful actin assembly factors. In addition, formins also modulate microtubule stabilization and dynamics (Bartolini and Gundersen 2010; Chesarone and Goode 2009; Goulimari et al. 2005, 2008) and are therefore particularly interesting targets for influencing cytoskeletal functions.

Mammalian tissues express 15 different formins plus various isoforms. Formins therefore represent the largest group of actin nucleators as well as of Rho-GTPase effector proteins (Higgs and Peterson 2005; Baarlink et al. 2010). Although little is known about distinct physiological roles of formin proteins in humans, a number of reports have provided evidence for their critical function in tumor cell motility and metastasis. Human diaphanous homolog 1 (DIAPH1) is necessary for invadopodia formation (Lizarraga et al. 2009) and invasive migration in a three-dimensional matrix (Kitzing et al. 2007). It is also required for bleb-associated cancer cell invasion (Kitzing et al. 2007; Shi et al. 2009), mediating a positive feedback towards RhoA-Rho kinase via the leukemia-associated Rho guanine nucleotide exchange factor (LARG). Single amoeboid-like invasion can also be driven by the formin-like protein 2 (FMNL2) and the metastasis-associated small Rho GTPase RhoC (Kitzing et al. 2010). Finally, SRC-transformed cells lacking diaphanous homolog 1 (Diap1, also known as mDia1) fail to form tumors and to invade surrounding tissues in vivo (Tanji et al. 2010).

These findings have been further corroborated by the analysis of protein expression profiles in a variety of malignant tumors. In particular, FMNL2 is overexpressed in metastatic colorectal cancer (Zhu et al. 2008), whereas FMNL1 was found to be upregulated in lymphatic, lymphoblastic, and acute myeloid leukemias (Schuster et al. 2007) and formin-2 (Fmn2) is overexpressed in pre-B-lineage acute lymphoblastic leukemia (Charfi et al. 2011). However, FMNL2 seems

to be downregulated in hepatocellular carcinoma, with lower FMNL2 expression being correlated with poor overall survival (Liang et al. 2011). Thus, the contribution of formins to tumor invasion and metastasis may be tumor-type sensitive and depend on the context of the interaction between tumor cells and their environment.

Two recent reports have identified small-molecule inhibitors of formin-mediated actin assembly. In the first report, 3-(2-amino-5-bromophenyl)-1H-quinoxalin-2-one (Table 1) was shown to inhibit actin assembly initiated by the FH2 domain of mDia1 and mDia2, with IC_{50} being approximately 2 μM (Gauvin et al. 2009). Surprisingly, mDia3-mediated actin assembly is not inhibited by this compound although it seems to bind solely within the highly conserved FH2 domain of mDia formins. Moreover, the potency of this compound against FMNL1 is much lower (IC_{50} ~ 15 μM), whereas another formin protein, inverted formin 2 (INF2), is only partially inhibited.

In contrast, a compound identified in another screen inhibits actin polymerization induced by FH2 domains derived from various species, thus apparently representing a general inhibitor of the FH2-mediated actin assembly (Rizvi et al. 2009). This compound, named small-molecule inhibitor of FH2 domains (SMIFH2) (Table 1), inhibits both nucleation activity (IC_{50} ~ 25 μM) and elongation (IC_{50} ~ 4 μM) activity of formins.

SMIFH2 reduces migration rates of NIH3T3 fibroblasts and induces non-apoptotic membrane blebbing (Rizvi et al. 2009). The latter finding is in concordance with reports demonstrating a profound plasma membrane blebbing upon mDia2 depletion (Eisenmann et al. 2007; Di Vizio et al. 2009). Similarly, in the carcinoma cell line A549, SMIFH2 induces bleb-like structures at cell periphery, indicative of cortical actin weakening upon inhibition of formin activity (Rizvi et al. 2009). Consistent with the role of mDia2 in cytokinesis (Watanabe et al. 2008), SMIFH2 further interferes with cytokinesis of mouse fibroblasts (Rizvi et al. 2009).

Moreover, SMIFH2 diminishes radial stress fibers and length of focal adhesions in U2OS cells and prevents fibronectin-remodeling by NIH3T3 cells (Oakes et al. 2012). Finally, SMIFH2 decreases the migration speed of highly metastatic MDA MB231 cells in Matrigel (Poincloux et al. 2011), further supporting the notion that formins can be useful targets for anti-invasive or anti-inflammatory therapies.

2.4 Targeting Actin Assembly and Bundling by Fascin in Cancer

Fascins are actin filament–binding proteins that crosslink actin filaments, promoting the formation of higher order actin filament bundles (Fig. 1). The assembly of linear actin filament structures underlies the formation of protrusions such as filopodia and invadopodia, which both rely on fascin-mediated actin filament bundling (Li et al. 2010; Stevenson et al. 2012). Being largely absent in normal adult epithelia, fascin-1 is often overexpressed in a variety of carcinomas, including

breast, prostate, and colorectal cancer. High expression of fascin-1 is associated with a more aggressive and invasive phenotype of these cancers. Thus, fascin-1 is an important player in cancer cell dissemination and metastasis—a finding, which is further supported by studies on cellular migration in vitro as well as by animal tumor models (Hashimoto et al. 2011).

In contrast to the synthetic actin nucleation inhibitors described previously, the fascin inhibitor migrastatin (Chen et al. 2010; Table 1) is a natural product isolated from a *Streptomyces* sp. strain, which was initially identified as a potent inhibitor of tumor cell migration (Nakae et al. 2000; Takemoto et al. 2001). Migrastatin does not affect cell viability and causes only a slight reduction of RNA and protein synthesis at 10–30 times higher concentrations than those required for the inhibition of cell migration. These effects of migrastatin as well as its more potent analogs such as macroketone have been further confirmed in several reports (Gaul et al. 2004; Shan et al. 2005; Ju et al. 2009; Lecomte et al. 2011). Intriguingly, treatment with 20 mg/kg of macroketone nearly abolishes the formation of lung metastases in a mice breast tumor model, without affecting the growth of the primary tumor (Shan et al. 2005). Similarly, migrastatin derivatives significantly reduce tumor metastasis in a human small-cell lung carcinoma model at doses of 10–49 mg/kg (Lecomte et al. 2011).

3 Conclusion

Metastasis is the transitional step into a malignant cancer disease that remains difficult to target by pharmacological intervention. The actin cytoskeleton plays an essential role during all steps of metastasis, such as migration in extracellular matrices and through endothelial or epithelial barriers. Actin filament turnover is highly dynamic and is subjected to strict and specific spatiotemporal regulation during cell migration. Hence, many of its regulators could serve as potential targets for future antitumor therapies.

Here, we have summarized evidence on diverse inhibitors of the actin nucleation and assembling machinery, which all interfere with actin-dependent processes in cellular systems. Clearly, further in vivo studies on mouse model systems are required to better understand possible approaches for actin assembly inhibition with future therapeutic relevance. In addition, the potency of these inhibitors is comparatively low. However, recent reports have demonstrated that chemical modifications of the core compounds can lead to profound potency improvement (Gaul et al. 2004; Gauvin et al. 2009; Baggett et al. 2012). It is therefore expected that inhibition of actin-based pathological cell migration will become an emerging field that may ultimately find its way into clinical applications for treatment of cancer as well as inflammatory diseases.

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The Role of mTORC1 in Regulating Protein Synthesis and Skeletal Muscle Mass in Response to Various Mechanical Stimuli

Craig A. Goodman

Abstract Skeletal muscle plays a fundamental role in mobility, disease prevention, and quality of life. Skeletal muscle mass is, in part, determined by the rates of protein synthesis, and mechanical loading is a major regulator of protein synthesis and skeletal muscle mass. The mammalian/mechanistic target of rapamycin (mTOR), found in the multi-protein complex, mTORC1, is proposed to play an essential role in the regulation of protein synthesis and skeletal muscle mass. The purpose of this review is to examine the function of mTORC1 in relation to protein synthesis and cell growth, the current evidence from rodent and human studies for the activation of mTORC1 signaling by different types of mechanical stimuli, whether mTORC1 signaling is necessary for changes in protein synthesis and skeletal muscle mass that occur in response to different types of mechanical stimuli, and the proposed molecular signaling mechanisms that may be responsible for the mechanical activation of mTORC1 signaling.

Contents

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1 Introduction

Skeletal muscle plays fundamental roles in the body, including the generation of limb and trunk movements and the control of breathing. Furthermore, due to the relatively large proportion of the body that is made up of skeletal muscle (~45 %), skeletal muscle also plays an important role in the regulation of whole-body metabolism (Lee et al. 2000; Izumiya et al. 2008). Thus, the maintenance of skeletal muscle mass is essential for mobility, disease prevention, and quality of life (Seguin and Nelson 2003; Lynch 2004; Srikanthan and Karlamangla 2011). For these reasons, a thorough understanding of the molecular mechanisms that regulate skeletal muscle mass is crucial to the development of effective exercise programs and potential pharmacological interventions aimed at preventing the loss of, or increasing, skeletal muscle mass.

Skeletal muscle mass is ultimately determined by the net difference in the rates of protein degradation and protein synthesis (Goodman et al. 2011c). For example, a net decrease in protein synthesis and/or a net increase in protein degradation leads to a reduction in muscle mass, otherwise known as muscle atrophy. Conversely, a net increase in protein synthesis and/or a net decrease in protein degradation leads to an increase in muscle mass, known as muscle hypertrophy. Importantly, the rates of protein synthesis and degradation can change quite rapidly, and these changes can be evoked by changes in a variety of stimuli including nutrients, neural activity, cytokines, growth factors, hormones, and mechanical loading (Bodine 2006; Frost and Lang 2007; Sandri 2008).

Mechanical loading is known to be a major regulator of skeletal muscle mass, with an increase in mechanical loading resulting in muscle hypertrophy and a decrease in mechanical loading resulting in muscle atrophy [for reviews, see (Goldberg et al. 1975; Adams and Bamman 2012)]. Furthermore, these mechanical load–induced changes in muscle mass are associated with changes in protein synthesis. For example, increased mechanical load–induced muscle hypertrophy is associated

with an increase in protein synthesis, while mechanical unloading–induced muscle atrophy is associated with a decrease in protein synthesis (Goldberg 1968a; Vandenburgh 1987; Adams and Bamman 2012). Although changes in protein synthesis appear to play a central role in the mechanical regulation of skeletal muscle mass, the molecular mechanisms through which changes in mechanical loading regulate protein synthesis, and thus muscle mass, remain to be fully determined. Furthermore, it is unclear whether different types of mechanical loading regulate protein synthesis and muscle mass in a similar or distinct manner.

Over the last decade, it has become apparent that the protein kinase known as mTOR (the mammalian/mechanistic target of rapamycin), which is found in the multi-protein complex called mTORC1, plays a fundamental role in the regulation of skeletal muscle mass (Sandri 2008; Frost and Lang 2011). In addition, mTORC1 has been identified as a master regulator of mRNA translation, and thus protein synthesis, with the potential to regulate both translational efficiency (i.e., the rate of mRNA translation) and translational capacity (i.e., the number of ribosomes) [for a review, see (Mahoney et al. 2009)]. To date, however, the necessity of mTORC1 signaling in the regulation of protein synthesis and skeletal muscle mass in response to different types of mechanical stimuli remains to be fully determined. Furthermore, the molecular mechanisms that regulate mTORC1 signaling in skeletal muscle in response to different mechanical loading paradigms are only beginning to be defined.

Thus, the purposes of this review are to: (1) briefly describe the discovery of mTOR and mTORC1, the cellular location of mTOR/mTORC1, and two of mTORC1's direct, and most studied, protein synthesis–related targets, $p70^{S6K1}$ and 4E-BP1; (2) briefly describe another potential mTORC1-regulated and protein synthesis–related molecule, eIF2Bε; (3) detail current evidence from rodent and human studies for the role of mTORC1 signaling in mechanical overload/resistance exercise–induced increases in protein synthesis and muscle mass; (4) summarize evidence from animal and human studies regarding whether mTORC1 signaling is also activated by acute bouts of endurance and sprint exercise; (5) examine the available evidence for a role of mTORC1 in the regulation of basal/resting protein synthesis and muscle mass; and (6) examine the main putative upstream molecular signaling mechanisms that are proposed to play a role in the activation of mTORC1 signaling in response to mechanical overload/resistance exercise.

Before beginning, it is important to note that while this review focuses on mTOR and mTORC1, other signaling mechanisms also play important roles in the regulation of protein synthesis and muscle mass [for recent reviews, see (Lynch and Ryall 2008; Glass 2010; Huang et al. 2011; Lee and MacLean 2011; McCarthy 2011; Phillips et al. 2012; Schiaffino and Mammucari 2011; Adams and Bamman 2012; Berdeaux and Stewart 2012; Dubois et al. 2012; Piccirillo et al. 2013)]. Furthermore, it should also be recognized that the activation of mTORC1 signaling could, in part, regulate skeletal muscle mass via the inhibition of autophagy-mediated protein degradation (Jung et al. 2010; Sandri 2013). Finally, due to space limitations, this review will not give a specific overview of the molecular regulation of protein synthesis (i.e., translation initiation, elongation and termination, and ribosome biogenesis);

however, the interested reader is directed to the following recent reviews: Mayer and Grummt 2006; Moss et al. 2007; Kelen et al. 2009; Mahoney et al. 2009; Topisirovic et al. 2011; and Hinnebusch and Lorsch 2012.

2 mTOR and mTORC1

2.1 A Brief History of Rapamycin, mTOR, and mTORC1

In 1965, an antifungal/antibiotic compound produced by microbes (*Streptomyces hygroscopicus*) found in soil from the island of Rapa Nui (Easter Island) was isolated and subsequently named rapamycin (Sehgal et al. 1975; Vezina et al. 1975; Baker et al. 1978). Later, rapamycin was found to also have immunosuppressant properties that required its binding to FK506-binding protein 12 (FKBP12) (Harding et al. 1989; Sehgal 2003). The mechanism behind this rapamycin-induced immunosuppression was shown to be via the inhibition of T cell proliferation (Dumont et al. 1990) and, specifically, the blocking of the transition from the G1 to the S phase of the cell cycle (Heitman et al. 1991). Rapamycin-induced cell cycle arrest was also found to be associated with the inhibition of the phosphorylation of the mitogen-activated 70 kDa ribosomal protein S6 kinase ($p70^{S6K}$) (Banerjee et al. 1990; Chung et al. 1992; Kuo et al. 1992; Price et al. 1992), indicating that rapamycin might suppress growth, not only by inhibiting cell proliferation but also by inhibiting mitogen-induced changes in cell size. In 1991, the growth-related targets of rapamycin were identified in yeast (*Saccharomyces cerevisiae*) and named the targets of rapamycin 1 and 2 (TOR1 and TOR2) (Heitman et al. 1991). Subsequent sequencing of TOR1 and TOR2 revealed that they were highly homologous to phosphoinositol lipid kinases but were later found to be serine/threonine protein kinases. By 1994, a single mammalian ortholog of the yeast TOR genes was discovered by several laboratories and given the names rapamycin target (RAPT1) (Chiu et al. 1994), FKBP-rapamycin-associated protein (FRAP) (Brown et al. 1994), and rapamycin and FKBP target 1 (RAFT1) (Sabatini et al. 1994). These names were later standardized to the mammalian target of rapamycin (mTOR) (Sabers et al. 1995); however, in 2009 the definition of the mTOR gene name was officially changed by the HUGO Gene Nomenclature Committee to the mechanistic target of rapamycin in an attempt to make the name more applicable across all species (Hall 2013).

Structurally, mTOR was shown to be a relatively large protein consisting of 2,549 amino acids, with a predicted molecular mass of 289 kDa (Sabers et al. 1995). Several important domains were identified in mTOR, including a C-terminal kinase domain, which is responsible for autophosphorylation and phosphorylation of downstream targets (Brown et al. 1995), and the FKBP12-rapamycin-binding (FRB) domain (Chen et al. 1995; Jungwon et al. 1996), as well as multiple N-terminal HEAT (Huntingtin, elongation factor 3, regulatory A subunit of PP2A, and Tor1)

motifs that are thought to mediate protein–protein interactions (Perry and Kleckner 2003). Evidence that mTOR did indeed participate in protein–protein interactions came from size exclusion chromatography experiments that showed that mTOR had an apparent molecular mass of ~1–2 MDa instead of 289 kDa (Fang et al. 2001; Kim et al. 2002). These reports strongly suggested the presence of other mTOR-associated proteins and, thus, the potential for mTOR to be part of a multicomponent protein complex. Indeed, in 2002, mTOR was reported to associate with a 150 kDa protein called the regulatory associated protein of mTOR (Raptor) (Hara et al. 2002; Kim et al. 2002). As well as binding to mTOR, Raptor was found to directly interact with known mTOR substrates, such as 4E-BP1 and $p70^{S6K1}$, via a short amino acid sequence called the TOS (mTOR Signaling) motif (Schalm and Blenis 2002; Nojima et al. 2003; Schalm et al. 2003). Around the same time, mTOR was also found to bind a 36 kDa protein called mammalian lethal with SEC13 protein 8 (mLST8; aka G-protein β-subunit-like protein, GβL), which functioned as a positive regulator of mTOR kinase activity (Loewith et al. 2002; Kim et al. 2003).

In 2004, the story of mTOR became significantly more complex with the identification of another mTOR-binding protein named Rictor (rapamycin-insensitive companion of mTOR) which had the effect of rendering mTOR essentially resistant to the inhibitory effect of rapamycin (Dos et al. 2004; Jacinto et al. 2004). Thus, now there were two mTOR complexes: the Raptor-associated rapamycin-sensitive mTOR complex 1 (mTORC1) and the Rictor-associated rapamycin-insensitive mTOR complex 2 (mTORC2) (Dos et al. 2004; Jacinto et al. 2004). Like mTORC1, mTORC2 was associated with mLST8 but was later shown to contain other distinct proteins such as mSIN1 (mammalian stress-activated protein kinase interacting protein 1) (Frias et al. 2006) and Protor-1 (protein observed with Rictor-1) (Pearce et al. 2007). Subsequent studies found that mTORC2 also had different downstream substrates than mTORC1, including members of the AGC kinase family, protein kinase B (PKB; aka Akt) (Sarbassov et al. 2005), protein kinase C (PKC) (Guertin et al. 2006), and serum- and glucocorticoid-induced protein kinase 1 (SGK1) (García-martínez and Alessi 2008). Furthermore, mTORC2 was shown to regulate processes distinct from mTORC1, such as actin cytoskeleton organization (Jacinto et al. 2004) and cell proliferation and survival (Goncharova et al. 2011). Although initially shown to be rapamycin resistant, more recent studies have shown that with prolonged exposure to rapamycin, the assembly of mTORC2, and thus mTORC2 signaling, can be inhibited, including in skeletal muscle (Sarbassov et al. 2006; Lamming et al. 2012).

In 2007, a previously identified insulin-sensitive protein called PRAS40 (proline-rich Akt substrate of 40 kDa) (Kovacina et al. 2003) was found to be another component of mTORC1 (Haar et al. 2007; Sancak et al. 2007; Wang et al. 2007). PRAS40 was shown to bind to Raptor via a modified TOS motif and inhibit mTORC1 kinase activity, possibly by inhibiting substrate binding to Raptor (Haar et al. 2007; Sancak et al. 2007; Wang et al. 2007). Upon stimulation by insulin, Akt-induced phosphorylation of PRAS40 resulted in its dissociation from mTORC1 and an increase in mTORC1 signaling (Haar et al. 2007; Sancak et al. 2007;

Wang et al. 2007). In addition, it was shown that activated mTORC1 could also phosphorylate PRAS40, thus further relieving PRAS40-mediated repression of mTORC1 signaling (Oshiro et al. 2007; Wang et al. 2008). Finally, in 2009, mTOR was found to associate with a 48 kDa protein named Deptor (dishevelled, egl-10, pleckstrin domain protein interacting with mTOR), which inhibited both mTORC1 and mTORC2 signaling (Peterson et al. 2009). Later, it was shown that activated mTORC1 and mTORC2 could phosphorylate Deptor, leading to its ubiquitination by the E3 ligase complex SCF βTrCP and subsequent degradation by the ubiquitin proteasome system (Duan et al. 2011; Gao et al. 2011; Zhao et al. 2011). Thus, activated mTORC1 and mTORC2 could mediate the degradation of Deptor and, in effect, create a positive feedback loop that further enhanced mTORC1 and mTORC2 signaling.

In summary, the unearthing of rapamycin led to the discovery of mTOR and has facilitated huge progress in our understanding of the regulation of cell growth and proliferation. Moreover, the identification of the accessory proteins that make up the rapamycin-sensitive mTORC1 (see Fig. 1) and the largely rapamycin-insensitive mTORC2 has further expanded our understanding of the roles mTOR plays in cell biology. It remains to be determined whether more mTOR-associated proteins will be discovered in the future, or even new mTOR complexes, which may further expand our understanding of cellular growth. Indeed, there is already speculation regarding the existence of an mTORC3 (e.g., (Alayev and Holz 2013)). Furthermore, future studies may also lead to the discovery of new functions and targets for mTOR that is free from any other associated proteins.

2.2 The Cellular Location of mTOR and mTORC1

Early non-muscle cell fractionation studies identified mTOR as a cytoplasmic protein that was associated with intracellular membranes (Withers et al. 1997; Sabatini et al. 1999). Consistent with these studies, mTOR has been shown to associate with the endoplasmic reticulum (ER) and Golgi apparatus in a variety of cell lines (Drenan et al. 2004). Another pool of mTOR has been found to be associated with the outer mitochondrial membrane, suggesting a potential role in the regulation of energy metabolism (Desai et al. 2002). Immunohistochemical studies have shown that mTOR is dispersed through the cytoplasm and, under certain conditions, could also adopt a more punctate appearance, perhaps indicating that certain conditions can induce mTOR translocation (Sabatini et al. 1999). A small amount of mTOR has also been found in the nucleus, and mTOR has been shown to shuttle between the cytoplasm and nucleus (Kim and Chen 2000; Bachmann et al. 2006). Further support for a nuclear pool of mTOR comes from studies showing an association between mTOR and the promoters of RNA polymerases I to III and other various transcription factors, including some involved in mitochondrial biogenesis (Cunningham et al. 2007; Kantidakis et al. 2010; Shor et al. 2010; Tsang et al. 2010).

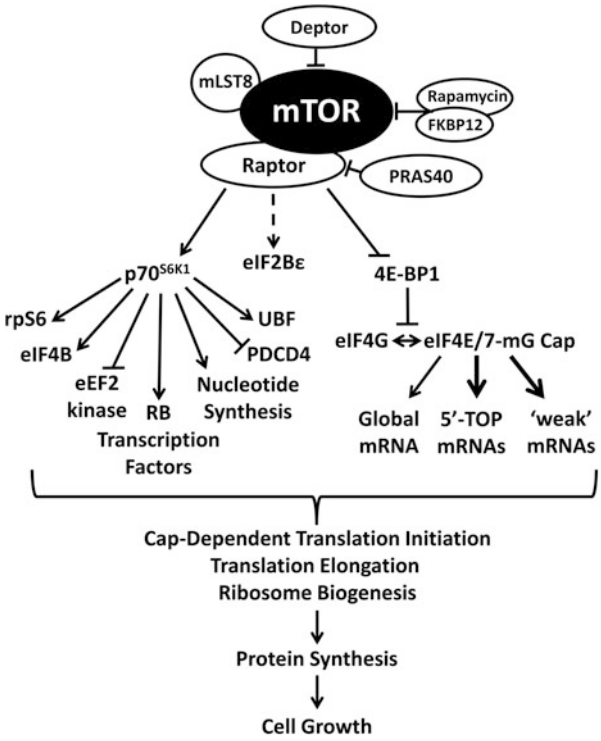

Fig. 1 *A summary of the protein components of mTORC1 and mTORC1's protein synthesis–related signaling targets.* mTORC1 (mTOR complex 1) is composed of mTOR (the mammalian/mechanistic target of rapamycin), Raptor (regulatory associated protein of mTOR), mLST8 (mammalian lethal with SEC13 protein 8), Deptor (dishevelled, egl-10, pleckstrin domain protein interacting with mTOR), and PRAS40 (proline-rich Akt substrate of 40 kDa). mTORC1 signaling can be inhibited by the rapamycin/FKBP12 (FK506-binding protein 12) complex and by PRAS40 and Deptor. Two of mTORC1's most studied protein synthesis–related targets are $p70^{S6K1}$ (ribosomal protein S6 p70 kinase 1) and 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1). $p70^{S6K1}$ is proposed to regulate protein synthesis, in part, by the direct phosphorylation of rpS6 (ribosomal protein S6), eIF4B (eukaryotic initiation factor 4B), eEF2 kinase (eukaryotic elongation factor kinase), PDCD4 (programmed cell death protein 4), and UBF (upstream binding factor) and, in part, by regulating the expression ribosome biogenesis (RB)-associated transcription factors and pyrimidine nucleotide synthesis. 4E-BP1 is proposed to regulate protein synthesis by inhibiting the interaction between the 7-mG (5′-7-methylguanosine) cap-bound eIF4E (eukaryotic initiation factor 4E) and eIF4G. mTORC1-induced phosphorylation of 4E-BP1 is proposed to relieve 4E-BP1's repression of eIF4E binding to eIF4G, leading to increased global cap-dependent translation initiation, and to increase the specific translation of mRNAs with highly structured 5′-untranslated regions (or "weak" mRNAs) and mRNAs that contain a 5′-tract of pyrimidines (5′TOP mRNAs). mTORC1 signaling has also been shown to increase the abundance of eIF2Bε, although the exact mechanism remains to be determined. Combined, mTORC1 signaling leads to an increase in cap-dependent translation initiation, translation elongation, and ribosome biogenesis, which, in turn, leads to an increase in protein synthesis and, potentially, cell growth

While these studies clearly indicate that mTOR is found in different cellular locations, they do not identify whether the mTOR being detected is part of mTORC1. More recent studies have, however, shed light on the possible locations of mTORC1. For instance, mTOR and Raptor are found in mitochondrial fractions and, interestingly, rapamycin treatment has been shown to significantly alter the mitochondrial phosphoproteome (Schieke et al. 2006). Raptor has also been found in the nucleus, suggesting the presence of nuclear mTORC1 (Rosner and Hengstschläger 2008). Moreover, mTORC1 has been found at the ER and has been proposed to play a role in ER stress signaling (Ramirez-Rangel et al. 2011; Appenzeller-Herzog and Hall 2012). Recent groundbreaking work has also shown that upon amino acid stimulation, mTOR and Raptor translocate to late endosomal/lysosomal (LEL) membrane structures where mTORC1 signaling is activated to transmit amino acid–induced anabolic signaling (Sancak et al. 2010).

To date, there is very little information regarding the cellular location of mTOR/mTORC1 in skeletal muscle cells. Recent work, however, has identified a pool of mTOR that is associated with LEL structures in mouse skeletal muscle. Moreover, this association was increased in response to mechanical activation (i.e., eccentric contractions), suggesting a possible mechanically induced translocation event (Jacobs et al. 2013b). It remains to be determined whether this LEL-associated mTOR is part of mTORC1 and whether the increased association with the LEL plays a significant role in the mechanical activation of mTORC1 signaling.

Overall, in non-muscle cells mTOR and mTORC1 are found in several different cellular compartments, which probably reflects their diverse and important range of functions within the cell (i.e., stress and nutrient sensing, gene transcription, energy metabolism, and protein degradation and synthesis). While some progress has been made, much more research is required to expand our understanding of the location/function relationship of mTORC1 in skeletal muscle and whether a specific pool of mTORC1 is activated by mechanical stimuli.

2.3 Protein Synthesis–Related mTORC1 Signaling Targets

To date, the two most studied direct targets of mTORC1 are the ribosomal protein S6 p70 kinase 1 ($p70^{S6K1}$) and the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Both of these mTORC1 targets are involved in the regulation of protein synthesis via their influence on translation initiation and elongation and ribosome biogenesis (Fig. 1). This section will briefly review the identification of $p70^{S6K1}$ and 4E-BP1 and how mTORC1 is proposed to regulate these two proteins and their downstream protein synthesis–related targets. Another potential protein synthesis–related target of mTORC1 signaling, which has received significantly less attention, is the abundance of the eukaryotic initiation factor 2B subunit ε (eIF2Bε). Thus, this section will also briefly describe the role of eIF2Bε in the regulation of protein synthesis and its possible regulation by mTORC1 in skeletal muscle.

2.3.1 Ribosomal Protein S6 p70 Kinase 1 ($p70^{S6K1}$)

In the late 1970s, insulin and the insulin-like growth factor were shown to stimulate the phosphorylation of the 40S ribosomal subunit–associated ribosome S6 protein (rpS6) in fibroblasts and pre-adipocytes (Haselbacher et al. 1979; Smith et al. 1979). Almost a decade later, a 70 kDa serine/threonine protein kinase was identified as a kinase that phosphorylated rpS6 and, as such, was named the p70 ribosomal protein S6 kinase ($p70^{S6K}$) but later changed to $p70^{S6K1}$ (Banerjee et al. 1990; Price et al. 1990; Shima et al. 1998). Similar to rpS6, $p70^{S6K1}$ was also found to be phosphorylated in response to insulin stimulation and, importantly, rapamycin was shown to inhibit insulin-stimulated $p70^{S6K1}$ phosphorylation (Chung et al. 1992; Kuo et al. 1992; Price et al. 1992). A search for the specific rapamycin-sensitive phosphorylation site(s) on $p70^{S6K1}$ revealed that, although the phosphorylation of the T389, T229, S404, and S411 residues was inhibited by rapamycin, the loss of T389 phosphorylation was most closely associated with the loss of $p70^{S6K1}$ activation (Pearson et al. 1995; Dennis et al. 1996). Indeed, the mutation of T389 to a non-phosphorylatable alanine residue (A389) completely abolished $p70^{S6K1}$ activity, providing further evidence that the phosphorylation of T389 by mTORC1 was a major event in the activation of $p70^{S6K1}$ (Pearson et al. 1995) [Note: It was later found that T229 phosphorylation by PDK1 (phosphoinositide-dependent kinase 1) was also required for full activation of $p70^{S6K1}$ activity (Alessi et al. 1998; Pullen et al. 1998)]. Combined, these data showed that the phosphorylation of the T389 residue is critical for $p70^{S6K1}$ function and that the T389 residue is a major rapamycin-sensitive phosphorylation site on $p70^{S6K1}$ and, thus, a major target of mTORC1 signaling. Because of the role of mTORC1 in the phosphorylation of $p70^{S6K1}$, combined with the relative ease of assessing T389 phosphorylation via a Western blot analysis, $p70^{S6K1}$ T389 phosphorylation remains one of the most commonly used readouts of changes in mTORC1 signaling in human, animal, and cell culture studies that investigate the mechanical activation of mTORC1 signaling in skeletal muscle. Interestingly, despite evidence that $p70^{S6K1}$ may play an important role in mTORC1-mediated muscle cell hypertrophy in response to IGF-1 and nutrients (Ohanna et al. 2005; Aguilar et al. 2007; Mieulet et al. 2007), it still remains to be determined whether mTORC1-mediated $p70^{S6K1}$ T389 phosphorylation is in fact necessary for mechanically induced increases in skeletal muscle protein synthesis and muscle mass.

2.3.2 $p70^{S6K1}$ Protein Synthesis–Related Signaling Targets

$p70^{S6K1}$ has the potential to regulate cell size, in part, due to its ability to regulate several factors involved in translation initiation and, thus, protein synthesis. For example, $p70^{S6K1}$ can phosphorylate the eukaryotic translation initiation factor 4B (eIF4B) on the S422 residue, which leads to an increased interaction with the

eIF3/preinitiation complex and the recruitment of the RNA helicase eIF4A (Holz et al. 2005; Shahbazian et al. 2006). Indeed, it has been shown that an S422D phosphomimetic mutant of eIF4B is sufficient to increase protein synthesis (Holz et al. 2005). Another $p70^{S6K1}$ target is the eukaryotic elongation factor 2 kinase (eEF2 kinase). eEF2 kinase phosphorylates eEF2 on the T56 residue, leading to its inhibition and the repression of the translation elongation process (Wang et al. 2001). $p70^{S6K1}$ inactivates eEF2 kinase by phosphorylating its S366 residue and relieving eEF2 kinase–induced inhibition of translation elongation (Wang et al. 2001). More recent evidence suggests that eEF2 kinase S366 phosphorylation may also be regulated in an mTORC1-dependent but $p70^{S6K1}$-independent manner in skeletal muscle cells (Mieulet et al. 2007). A third $p70^{S6K1}$ target with the potential to regulate protein synthesis is the tumor suppressor, the programmed cell death protein 4 (PDCD4). PDCD4 binds to the RNA helicase eIF4A and inhibits translation initiation (Suzuki et al. 2008; Loh et al. 2009). $p70^{S6K1}$-induced phosphorylation of PDCD4 on the S67 residue has been shown to lead to the degradation of PDCD4 by the ubiquitin proteasome system, resulting in an increase in protein synthesis (Dorrello et al. 2006; Zargar et al. 2011).

As mentioned above, another target of $p70^{S6K1}$ with the potential to regulate protein synthesis and cell size is rpS6. rpS6 is predominantly phosphorylated on five residues in response to serum stimulation: S235, S236, S240, S244, and S247 (Krieg et al. 1988; Ferrari et al. 1991; Bandi et al. 1993). As the phosphorylation of these sites is almost completely inhibited by rapamycin, mTORC1-activated $p70^{S6K1}$ is considered the predominant kinase responsible for rpS6 phosphorylation (Blenis et al. 1991; Chung et al. 1992). Thus, rpS6 phosphorylation (S235/236 and S240/244) is frequently used as a marker of changes in mTORC1 signaling. Recent studies have demonstrated, however, that relatively smaller contributions to rpS6 S235/236 phosphorylation are also made by Ras/ERK/RSK and cAMP-dependent kinase signaling pathways (Pende et al. 2004; Roux et al. 2007; Moore et al. 2009). The exact role that rpS6 plays in the regulation of protein synthesis and cell size remains unclear. Initially, because rpS6 was shown to interact with mRNA and translation initiation and elongation factors (Nygard and Nilsson 1990) and was located at the interface between the 40S and 60S ribosomal subunits, it was thought that rpS6 played a positive role in the regulation of protein synthesis (Uchiumi et al. 1986). Other data, however, showed that rpS6 phosphorylation was not sufficient to stimulate protein synthesis (Kruppa and Clemens 1984; Tas and Martini 1987; Montine and Henshaw 1990). Moreover, cells from a knock-in mouse model that expresses an rpS6 mutant that cannot be phosphorylated display an increase in protein synthesis (Ruvinsky et al. 2005), suggesting that phosphorylated rpS6 may in fact play an inhibitory role in the regulation of translation initiation and protein synthesis. Phosphorylated $p70^{S6K1}$ and rpS6 were also thought to play a role in the translation of a group of mRNAs, known as 5′-tract of pyrimidine (5′-TOP) mRNAs, that encode for translation factors and ribosomal proteins (Jefferies et al. 1994, 1997); however, this was not supported by later evidence (Pende et al. 2004; Ruvinsky et al. 2005). Thus, while rpS6 phosphorylation is

commonly used as an indirect marker of mTORC1 activation, the exact role that rpS6 plays in the regulation of protein synthesis and cell size remains to be determined.

Finally, mTORC1 and $p70^{S6K1}$ not only play a role in the acute regulation of translation initiation but may also play a role in the regulation of the number of ribosomes (i.e., ribosome biogenesis) and, thus, translational capacity (Mayer and Grummt 2006; Jastrzebski et al. 2007). Indeed, $p70^{S6K1}$ has been shown to be necessary and sufficient to increase ribosomal DNA transcription via the phosphorylation of the rDNA transcription factor, UBF (upstream binding factor) (Hannan et al. 2003). Furthermore, recent data shows that $p70^{S6K1}$ regulates the expression of a large proportion of genes involved in the ribosome biogenesis transcriptional program (Chauvin et al. 2013). Lastly, mTORC1-mediated signaling through $p70^{S6K1}$ is also required for growth signaling–induced increases in the de novo synthesis of pyrimidine nucleotides (i.e., cytosine, thymine, and uracil) (Ben-Sahra et al. 2013). This mechanism would appear to nicely link mTORC1-mediated DNA and RNA synthesis with an increase in the pool of the nucleotides required for these processes.

In summary, $p70^{S6K1}$ T389 phosphorylation is one of the most widely used markers of mTORC1 signaling. While the functional role of $p70^{S6K1}$-induced phosphorylation of rpS6 remains to be determined, $p70^{S6K1}$ activation by mTORC1 has the potential to play a significant role in the acute, and longer-term, regulation of protein synthesis by phosphorylating substrates involved in translation initiation and elongation (i.e., eIF4B, eEF2 kinase, and PDCD4) and by regulating the synthesis of rRNA and various transcription factors involved in ribosome biogenesis (Fig. 1). Despite these reported roles in the regulation of protein synthesis, it still remains to be determined whether mTORC1-mediated $p70^{S6K1}$ phosphorylation or $p70^{S6K1}$-mediated phosphorylation of rpS6, eIF4B, eEF2 kinase, or PDCD4 is necessary for mechanically induced increases in protein synthesis and skeletal muscle mass.

2.3.3 The Eukaryotic Initiation Factor 4E-Binding Protein 1 (4E-BP1)

In 1980, a hyper-phosphorylated protein, with an apparent mass of 22 kDa (later determined to be ~12.5 kDa), was isolated from insulin-treated rat adipose cells and shown to remain soluble after boiling and acid treatment (Belsham and Denton 1980). Because of these unusual properties, this protein was given the name PHAS-I (phosphorylated heat- and acid-stable protein regulated by insulin) (Hu et al. 1994). Later, the rat PHAS-I protein was found to be homologous with a human protein that bound to eIF4E, named 4E-BP1 (eIF4E-binding protein 1) (Pause et al. 1994) [Note: Two other 4E-BP isoforms, 4E-BP2 and 3, have also been discovered in mammals with similar functions to 4E-BP1 (Pause et al. 1994; Poulin et al. 1998)].

4E-BP1 has since been shown to play a crucial role in the regulation of cap-dependent translation initiation and, thus, protein synthesis. Specifically,

hypo-phosphorylated 4E-BP1 binds to eIF4E (which binds to the 7-methylguanosine "cap" structure found on the 5′-end of all nuclear-encoded mRNAs) and, in turn, inhibits cap-bound eIF4E from interacting with the scaffold protein eIF4G, the subsequent formation of the eIF4F complex, and the recruitment of the 40S ribosomal subunit (Mahoney et al. 2009). Thus, hypo-phosphorylated 4E-BP1 effectively suppresses the initiation of cap-dependent translation. Importantly, insulin and growth factors were shown to induce 4E-BP1 phosphorylation, which inhibited its ability to bind to eIF4E and relieved its repression of cap-dependent translation initiation. Furthermore, this insulin/growth factor–induced phosphorylation of 4E-BP1 was found to be inhibited by rapamycin (Graves et al. 1995; Azpiazu et al. 1996; von Manteuffel et al. 1996, 1997; Brunn et al. 1997b; Burnett et al. 1998). Significantly, this provided the first evidence that mTORC1 was responsible for the hyper-phosphorylation of 4E-BP1 and the associated increase in translation initiation.

4E-BP1 has numerous phosphorylation sites, and of these, T36, T45, S64, and T69 in rodent cells and T37, T46, S65, and T70 in humans are the most responsive to insulin stimulation (Fadden et al. 1997). While the phosphorylation of all these sites has been shown to be inhibited by rapamycin, albeit with differing sensitivities, mTORC1 has been reported to preferentially phosphorylate the T36 and T45 residues (Brunn et al. 1997a; Burnett et al. 1998; Gingras et al. 1999; Yang et al. 1999). Thus, 4E-BP1 is a major target of mTORC1 signaling and plays a significant role in the regulation of translation initiation and protein synthesis. While mechanically induced increases in 4E-BP1 phosphorylation are currently used as a marker of the activation of mTORC1 signaling in skeletal muscle, relatively little is known about the necessity of mTORC1-mediated phosphorylation of 4E-BP1 for increases in translation initiation, protein synthesis, and skeletal muscle mass in response to different types of mechanical activation.

2.3.4 Role of 4E-BP1 in Inhibiting the Translation of "Weak" and 5′TOP mRNAs

mTORC1-mediated phosphorylation of 4E-BP1 is proposed to not only play a role in the acute upregulation of translation initiation, and thus global rates of protein synthesis, but also in the selective translation of two groups of mRNAs that are crucial for cell growth and proliferation. The first of these groups is mRNAs that contain relatively long, highly structured, guanine/cytosine (GC)-rich 5′-untranslated regions (5′-UTR). It is proposed that because of the presence of the complex 5′-UTR structure, these mRNAs are translated with much less efficiency under basal conditions than mRNAs with relatively short and uncomplicated 5′-UTR structures, such as those found for "housekeeping" mRNAs [for a review, see (De Benedetti and Graff 2004)]. Indeed, by virtue of the fact that they are translated with less efficiency, mRNAs with highly structured 5′-UTRs are sometimes called "weak" mRNAs, while those with simple 5′-UTRs, which are translated more efficiently, are called "strong" mRNAs.

Importantly, "weak" mRNAs are reported to be particularly sensitive to changes in the availability of eIF4E (De Benedetti and Graff 2004). Thus, mTORC1-mediated phosphorylation of 4E-BP1, and its subsequent dissociation from eIF4E, effectively increases eIF4E availability and may induce an increase in the translation of these "weak" mRNAs that facilitate cell growth. Relevant examples of "weak" mRNAs that are known to play a significant role in cell growth are cyclin D1, c-Myc, and ornithine decarboxylase. Thus, mechanical stimuli may, in part, regulate skeletal muscle mass by increasing mTORC1/eIF4E-mediated translation of "weak" growth-related mRNAs. Interestingly, cyclin D1, c-Myc, and ornithine decarboxylase have previously been proposed to play a role in mechanically induced skeletal muscle hypertrophy (Alway 1997; Armstrong and Esser 2005; White et al. 2009; Lee and MacLean 2011).

The second group of mRNAs that are regulated by the mTORC1-mediated phosphorylation of 4E-BP1 are those that contain a tract of pyrimidines (TOP) in their 5′-UTR, known as 5′-TOP mRNAs (Huo et al. 2012; Thoreen et al. 2012). 5′-TOP mRNAs are characterized as containing a cytidine nucleoside immediately downstream of the 7-methylguanosine cap structure, which is followed by a sequence, or tract, of 4–15 pyrimidine nucleotides (cytosine or uracil). Importantly, 5′TOP mRNAs typically encode for translation initiation and elongation factors and ribosomal proteins (Meyuhas and Dreazen 2009; Thoreen et al. 2012). Thus, an increase in the translation of 5′-TOP mRNAs has the potential to increase protein synthesis by increasing both translation initiation and ribosome biogenesis. The translation of 5′-TOP mRNAs is increased by serum, amino acid, and growth factor stimulation and is partially inhibited by rapamycin (Jefferies et al. 1994; Tang et al. 2001; Stolovich et al. 2002). Initially, an increase in the translation of 5′-TOP mRNAs was thought to be correlated with increased $p70^{S6K1}$ and rpS6 phosphorylation (Jefferies et al. 1994, 1997); however, rapamycin-sensitive 5′-TOP mRNA translation was later found to be normal in $p70^{S6K1}\&^{2}$-deficient and rpS6 phosphorylation–deficient cells (Pende et al. 2004; Ruvinsky et al. 2005). More recently, however, 5′-TOP mRNA translation has been found to be highly sensitive to changes in mTORC1-mediated 4E-BP1 phosphorylation. For example, it has recently been shown that while the ATP-competitive mTOR inhibitor Torin1 markedly inhibited protein synthesis in wild-type cells, it had a very minimal effect on protein synthesis in cells deficient in both 4E-BP1 and 2 (Thoreen et al. 2012). Moreover, Torin1 also reduced 5′-TOP mRNA translation in wild-type, but not 4E-BP1/2-deficient, cells (Thoreen et al. 2012). This data indicates that, at least in non-muscle cells, mTORC1-mediated phosphorylation of 4E-BPs plays a major role in the regulation of 5′-TOP mRNA translation and global protein synthesis. However, the importance of mTORC1-mediated 5′-TOP mRNA translation for mechanically induced increases in protein synthesis and skeletal muscle mass still remains to be determined.

In summary, mTORC1-mediated 4E-BP1 phosphorylation has the potential to increase global rates of protein synthesis not only by an acute increase in

eIF4E-mediated cap-dependent translation initiation but also by a selective increase in the translation of growth-related "weak" and 5′-TOP mRNAs that are vitally important for facilitating cell growth (Fig. 1). Significantly more research is required to determine the necessity of these events for increases in protein synthesis and muscle mass in response to mechanical stimuli.

2.3.5 The Eukaryotic Initiation Factor 2B Subunit ε (eIF2Bε)

The eIF2Bε is a subunit of the eIF2B holoenzyme which, in turn, makes up part of the larger eIF2 complex. The eIF2 complex plays a key role in the process of translation initiation. Specifically, in a GTP-dependent manner, eIF2 delivers the initiator tRNA (Met-tRNA$_i$) to the ribosome during the formation of the 48S preinitiation complex [for reviews, see (Kimball 1999; Proud 2005)]. The GTP on eIF2 is subsequently hydrolyzed to GDP, causing the release of GDP-bound eIF2 and other initiation factors from the 48S preinitiation complex, which ultimately allows the formation of the functional 80S ribosome and the commencement of translation elongation (Kimball 1999; Proud 2005). In order to participate in another round of translation initiation, the GDP bound to eIF2 is exchanged for GTP, a reaction that is catalyzed by the eIF2B holoenzyme (Kimball 1999; Proud 2005). Importantly, the eIF2Bε subunit serves the catalytic function of eIF2B and its activity can be regulated by a variety of stimuli, including hormones, nutrients, and mechanical loading (Gilligan et al. 1996; Welsh et al. 1996; Kimball et al. 1998; Kostyak et al. 2001; Bush et al. 2003).

Recent studies have shown that the overexpression of eIF2Bε is sufficient to induce an increase in eIF2B activity and protein synthesis, while knockdown of eIF2Bε results in a decrease in protein synthesis (Gallagher et al. 2008; Kubica et al. 2008). Together, these observations suggest that the abundance of eIF2Bε may be a rate-limiting factor for protein synthesis. Several studies have also shown that, in skeletal muscle, the activity of eIF2B correlates well with the changes in protein synthesis that occur in response to various conditions including increased mechanical loading (Kimball et al. 1998; Kostyak et al. 2001; Tuckow et al. 2010). Moreover, previous studies have shown that eIF2Bε abundance is elevated by increased mechanical loading (Kostyak et al. 2001; Kubica et al. 2004, 2005; Fluckey et al. 2006; Mayhew et al. 2011) and that overexpression of eIF2Bε is sufficient to induce muscle fiber hypertrophy in vivo (Mayhew et al. 2011). Importantly, recent reports also show that rapamycin is sufficient to inhibit leucine-, IGF-1-, and resistance exercise–induced increases in the abundance of eIF2Bε, indicating that mTORC1 signaling is necessary to increase eIF2Bε abundance (Kubica et al. 2005, 2008); however, the mechanism via which mTORC1 regulates eIF2Bε remains to be determined. Nevertheless, mTORC1-mediated increases in the abundance of eIF2Bε and a subsequent increase in eIF2B activity may play a significant role in regulating mechanical load–induced increases in protein synthesis and muscle mass.

2.4 New-Generation ATP-Competitive mTOR Inhibitors and Their Implications for Understanding mTORC1-Mediated Processes

As indicated above, rapamycin forms a complex with FKBP12, which then binds to the FRB domain of mTOR and acts as an allosteric inhibitor of mTOR kinase activity. The mechanism behind rapamycin's inhibitory effect has been suggested to involve the dissociation of Raptor from mTOR leading to reduced mTOR interaction with its Raptor-bound substrates (Oshiro et al. 2004), competition between the inhibitory rapamycin/FKBP12 complex and the mTOR activator phosphatidic acid (PA) for binding to the mTOR FRB domain (Fang et al. 2001), and/or reduced mTOR-mediated repression of protein phosphatase activity (Peterson et al. 1999). A recent crystallography study has shed further light on this issue, demonstrating that rapamycin/FKBP12 complex binding to the FRB domain effectively blocks the binding of mTOR substrates to mTOR and restricts their access to the active catalytic site (Yang et al. 2013).

While rapamycin has played a pivotal role in the discovery of various mTORC1 substrates, the difference in the efficacy of rapamycin to inhibit protein synthesis and cell cycle progression in yeast compared to mammalian cells prompted the suggestion that some mTORC1-mediated events may be resistant to the inhibitory effects of rapamycin in mammalian cells (Thoreen and Sabatini 2009). This observation, in part, led to the development of a new class of small-molecule mTOR inhibitors that function by directly competing with ATP binding to mTOR (e.g., PP242, Torin1, and WYE-354) and, thus, directly inhibiting mTOR kinase activity (Feldman et al. 2009; Thoreen et al. 2009; Yu et al. 2009). Although these drugs inhibit mTOR in both mTORC1 and mTORC2, using cells that lack Rictor and, thus, mTORC2, Thoreen et al. (2009) showed that Torin1 inhibited protein synthesis to a greater extent than rapamycin and confirmed the presence of rapamycin-resistant, but mTORC1-dependent, events within mammalian cells (Thoreen et al. 2009). This finding is potentially very important because events that have previously been reported to be partially rapamycin sensitive, or even mTORC1 independent, may in fact be mediated by mTORC1.

Another important implication of these new findings is that caution should be used when using a marker of mTORC1 signaling (e.g., $p70^{S6K1}$ T389 phosphorylation) as the only readout to indicate that rapamycin has fully inhibited mTORC1 signaling. This caution is warranted because studies have shown that rapamycin has differential effects on the phosphorylation of $p70^{S6K1}$ compared to 4E-BP1, such that $p70^{S6K1}$ T389 phosphorylation is strongly inhibited by rapamycin whereas 4E-BP1 T37/46 phosphorylation is largely resistant to rapamycin-induced inhibition [e.g., (Choo et al. 2008; Thoreen et al. 2009)]. Indeed, Kang et al. (2013) recently characterized the $p70^{S6K1}$ T389 and 4E-BP1 S64 residues as "poor" mTORC1 substrates and, thus, easily inhibited by rapamycin. Conversely, the 4E-BP1 T37/46 residues were characterized as "good" mTORC1 substrates and, thus, markedly less sensitive to rapamycin inhibition (Kang et al. 2013).

The implication of this new data is that although rapamycin may fully inhibit $p70^{S6K1}$ T389 phosphorylation, depending on the dose and duration of exposure, this does not necessarily mean that mTORC1 activity toward "good" targets like 4E-BP1 T37/46 has been fully inhibited. While the use of the ATP-competitive inhibitors may seem like a potential solution to the limitations of rapamycin, these could prove to be just as problematic as they inhibit both mTORC1 and mTORC2 signaling which could introduce a range of confounding effects that severely limit data interpretation. Overall, the findings obtained with the new ATP-competitive mTOR inhibitors show that rapamycin does not completely inhibit all of mTORC1's signaling events and suggest that mTORC1 may have more downstream, rapamycin-insensitive, targets than previously recognized. Moreover, the relative role(s) of "poor" versus "good" mTORC1 substrates in mechanically induced increases in protein synthesis and skeletal muscle mass remain to be determined.

3 The Role of mTORC1 in the Regulation of Mechanical Stimuli–Induced and Basal Rates of Protein Synthesis

It is well known that mTORC1 signaling regulates protein synthesis and cell size in a variety of cell types, including muscle cells, in response to mitogens such as insulin or IGF-1 [e.g., (Dardevet et al. 1996; Rommel et al. 2001; Glass 2010; Schiaffino and Mammucari 2011)]. Over the last ~15 years, a significant body of evidence has also developed describing the role that mTORC1 plays in the regulation of skeletal muscle mass and protein synthesis in response to different types of mechanical activation. In this section, we will review the evidence, from rodent and human studies, for whether mTORC1 is activated by overload/resistance, endurance, and sprint types of exercise. Furthermore, we will examine whether there is evidence showing that mTORC1 signaling is necessary for changes in protein synthesis and muscle mass in response to these types of exercise. Finally, the role of mTORC1 signaling in basal protein synthesis and muscle mass will also be explored.

3.1 Evidence for the Role of mTORC1 in the Regulation of Muscle Mass and Protein Synthesis in Response to Increased Mechanical Loading or Resistance Exercise

Resistance exercise is the most commonly used model of mechanical overload for increasing strength and skeletal muscle mass. Using this model, numerous human studies have shown that acute resistance exercise induces an increase in muscle protein synthesis rates for up to 48 h post exercise [e.g., (Chesley et al. 1992; MacDougall et al. 1995; Phillips et al. 1997; Welle et al. 1999; Trappe et al. 2002; Dreyer et al. 2008)] and that prolonged resistance training results in significant

whole muscle and muscle fiber hypertrophy [e.g., (Cureton et al. 1988; Staron et al. 1990; McCall et al. 1996; Kraemer et al. 2004; Leger et al. 2006; Thalacker-Mercer et al. 2013)]. Given mTORC1's proposed roles in the regulation of translation and cell growth, we will next examine evidence from human and rodent studies for whether mTORC1 signaling plays a role in inducing an increase in protein synthesis and muscle mass in response to resistance-type exercise.

3.1.1 Rodent Studies

Baar and Esser (1999) performed the first study to examine whether the increase in muscle mass that occurs in response to high-resistance exercise training was associated with an increase in mTORC1 signaling (Baar and Esser 1999). This study showed that the increase in the mass of rat muscles subjected to 6 weeks of high-intensity eccentric contraction training was highly correlated with the magnitude of the increase in $p70^{S6K1}$ phosphorylation at 6 h after the first exercise bout. Furthermore, they found that eccentric contractions induced an increase in the size of the polysome pool, an indicator of increased translation initiation. Thus, this study provided the first evidence that increased mechanical loading was associated with increased mTORC1 signaling, protein synthesis, and muscle hypertrophy. Since this seminal study, numerous rodent studies have reported increased mTORC1 signaling in response to various forms of acute and chronic mechanical overload models, including stretched cultured myoblasts and myotubes (Hornberger et al. 2005a; Frey et al. 2009; Nakai et al. 2010; Sasai et al. 2010), ex vivo passive stretch of isolated muscles (Hornberger et al. 2004, 2005b, 2006; Hornberger and Chien 2006; You et al. 2012), in vivo maximal isometric contractions (Ogasawara et al. 2013), in vivo high-resistance eccentric and concentric contractions (Parkington et al. 2003; Burry et al. 2007; Thomson et al. 2008; O'Neil et al. 2009; Witkowski et al. 2010), and chronic mechanical overload induced by synergist ablation (SA) surgery (Hornberger et al. 2003; Thomson and Gordon 2006; Spangenburg et al. 2008; Thomson et al. 2009; Goodman et al. 2011a; Miyazaki et al. 2011). However, the first evidence that mTORC1 signaling was not only associated with, but also necessary for, mechanical load–induced growth came from the study of Bodine et al. (2001). In this study, rapamycin was shown to inhibit SA-induced muscle hypertrophy, and this was associated with an inhibition of $p70^{S6K1}$ phosphorylation, an increase in 4E-BP1/eIF4E binding, and a decrease in eIF4E/eIF4G interaction (Bodine et al. 2001).

One issue that remained after the study of Bodine et al. (2001) was that, because systemic administration of rapamycin would be expected to inhibit mTORC1 signaling in all cells throughout the body, it was not clear if the anti-hypertrophic effects of rapamycin were due to the inhibition of mTORC1 signaling in skeletal muscle cells per se, or other cell types within skeletal muscle tissue (e.g., immune cells), or distant effects in other tissues. This was an important point because several studies had shown that immune cells may play an important role in mechanically induced skeletal muscle hypertrophy (DiPasquale et al. 2007; Marino et al. 2008; Novak et al. 2009; Dearth et al. 2013), and rapamycin potentially could inhibit their

ability to proliferate and function normally. Thus, using mice with skeletal muscle cell-specific expression of rapamycin-resistant (RR) or rapamycin-resistant kinase dead (RRKD) mutants of mTOR, Goodman et al. (2011a) investigated this issue and demonstrated that, like Bodine et al. (2001), rapamycin inhibited SA-induced increase in $p70^{S6K1}$ T389 phosphorylation and muscle fiber hypertrophy in wild-type mice (Goodman et al. 2011a). Importantly, however, both of these effects of rapamycin were rescued in RR mice but not in RRKD mice. These results clearly demonstrated that mTOR/mTORC1 kinase activity, specifically in skeletal muscle cells, and not in other cell types (e.g., immune cells), was required for the SA-induced hypertrophic response (Goodman et al. 2011a). More recently, 7 and 28 days of SA-induced chronic mechanical overload failed to induce muscle fiber hypertrophy in mice lacking Raptor expression, and thus mTORC1, specifically in skeletal muscle (Bentzinger et al. 2013). Combined, these studies show that increased mechanical loading is sufficient to activate skeletal muscle mTORC1 signaling and that skeletal muscle cell-specific mTORC1 kinase activity is necessary for muscle fiber hypertrophy induced by increased mechanical loading.

It has long been known that increased mechanical loading of rodent skeletal muscle results in an increase in protein synthesis and that this is, in part, responsible for load-induced muscle hypertrophy (Goldberg et al. 1975; Booth et al. 1982). This suggests that mTORC1 signaling may play a role in the acute mechanical load–induced increases in protein synthesis. Indeed, the activation of mTORC1 signaling by the GTPase Rheb, one of the most proximal activators of mTOR, has been shown to be sufficient to increase muscle fiber protein synthesis and cross-sectional area in vivo (Goodman et al. 2010, 2011b), providing further support for the idea that mechanical activation of mTORC1 may play an important role in the regulation of protein synthesis. More direct evidence to support this idea comes from Hornberger et al. (2004) who showed that the increase in mTORC1 signaling and protein synthesis, induced by ex vivo passive stretch of mouse EDL muscles, was completely inhibited in the presence of rapamycin (Hornberger et al. 2004). Similarly, Kubica et al. (2005) demonstrated that rapamycin inhibited the in vivo resistance exercise–induced increase in mTORC1 signaling, translation initiation, and protein synthesis at 16 h post exercise in rat skeletal muscle (Kubica et al. 2005). Combined, these few studies show that mTORC1 signaling is necessary for the increase in protein synthesis in rodent skeletal muscle, in response to increased mechanical loading.

In summary, studies in rodents clearly show that (1) increased mechanical loading is sufficient to activate mTORC1 signaling, (2) activation of mTORC1 is sufficient to induce an increase in protein synthesis and muscle fiber hypertrophy, and (3) mTORC1 kinase activity is necessary for mechanical load–induced increases in protein synthesis and muscle hypertrophy.

3.1.2 Human Studies

As mentioned above, many studies have shown that resistance exercise is sufficient to induce an increase in protein synthesis and stimulate skeletal muscle

hypertrophy. Moreover, many studies have also shown that acute heavy resistance exercise is sufficient to induce a significant increase in markers of mTORC1 signaling (typically, $p70^{S6K1}$ or 4E-BP1 phosphorylation) [e.g., (Deldicque et al. 2005; Cuthbertson et al. 2006; Dreyer et al. 2006; Eliasson et al. 2006; Glover et al. 2008; Witard et al. 2009; Holm et al. 2010)]. Indeed, similar to the rodent study of Baar and Esser (1999), Terzis et al. (2008) showed in humans that the increase in $p70^{S6K}$ T389 phosphorylation, 30 min after the first resistance training bout, was highly correlated with measures of whole muscle and muscle fiber hypertrophy induced by 14 weeks of high-intensity resistance training (Terzis et al. 2008). These data show that mTORC1 signaling is activated by resistance exercise in human skeletal muscle and, similar to rodent studies, suggests that mTORC1 signaling may also play a major role in mediating resistance exercise–induced increases in skeletal muscle mass in humans. To date, however, there are currently no studies that have used rapamycin to investigate whether mTORC1 signaling is necessary for resistance training–induced muscle hypertrophy. While more studies are needed to clarify this question, there may be significant issues in the approval of studies that aim to administer rapamycin, or another mTOR/mTORC1 inhibitor, for a prolonged period of time to human subjects.

In regard to protein synthesis, to date, only one human study has used rapamycin to determine whether mTORC1 signaling regulates skeletal muscle protein synthesis in response to an acute bout of resistance exercise. This study by Drummond et al. (2009) found that rapamycin completely inhibited the early (1–2 h) resistance exercise–induced increase in protein synthesis (Drummond et al. 2009). Unlike the rodent studies, however, rapamycin only delayed or partially inhibited markers of mTORC1 signaling over this time period. Indeed, rapamycin inhibited the resistance exercise–induced increase in $p70^{S6K1}$ T389 phosphorylation 1 h, but not 2 h, post exercise, and had no effect on 4E-BP1 T37/46 phosphorylation. The reason for the apparent discrepancy between this study and the in vivo rat study of Kubica et al. (2005) in the ability of rapamycin to inhibit markers of mTORC1 signaling could be related to the dose of rapamycin (~0.15 mg/kg vs. 0.75 mg/kg, respectively) and time point after exercise (1–2 h vs. 16 h, respectively). The study of Drummond et al. may also suggest that a complete inhibition of mTORC1 signaling may not be required to fully inhibit the resistance exercise–induced increase in protein synthesis. Indeed, perhaps acute mechanically induced increases in protein synthesis are predominantly driven by mTORC1 signaling targets that are highly sensitive to rapamycin, such as $p70^{S6K1}$ T389 phosphorylation, and not by the more rapamycin-resistant mTORC1 substrates, such as 4E-BP1 T37/46 phosphorylation. Alternatively, the post-resistance-exercise-induced increase in protein synthesis in humans may be mediated, in part, by a rapamycin-sensitive mTOR that is not part of the traditional mTORC1. Further studies are therefore required to better determine the requirement of mTORC1 signaling for resistance exercise–induced increases in protein synthesis and muscle mass in humans.

In summary, heavy resistance exercise in humans is sufficient to increase post-exercise markers of mTORC1 signaling, protein synthesis, and induce skeletal

muscle hypertrophy. To date, one study has reported that the acute resistance exercise–induced increase in muscle protein synthesis is inhibited by a relatively low dose of rapamycin, suggesting that mTORC1 signaling is necessary for this event. Furthermore, no human studies have investigated the necessity of mTORC1 for resistance training–induced skeletal muscle hypertrophy. Clearly, more studies are required to establish the role of mTORC1 signaling in response to resistance exercise in humans.

3.2 Evidence for the Role of mTORC1 Signaling in the Regulation of Protein Synthesis in Recovery from Acute Endurance Exercise

In contrast to resistance exercise, endurance exercise is characterized by the repetition of low to moderate force contractions over a prolonged period of time. While there is significant evidence that protein synthesis is depressed during acute endurance exercise [for a review, see (Rose and Richter 2009)], several studies have shown an increase in muscle protein synthesis for many hours after endurance exercise [e.g., (Carraro et al. 1990; Sheffield-Moore et al. 2004; Miller et al. 2005; Harber et al. 2009a; Mascher et al. 2011)]. This increase in protein synthesis during recovery from endurance exercise may be due to the increased synthesis of mitochondrial proteins and/or the turnover of proteins damaged during the exercise bout [e.g., calcium-activated protease- or oxidative stress–induced damage (Overgaard et al. 2004; Seene et al. 2011)]. Moreover, endurance training has also been shown to increase resting muscle protein synthesis (Short et al. 2004; Pikosky et al. 2006) and induce whole muscle and muscle fiber hypertrophy in relatively sedentary subjects [e.g., (Verney et al. 2008; Harber et al. 2009b; Hudelmaier et al. 2010; Konopka et al. 2010; Lovell et al. 2010; McPhee et al. 2010)], albeit to a lesser extent than that induced by resistance training. Given the role of mTORC1 in the regulation of resistance exercise–induced increases in protein synthesis, could there also be a role for mTORC1 in mediating post-endurance-exercise increases in protein synthesis? Unfortunately, to date, there are no studies that have performed acute endurance exercise in combination with rapamycin administration to determine whether mTORC1 plays a necessary role in endurance exercise–induced increases in post-exercise protein synthesis. Nevertheless, some progress has been made in relation to the effect of endurance exercise on mTORC1 signaling and/or protein synthesis in rodents and humans.

3.2.1 Rodent Studies

To date, only a few studies have examined the effect of prolonged repeated contractions or endurance-type exercise on post-exercise mTORC1 signaling and/or protein

synthesis in rodent skeletal muscle. Some of the first studies showed that skeletal muscle protein synthesis rates were depressed 1 h after a 2 h bout of treadmill running in rats that were fasted or fed a carbohydrate meal immediately post exercise (Gautsch et al. 1998; Anthony et al. 2007). This depressed protein synthesis was associated with either no change or a decrease in $p70^{S6K1}$ phosphorylation, a reduction in 4E-BP1 phosphorylation, a reduction in the eIF4E/eIF4G binding, and an increase in eIF4E/4E-BP1 binding (Gautsch et al. 1998; Anthony et al. 2007). Later, using prolonged (3 h) ex vivo low frequency (10 Hz) stimulation of rat skeletal muscle, Atherton et al. (2005) found a trend for increases in myofibrillar and sarcoplasmic protein synthesis 3 h post exercise. Interestingly, this was associated with reduced $p70^{S6K1}$ T389 and 4E-BP1 T37/46 phosphorylation, indicating an inhibition of mTORC1 signaling (Atherton et al. 2005). While this study suggests a dissociation between post-stimulation protein synthesis and mTORC1 signaling, the reduced mTORC1 signaling may, in part, be due to the absence of amino acids in the incubating medium (Atherton et al. 2005). More recently, Edgett et al. (2013) reported that over 2 h of exhaustive treadmill running in rats induced an increase in $p70^{S6K1}$ T389, but not 4E-BP1 T37/46, phosphorylation immediately post exercise, an effect that had largely receded by 3 h post exercise (Edgett et al. 2013). Finally, Ito et al. (2013a) recently reported a significant increase in $p70^{S6K1}$ T389 phosphorylation immediately after 30 min of forced incremental-speed treadmill running in mice (Ito et al. 2013a). Overall, these few studies highlight that there is currently no clear picture for the effect of acute endurance exercise on post-exercise mTORC1 signaling and protein synthesis in rodents. The differences in results between these studies may be due to differences in species, contraction/exercise protocols, nutritional status, and/or sample timing. Clearly, more studies are required to obtain a more definitive answer to the question of whether mTORC1 plays any role in the acute response, and more chronic adaptation, to endurance exercise in rodents.

3.2.2 Human Studies

Several human studies have reported that acute endurance exercise (one- or two-legged cycling) is sufficient to increase mTORC1 signaling at various early time points after exercise (0.5–3 h) in relatively well-trained subjects [e.g., (Coffey et al. 2006; Mascher et al. 2007; Benziane et al. 2008; Camera et al. 2010; Wang et al. 2011)]. Furthermore, a few human studies have examined changes in both protein synthesis and markers of mTORC1 signaling after acute endurance exercise. Of these, Wilkinson et al. (2008) found that $p70^{S6K1}$ T389 phosphorylation was elevated approximately twofold immediately after 45 min of one-legged cycling (75 % VO_{2peak}) in fed trained and untrained subjects. Moreover, this increase in mTORC1 signaling was associated with an increase in mitochondrial, but not myofibrillar, protein synthesis, over the first 4 h post exercise (Wilkinson et al. 2008). Also, Mascher et al. (2011) reported an increase in mixed muscle protein synthesis at 3 h after a 60 min bout of one-legged cycling (65–70 % VO_{2max}) in fasted trained subjects which was associated with increased $p70^{S6K1}$

T389 phosphorylation at 90 min and 3 h post exercise (Mascher et al. 2011). Furthermore, Beelen et al. (2011) reported an increase in mixed muscle protein synthesis during 2 h of cycling (55 % W_{max}) in fed trained subjects which corresponded with increased $p70^{S6K1}$ T389, but not 4E-BP1 T37, phosphorylation (Beelen et al. 2011). Finally, it was recently shown that 1 h of cycling (72 % VO_{2peak}) was sufficient to increase mixed muscle protein synthesis (~60 %) at 2 h post exercise in active, but untrained, subjects (Harber et al. 2010; Reidy et al. 2013). Interestingly, at this time point there was no significant exercise-induced increase in $p70^{S6K1}$ T389 or 4E-BP1 T37/46 phosphorylation (Harber et al. 2010; Reidy et al. 2013). While these results suggest that mTORC1 signaling was not activated by the endurance exercise, mTORC1 signaling may have been elevated at an earlier time and subsequently subsided.

Overall, unlike the rodent studies, many human studies show that acute endurance cycling exercise is sufficient to induce an increase in post-exercise mTORC1 signaling, while a limited number of studies have found an associated increase in post-exercise muscle protein synthesis. This increase in muscle protein synthesis may, in part, be due to an increase in the synthesis of new mitochondrial proteins and/or the replacement of proteins damaged by reactive oxygen species or Ca^{2+}-activated proteases. Whether mTORC1 signaling is required for the increase in protein synthesis and for endurance training–induced muscle fiber hypertrophy still remains to be determined. Lastly, there are currently no human studies that have examined the effect of prolonged running or swimming exercise on post-exercise mTORC1 signaling or protein synthesis. Thus, significantly more research is required to obtain a better understanding of the role of mTORC1 signaling in mediating the acute responses and chronic adaptations to endurance exercise.

3.3 Evidence for the Activation of mTORC1 Signaling by High-Intensity Sprint Exercise

Sprint exercise is characterized by high-velocity, high-power-output contractions over relatively short periods of time. Some, but not all, studies have reported that sprint training can induce increases in muscle fiber size [for a review, see (Ross and Leveritt 2001)]. Thus, sprint training–induced muscle fiber hypertrophy could, in part, be due to an mTORC1-mediated increase in protein synthesis. Unfortunately, however, to date no studies have examined whether single or repeated sprints are sufficient to increase muscle protein synthesis rates above basal levels. There are, however, a few recent studies that have examined changes in mTORC1 signaling in response to acute sprint exercise. For example, Gibala et al. (2009) reported no change in $p70^{S6K1}$ T389 or 4E-BP1 T37/46 phosphorylation in male subjects immediately after a single maximal 30 s cycling sprint, immediately after four 30 s sprints separated by 4 min rest periods, or 3 h after the fourth sprint (Gibala et al. 2009). Coffey et al. (2011) showed that ten 6 s cycling sprints in fasted male subjects (placebo group) tended to increase $p70^{S6K1}$ T389 phosphorylation

and induced a decrease in 4E-BP1 T37/46 phosphorylation 15 min post exercise, with both of these returning to basal levels by 4 h post exercise (Coffey et al. 2011). More recently, Esbjörnsson et al. (2012) reported a significant increase in $p70^{S6K1}$ T389 phosphorylation in fasted physically active men and women 2 h after performing three 30 s cycling sprints with 20 min rest periods between sprints (Esbjörnsson et al. 2012). Moreover, the increase in $p70^{S6K1}$ T389 phosphorylation was greatest in female subjects, suggesting sex-based differences in sprint exercise–induced mTORC1 signaling. Finally, Rundqvist et al. (2013) had physically active subjects (predominantly male) perform three 30 s maximal cycling sprints, each separated by a 20 min rest period, combined with either repeated ingestion of a nutritional supplement (essential amino acids and carbohydrate) or a placebo. The results showed that at 140 min post exercise, $p70^{S6K1}$ T389 phosphorylation remained unchanged in the placebo condition but increased ~15-fold with ingestion of the nutritional supplement (Rundqvist et al. 2013).

Overall, significant variation exists in the limited results from a few studies that have investigated the effect of acute sprint exercise on skeletal muscle mTORC1 signaling and protein synthesis. This variability is likely due, in part, to differences in the duration and number of sprint bouts, rest period duration, nutritional status, the timing of post-exercise sampling, and the sex, age, and training status of the subjects. Thus, more studies are required to investigate whether sprint exercise is sufficient to increase skeletal muscle protein synthesis and, if so, whether mTORC1 signaling is necessary for this to occur.

3.4 Does mTORC1 Signaling Regulate Basal Protein Synthesis and Resting Skeletal Muscle Mass?

With the exception of during sleep, even basic daily activities involve performing muscle contractions of varying number, intensity, and duration, and these contractions have the potential to play a role in the regulation of protein synthesis and muscle mass. Thus, it is also of interest to ask whether mTORC1 signaling plays a role in maintaining basal protein synthesis rates and/or muscle mass.

3.4.1 Protein Synthesis

Insights into the question of whether mTORC1 signaling is necessary for the regulation of basal protein synthesis rates in skeletal muscle come from a limited number of rodent and human studies that have used the mTORC1 inhibitor, rapamycin. For example, Hornberger et al. (2004) reported that 2 h ex vivo incubation of mouse EDL muscles with a dose of rapamycin that was sufficient to inhibit passive stretch–induced increases in mTORC1 signaling and protein synthesis did not significantly reduce basal protein synthesis despite completely inhibiting basal

$p70^{S6K1}$ T389 phosphorylation (Hornberger et al. 2004). Furthermore, Kubica et al. (2005) showed that an in vivo injection of rapamycin, at a dose sufficient to inhibit resistance exercise–induced increases in mTORC1 signaling and protein synthesis, had no effect on basal protein synthesis despite significantly reducing basal $p70^{S6K1}$ and 4E-BP1 phosphorylation (Kubica et al. 2005). Similar to these rodent studies, human studies from the Rasmussen laboratory found that oral doses of rapamycin (administered 2 h prior to basal sample collection) that were sufficient to inhibit resistance exercise–induced and essential amino acid–induced increases in mTORC1 signaling and protein synthesis had no effect on basal protein synthesis (Drummond et al. 2009; Dickinson et al. 2011, 2013). It is worth noting, however, that although the dose of rapamycin used in these human studies did not affect basal protein synthesis, it also did not fully inhibit mTORC1 signaling. In a recent longer-term study, Drake et al. (2013) reported that 12 weeks of a rapamycin-containing diet induced a small but significant decrease in skeletal muscle protein synthesis in mixed and cytoplasmic fractions, but not in a mitochondrial fraction of mouse skeletal muscles (Drake et al. 2013). Unfortunately, it was not reported whether this rapamycin-induced decrease in protein synthesis was associated with a reduction in skeletal muscle mass. Thus, while short-term rapamycin administration does not seem to affect skeletal muscle protein synthesis, longer-term administration may induce a small decrease in protein synthesis. While these studies suggest that mTORC1-independent mechanisms may play a more dominant role in regulating basal protein synthesis in skeletal muscle, it cannot be ruled out that the rapamycin used in these studies did not fully inhibit mTORC1 signaling.

3.4.2 Muscle Mass

In regard to whether mTORC1 signaling is required for the maintenance of resting skeletal muscle mass, no studies have specifically attempted to address this question. Nonetheless, recent studies have shown that relatively short-term (7–14 days) daily rapamycin injections (0.6–1.0 mg/kg), which are sufficient to inhibit Rheb- and SA-induced $p70^{S6K1}$ T389 phosphorylation and muscle fiber hypertrophy, had no effect on resting muscle fiber size in control mice (Goodman et al. 2010, 2011a). Furthermore, longer-term studies (4–6 months) using diet-based rapamycin ingestion have reported no effect on the mass of various hind limb muscles of young and aged mice (Fok et al. 2013; Neff et al. 2013; Zhang et al. 2013). Thus, notwithstanding the question of the ability of rapamycin to completely inhibit mTORC1 signaling, these rapamycin studies suggest that basal skeletal muscle mass is not regulated by mTORC1.

Recently, Bentzinger et al. (2008) developed a muscle-specific Raptor knockout (KO) mouse and, thus, a model of inhibited muscle mTORC1 signaling that could give insights into whether mTORC1 signaling plays a role in regulating basal muscle mass. This study showed that the absence of Raptor, and thus mTORC1 signaling, throughout development and maturation (up to ~90–140 days) resulted in a significant reduction in whole muscle mass and muscle fiber size compared to controls

(Bentzinger et al. 2008). This was associated with severely reduced rpS6 S235/236 and 4E-BP1 T65 phosphorylation. These observations suggest that mTORC1 signaling may play a role in the full development or maintenance of basal muscle mass; however, the fact that muscle mass was only ~20–30 % lower in the Raptor KO mice compared to controls indicates that other mTORC1-independent factors play a larger role in determining the basal levels of skeletal muscle mass. It should also be noted that the interpretation of data from these Raptor KO mice is complicated by the fact that these mice have a severely reduced life span, such that they begin to die at ~110 days (all are dead at 190 days), while healthy control mice usually live for 2–3 years (Bentzinger et al. 2008). Thus, the observations from these Raptor KO mice may, in large part, reflect accelerated aging and, thus, the early development of sarcopenia. To help avoid the extra variables introduced by the ablation of Raptor during development and the issues of premature aging, future studies on the necessity of Raptor and mTORC1 signaling for maintaining basal muscle mass could be performed by using an inducible muscle-specific Raptor KO model in young adult mice.

Overall, a very limited amount of short- and medium-term studies in rodents and humans suggest that mTORC1 signaling may not be necessary for maintaining basal rates of protein synthesis and basal levels of skeletal muscle mass. It is possible, however, that these findings may be affected by the inability of rapamycin to fully inhibit mTORC1 signaling (Thoreen et al. 2009, 2012). While data from Raptor knockout mice suggest that mTORC1 signaling may play a small role in determining basal skeletal muscle mass, this model also suggests that mTORC1-independent mechanisms play a more dominant role in the regulation of basal skeletal muscle mass. Thus, more studies are required to fully clarify the role, if any, of mTORC1 signaling in the regulation of basal protein synthesis and skeletal muscle mass.

4 Potential Activators of mTORC1 Signaling Induced by Mechanical Overload or Resistance Exercise

Given that the current evidence that mTORC1 signaling is necessary for the increases in protein synthesis, and possibly muscle mass, that occur in response to mechanical overload/resistance exercise, this final section will briefly examine the evidence for some of the putative mechanisms (Figs. 2, 3, and 4) that have been proposed to play a role in the activation of mTORC1 signaling by this form of mechanical stimuli.

4.1 Insulin-Like Growth Factor 1 (IGF-1)

More than two decades after early studies had shown that humoral factors, such as growth hormone and insulin, were not required for mechanical overload–induced

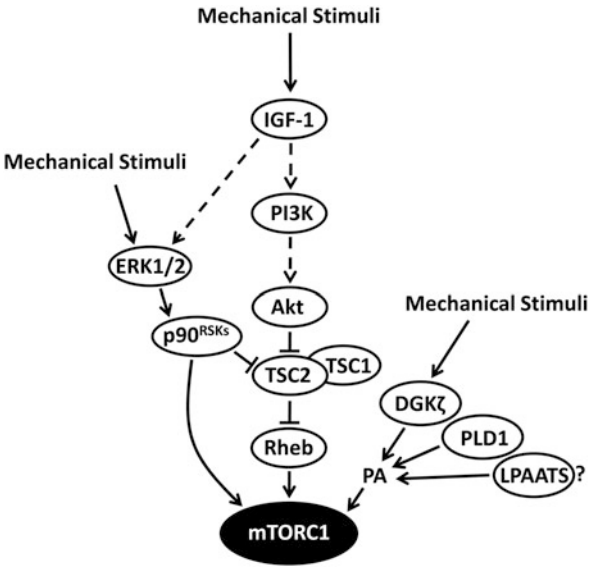

Fig. 2 *Proposed molecular mechanisms for the mechanical activation of mTORC1 by IGF-1/PI3K/Akt, ERK1/2, and PDL1/PA signaling pathways*. Current evidence suggests that the mechanical overload/resistance exercise–induced activation of mTOR complex 1 (mTORC1) signaling in skeletal muscle occurs through insulin-like growth factor 1(IGF-1)/phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal–regulated kinase 1/2 (ERK1/2) independent pathways. Evidence obtained from rodent models strongly suggests phosphatidic acid (PA), produced by the zeta isoform of diacylglycerol kinase (DGKζ), plays a significant role in the mechanical activation of mTORC1 signaling. See text for further details. *Dashed lines* (– – –) indicate multiple steps between signaling molecules. *Question mark* (?) indicates that it is currently unknown whether mechanical stimuli are sufficient to activate mTORC1 signaling via PA produced by lysophosphatidic acid acyltransferases (LPAATs)

skeletal muscle hypertrophy (Goldberg 1967, 1968b), it was discovered that IGF-1 expression was induced in animal skeletal muscle by increased mechanical loading [e.g., (DeVol et al. 1990; Yan et al. 1993; Czerwinski et al. 1994; Goldspink et al. 1995; Adams and Haddad 1996)]. Indeed, mechanical stimuli were shown to increase the expression of several isoforms of IGF-1 in animal and human skeletal muscle, including one splice variant known as the mechano-growth factor (MGF), which was proposed to act in an autocrine manner (Perrone et al. 1995; Yang et al. 1996; McKoy et al. 1999; Hameed et al. 2003). Importantly, IGF-1 was shown to be sufficient to increase muscle protein synthesis (Monier et al. 1983; Gulve and Dice 1989) and to induce skeletal muscle hypertrophy (Coleman et al. 1995; Barton-Davis et al. 1998; Musaro et al. 2001). Moreover, IGF-1 was shown to activate the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR pathway in muscle cells in a rapamycin-sensitive manner (Fig. 2), indicating that IGF-1 activated mTORC1 signaling (Dardevet et al. 1996; Frost and Lang 1999; Rommel et al. 2001; Latres et al. 2005; Park et al. 2005). Thus, it was proposed

that mechanically induced IGF-1 expression may stimulate an increase in protein synthesis and induce muscle hypertrophy, in part, by the activation of mTORC1 signaling [Note: IGF-1 may also induce skeletal muscle hypertrophy via the activation and differentiation of quiescent satellite cells and the incorporation of new myonuclei into existing muscle fibers (Adams 1998); however, this is a controversial topic with recent data casting doubts on the function of the MGF and on the role of satellite cells in mechanically induced muscle hypertrophy. For further information on this topic, the reader is referred to the following papers: Adams 1998; Hill et al. 2003; McCarthy and Esser 2007; O'Connor and Pavlath 2007; O'Connor et al. 2007; Matheny et al. 2010; McCarthy et al. 2011; Fornaro et al. 2013].

While this IGF-1-centric hypothesis for explaining mechanically induced increases in mTORC1 signaling, protein synthesis, and skeletal muscle mass seemed very attractive, serious doubts have since emerged to challenge its validity (Philp et al. 2011). For example, several rodent studies have shown that skeletal muscle mTORC1 signaling can be activated by mechanical stimuli in a manner that is independent of PI3K/Akt signaling (Parkington et al. 2003; Hornberger et al. 2004; Hornberger and Chien 2006; O'Neil et al. 2009). Furthermore, studies using a transgenic mouse model that expresses a dominant-negative IGF-I receptor specifically in skeletal muscle (MKR) have shown that, in the absence of IGF-1 signaling, chronic mechanical overload–induced mTORC1 signaling and muscle hypertrophy were identical to that in wild-type mice (Spangenburg et al. 2008). In addition, acute eccentric contractions in these MKR mice were sufficient to activate mTORC1 signaling (Witkowski et al. 2010). Further doubt comes from studies showing that acute high-frequency electrical stimulation, which is sufficient to activate mTORC1 signaling in mouse muscle, does not increase muscle IGF-1 receptor, or PI3K p85 subunit, tyrosine phosphorylation, which are markers of IGF-1 receptor activation (Hamilton et al. 2010). Moreover, mice with a muscle-specific deletion of phosphatase and tensin homologue (PTEN), which should lead to an increase in the PI3K substrate phosphatidylinositol-3,4,5 trisphosphate (PIP3) and thus larger PI3K/Akt activation, had no greater SA-induced activation of mTORC1 signaling or muscle hypertrophy compared to wild-type mice (Hamilton et al. 2010). Finally, it was recently shown that the PI3K inhibitor, wortmanin, did not inhibit the early (24 h) SA-induced increase in skeletal muscle mTORC1 signaling (Miyazaki et al. 2011). Combined, these data strongly suggest that the initial mechanical stimuli–induced activation of mTORC1 signaling occurs in an IGF-1/PI3K/Akt-independent manner.

In summary, at least in rodent skeletal muscle, current evidence suggests that IGF-1 signaling does not play a significant role in the initial activation of mTORC1 signaling in response to mechanical stimuli. While there is some limited data from human studies that supports this contention [e.g., (Deldicque et al. 2008; West et al. 2009)], more research is required to establish that an IGF-1/PI3K/Akt-independent activation of mTORC1 signaling also occurs in human muscle in response to acute resistance exercise.

4.2 Extracellular Signal–Regulated Kinase 1/2 (ERK1/2)

ERK isoforms 1 and 2 are members of the large mitogen-activated protein kinase (MAPK) family and form part of the Ras/Raf/MEK/ERK1/2 signaling pathway (Morrison 2012). ERK1/2 signaling is activated under a range of conditions including growth factor stimulation (e.g., IGF-1) and various types of stresses, including mechanical stress (D'Angelo et al. 2011; Morrison 2012). Moreover, ERK1/2-mediated signaling has the potential to regulate a range of cellular processes, including cap-dependent translation initiation and elongation and ribosome biogenesis (Anjum and Blenis 2008). For example, ERK1/2 can phosphorylate the rDNA transcription factor UBF (Stefanovsky et al. 2001) and the MAPK-interacting kinases 1 and 2 (Mnk1 and Mnk2), which, in turn, phosphorylate eIF4E (Buxade et al. 2008). Furthermore, ERK1/2 phosphorylates and activates the 90 kDa ribosomal S6 kinases ($p90^{RSK}$). In turn, ERK1/2-activated $p90^{RSK}$ has been shown to phosphorylate and regulate a range of protein synthesis–related factors, including eIF4B (Shahbazian et al. 2006), eEF2 kinase (Wang et al. 2001), rpS6 (Roux et al. 2007), the RNA polymerase I–specific transcription initiation factor, TIF-IA (Zhao et al. 2003), and GSK3β (Stambolic and Woodgett 1994). Importantly, ERK1/2-activated $p90^{RSK}$ signaling has also been shown to positively regulate mTORC1 activity via the phosphorylation of TSC2 and Raptor (Roux et al. 2004; Ma et al. 2005; Carrière et al. 2008; Fonseca et al. 2011). Therefore, ERK1/2/$p90^{RSK}$ signaling has the potential to play an important role in the PI3K/Akt-independent activation of skeletal muscle mTORC1 signaling in response to mechanical overload/resistance exercise (Fig. 2).

ERK1/2 is known to be phosphorylated on the T202/Y204 residues, and thus activated, after various types of mechanical overload/resistance exercise in cultured cells, and in rodent and human skeletal muscle [e.g., (Martineau and Gardiner 2001; Drummond et al. 2009; Tannerstedt et al. 2009; Sasai et al. 2010; Miyazaki et al. 2011; Hulmi et al. 2012; You et al. 2012)]. While these studies suggest that ERK1/2 signaling could play a role in the mechanical activation of mTORC1 signaling, protein synthesis, and muscle hypertrophy, to date, only two studies have examined whether ERK1/2 signaling is necessary for these responses (Sasai et al. 2010; You et al. 2012). Firstly, Sasai et al. (2010) found that rapamycin, but not the MEK/ERK inhibitor, U0126, inhibited cyclic stretch–induced hypertrophy of cultured myotubes, indicating that mTORC1, and not ERK1/2, signaling was necessary for mechanically induced hypertrophy (Sasai et al. 2010). Secondly, You et al. (2012) used ex vivo passive stretch of isolated muscles to show that, while U0126 completely eliminated both basal and stretch-induced ERK1/2 T202/Y204 phosphorylation, and reduced basal $p70^{S6K}$ T389 and 4E-BP1 T36/45 and S64 phosphorylation, it did not inhibit the magnitude of the stretch-induced increase in $p70^{S6K}$ T389 or 4E-BP1 S64 phosphorylation (You et al. 2012). Thus, in the absence of ERK1/2 signaling, mechanical stimuli were still able to activate mTORC1 signaling (You et al. 2012). Furthermore, U0126 had no effect on either basal or stretch-induced rates of protein synthesis (You et al. 2012). Thus, at least in these stretch models, it appears that

ERK1/2 signaling is not necessary for the mechanically induced activation of mTORC1 signaling, protein synthesis, or muscle hypertrophy. Further in vivo research in rodents and humans is needed to confirm these findings.

4.3 Phosphatidic Acid (PA)

In 2001, the glycerophospholipid PA was found to activate mTOR signaling in a rapamycin-sensitive manner (Fang et al. 2001; Park et al. 2002). Since then, numerous studies have shown that the addition of exogenous PA to cultured cells, including muscle cells, is sufficient to activate mTORC1 signaling and increase protein synthesis in a PI3K/Akt-independent manner [e.g., (Foster 2007; O'Neil et al. 2009; You et al. 2012)]. Mechanistically, PA's ability to activate mTOR signaling has been shown to be due to its direct binding to mTOR's FRB domain and the subsequent activation of mTOR kinase activity (Fang et al. 2001; Veverka et al. 2008; You et al. 2012). Intracellular PA can be regulated by a number of different classes of enzymes that include phospholipase D (PLD), which synthesizes PA from phosphotidylcholine (PC); lysophosphatidic acid acyltransferases (LPAAT), which synthesize PA from lysophosphatidic acid (LPA); and the diacylglycerol kinases (DGK), which synthesize PA from diacylglycerol (DAG) (Wang et al. 2006; Foster 2007). Indeed, the overexpression of PLD1 (Jaafar et al. 2013), LPAATθ (Tang et al. 2006), and DGKζ (Avila-Flores et al. 2005) has been shown to be sufficient to activate mTORC1 signaling in various cell types. Alternatively, PA can also be controlled by enzymes that degrade PA, including A-type phospholipases (PLA), which convert PA to LPA, and phosphatidic acid phosphatases (PAP), which convert PA to DAG (Carman and Han 2006; Wang et al. 2006; Aoki et al. 2007).

To date, the role of PA produced by PLD1 has received the most investigation in regard to its potential role for regulating mTORC1 signaling and skeletal muscle mass. For example, PLD1 overexpression has been shown to induce hypertrophy of cultured myotubes, while the knockdown of PLD1 leads to a decrease in myotube size (Jaafar et al. 2013). Furthermore, the overexpression of PLD1 is sufficient to activate mTORC1 signaling and induce mouse skeletal muscle fiber hypertrophy in vivo (Jaafar et al. 2013). The first study to implicate PA in the mechanically induced activation of mTORC1 signaling in skeletal muscle showed that an increase in PA was sufficient to activate basal mTORC1 signaling, that passive stretch increased PLD activity and PA, and that the PLD inhibitor, 1-butanol, inhibited the stretch-induced increase in PA and mTORC1 signaling (Hornberger et al. 2006). Later studies also showed that in vivo eccentric contractions were also sufficient to increase muscle PA and that inhibiting the PLD-induced increase in PA with 1-butanol prevented the activation of mTORC1 signaling (O'Neil et al. 2009).

While these studies suggested that PLD1 played an important role in the mechanically induced activation of mTORC1 signaling, some investigators had questioned the specificity of 1-butanol as a PLD inhibitor. Specifically, recent studies had reported that many of the biological effects of 1-butanol could not be attributed to

the inhibition of the synthesis of PA by PLD (Su et al. 2009; Yanase et al. 2010; Sato et al. 2013). In light of this data, You et al. (2013) reexamined the role of PLD in the mechanically-induced increase in PA using a more specific PLD inhibitor, 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) (Su et al. 2009). Surprisingly, it was found that FIPI did not inhibit passive stretch–induced increases in PA or mTORC1 signaling, suggesting that PLD was in fact not necessary for these events (You et al. 2013). Instead, it was shown that passive stretch induced an increase in DAG, suggesting that there could be corresponding increases in DGK activity. Indeed, passive stretch was sufficient to increase membrane DGK activity, but further experiments showed that the α, β, γ, and θ isoforms of DGK were not necessary for the mechanically induced increase in PA and mTORC1 signaling (You et al. 2013). These findings then prompted the investigation of the potential role of DGKζ, and using DGKζ knockout mice, it was found that the passive stretch–induced increase in PA was almost completely abolished and mTORC1 signaling markedly impaired (You et al. 2013). Furthermore, the in vivo overexpression of DGKζ was sufficient to induce muscle fiber hypertrophy in a kinase-dependent manner, and this hypertrophic response was largely inhibited by rapamycin (You et al. 2013). Combined, the results of this recent study strongly suggest that DGKζ, and not PLD1 as previously thought, is largely responsible for the passive stretch–induced increase in PA and mTORC1 signaling. Further research is now required to confirm the role of DGKζ in other models of mechanical stimulation (e.g., eccentric contractions and SA-induced chronic mechanical overload).

Overall, these data strongly suggest that PA plays an important role in the activation of mTORC1 signaling in response to mechanical stimuli. Moreover, the mechanically induced increase in PA may, in part, explain the previously observed PI3K/Akt-independent activation of mTORC1 (Fig. 2).

While mechanically induced increases in PA appear to play a significant role in the activation of mTORC1 signaling, several questions remain to be answered. For example, are mechanically induced increases in PA sufficient to increase protein synthesis in an mTORC1-dependent manner in skeletal muscle in vivo? Also, what is the upstream mechanically sensitive element(s) that induces an increase in PA? Finally, to date, all of the data regarding the role of PA in the mechanically induced activation of mTORC1 comes from rodent studies. Thus, more studies are needed to determine whether this proposed mechanism is conserved in human skeletal muscle.

4.4 Tuberin (TSC2) Translocation and Ras Homologue Expressed in the Brain (Rheb) GTP-Loading Status

TSC2 and Rheb form part of the canonical insulin/IGF-1 signaling pathway, with Rheb being located immediately upstream of mTOR, while TSC2, in a complex with TSC1 (Hamartin), is located immediately upstream of Rheb (Fig. 2)

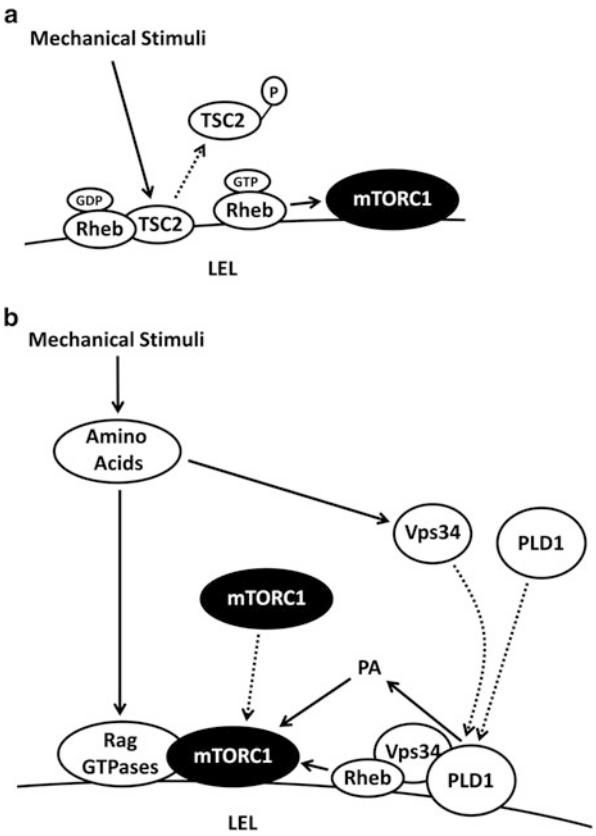

Fig. 3 *Proposed molecular mechanisms for the mechanical activation of mTORC1 by changes in Rheb GTP-loading state and by amino acids.* Emerging evidence suggests that mechanical overload/resistance exercise could (**a**) induce the translocation of late endosome/lysosome (LEL) membrane-bound tuberin (TSC2) away from Ras homologue enriched in the brain (Rheb) GTPase, leading to an increase in Rheb GTP loading and the activation of mTORC1, or (**b**) induce a Rag GTPase-mediated translocation of mTORC1 to LEL membrane-bound Rheb that may also be accompanied by an increase in phosphatidic acid (PA) via a vacuolar sorting protein 34 (Vps34)-mediated translocation of phospholipase D1 (PLD1) to the LEL. See text for further details. *Dotted lines* (. . .. . .) indicate potential translocation events

(Huang and Manning 2008). Rheb is a GTP-binding protein that possesses GTPase activity, and its GTP/GDP-binding status is regulated by the GTPase activating protein (GAP) activity of TSC2 (Aspuria and Tamanoi 2004). Importantly, Rheb has been shown to interact with mTOR's catalytic domain, and when it is in its GTP-bound state, Rheb can directly activate mTOR kinase activity (Long et al. 2005; Sato et al. 2009). Thus, the current dogma proposes that under basal conditions, the GAP domain of TSC2 stimulates Rheb's intrinsic GTPase activity, which, in turn, converts active GTP-Rheb into inactive GDP-Rheb, leading to a repression of mTORC1 signaling (Inoki et al. 2003; Tee et al. 2003; Zhang et al. 2003). Conversely, when

stimulated by factors such as insulin, TSC2 GAP activity is proposed to be inhibited in a phosphorylation-dependent manner, leading to an increase in GTP-Rheb and the activation of mTORC1 signaling (Huang and Manning 2008).

In skeletal muscle, the overexpression of Rheb is sufficient to activate mTORC1 signaling, induce an increase in protein synthesis, and stimulate muscle fiber hypertrophy (Goodman et al. 2010, 2011b). Conversely, the depletion of Rheb in cultured myoblasts reduces mTOCR1 signaling (Ge et al. 2011). Although relatively little is known regarding a possible role for TSC2 and Rheb in the mechanically induced activation of mTORC1 signaling in skeletal muscle, a recent study may have shed some light on this issue by suggesting that mechanical stimuli could regulate mTORC1 signaling, in part, by controlling the amount of GTP-Rheb (Jacobs et al. 2013b). In this study, it was shown that an acute bout of high-intensity eccentric contractions almost completely abolished the association of TSC2 with late endosomal/lysosomal (LEL) membrane structures (Jacobs et al. 2013b). Importantly, because a population of mTOR and Rheb is also found at LEL structures [e.g., (Saito et al. 2005; Sancak et al. 2008; Flinn et al. 2010; Yoon et al. 2011; Zhao et al. 2012; Jacobs et al. 2013b)], it is possible that mechanical stimuli could increase the amount of GTP-loaded Rheb, and thus mTORC1 signaling, by promoting the translocation of TSC2 away from LEL structures so that TCS2 can no longer act as a GAP toward Rheb (Fig. 3a). Furthermore, this mechanically induced translocation of TSC2 may be a phosphorylation-mediated event, although the kinase(s) responsible remains to be determined (Jacobs et al. 2013b). Thus, a mechanically induced translocation of TSC2 away from its GAP target Rheb could play an important role in the mechanically induced activation of mTORC1 signaling (Jacobs et al. 2013a) (Fig. 3a); however, whether this putative mechanism is PI3K/Akt independent remains to be determined. While this is an attractive hypothesis, significantly more work is required to confirm this mechanism in rodent muscle and to obtain evidence for such a mechanism in human skeletal muscle.

4.5 Amino Acids

It has long been known that mTOR signaling is regulated by amino acids, with amino acid depletion reducing mTOR signaling and increased amino acid availability leading to increased mTOR signaling [for a review, see (Jewell et al. 2013)]. However, it has only been relatively recently that the molecular mechanism behind the effect of amino acids has begun to be elucidated. For example, it was recently demonstrated in non-muscle cells that amino acids regulate the association of mTOR with LEL structures via a mechanism that is dependent on the Rag family of GTPases (Sancak et al. 2008, 2010). Specifically, it was shown that amino acid stimulation increases the association of mTOR with LEL structures by regulating the activity/GTP-loading state of the Rag GTPases [for a more comprehensive review of the role of Rag GTPases, see (Efeyan et al. 2012)]. Thus, it has been proposed that

amino acid–induced changes in mTORC1 signaling are primarily regulated by spatially controlling the ability of mTOR to interact with the LEL-associated activator, Rheb. Furthermore, PA could also play a role in amino acid–induced mTORC1 signaling. Indeed, a recent study has demonstrated that amino acids promote an increase in PLD1 activity and PLD1's association with the LEL and that PLD1 contributes to the amino acid–induced increases in mTORC1 signaling (Yoon et al. 2011). Thus, at least in cell culture models, the current evidence suggests that amino acids may control mTORC1 signaling by regulating both the association of mTOR with the LEL and the concentration of PA at the LEL (Fig. 3b).

In regard to skeletal muscle, it has long been known that exogenous amino acids are sufficient to induce an increase in protein synthesis in isolated muscles (Fulks et al. 1975) and that chronic mechanical overload induces an increase in the uptake of amino acids (Goldberg and Goodman 1969). More recently, amino acids (especially the branched-chain amino acid, leucine) have been shown to be sufficient to activate mTORC1 signaling and increase protein synthesis in rodent and human skeletal muscle in a rapamycin-sensitive manner [e.g., (Anthony et al. 2000; Dickinson et al. 2011)]. Thus, a mechanically induced uptake of amino acids could, in part, play a role in the mechanical activation of mTORC1 signaling in skeletal muscle. While there is certainly evidence that the ingestion of amino acids prior to, or immediately post, resistance exercise can potentiate the post-exercise increase in mTORC1 signaling and protein synthesis [for reviews, see (Blomstrand et al. 2006; Koopman et al. 2007; Drummond and Rasmussen 2008)], there is currently no direct evidence to support the hypothesis that amino acids are involved in the initial mechanically-induced activation of mTORC1. Nevertheless, it has recently been shown that an acute bout of resistance exercise that was sufficient to activate mTORC1 signaling also induced an increase in the leucine content of rat muscle (MacKenzie et al. 2007). Furthermore, this bout of resistance exercise increased the post-exercise activity of the class III PI3K, Vps34, which has been implicated in the amino acid–induced activation of PLD1 activity and mTORC1 signaling [e.g., (Gulati et al. 2008; Gran and Cameron-Smith 2011; Xu et al. 2011; Yoon et al. 2011)]. Thus, amino acids could potentially play a role in the mechanically induced PI3K/Akt-independent increase in mTORC1 signaling via the activation of PLD1 and an increase in PA synthesis (Fig. 3b). While promising, significantly more research is required to gain further insights into this hypothesis in both rodent and human skeletal muscle, and to reconcile this potential mechanism with the recent data suggesting that PLD1 does not play a role in the mechanically-induced activation of mTORC1 signaling (You et al. 2013).

4.6 Peroxynitrite-Induced Calcium Release

Previous studies in non-muscle cells have shown that mTORC1 signaling may be regulated by changes in intracellular calcium concentration ($[Ca^{2+}]_i$), with an increase in $[Ca^{2+}]_i$ activating mTORC1 signaling and a decrease in $[Ca^{2+}]_i$ leading to an inhibition of mTORC1 signaling (Graves et al. 1997; Conus et al. 1998).

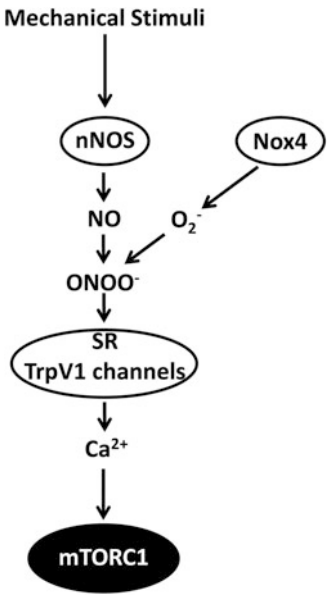

Fig. 4 *The proposed molecular mechanism for the mechanical activation of mTORC1 by peroxynitrite-induced Ca^{2+} release*. Recent evidence suggests that mechanical stimuli induce a rapid and transient activation of neuronal nitric oxide synthase (nNOS) activity and an increase in nitric oxide (NO) production. This mechanically induced NO is proposed to react with NADPH oxidase 4 (Nox4)-derived superoxide (O_2^-) to form peroxynitrite ($ONOO^-$), which, in turn, induces calcium (Ca^{2+}) release from sarcoplasmic reticulum (SR)-transient receptor potential cation channel subfamily V (TrpV1) channels, leading to the activation of mTORC1. See text for further details

Furthermore, depending on the conditions, changes in the cellular redox state have been shown to both positively and negatively regulate mTORC1 signaling (Corradetti and Guan 2006). Importantly, it is well known that mechanical stimulation of skeletal muscle results in increases in $[Ca^{2+}]_i$ and a change in the cellular redox state via the production of reactive oxygen species [ROS; e.g., superoxide (O_2^-) and hydrogen peroxide (H_2O_2)] and reactive nitrogen species [RNS; e.g., nitric oxide (NO) and peroxynitrite ($ONOO^-$)] [for reviews, see (Allen et al. 2008; Powers and Jackson 2008; Baylor and Hollingworth 2012; McConell et al. 2012)]. Thus, it is possible that increases in $[Ca^{2+}]_i$ and/or ROS/RNS may play a role in the mechanical activation of mTORC1 signaling and muscle hypertrophy. In support of this hypothesis, NO has been shown to play a role in mechanical overload–induced skeletal muscle hypertrophy, with the nitric oxide synthase (NOS) inhibitor, L-NAME, being sufficient to inhibit SA-induced muscle hypertrophy in rats (Smith et al. 2002; Soltow et al. 2006); however, against this hypothesis, one study has reported that the Ca^{2+} chelator, BAPTA-AM, did not inhibit passive stretch–induced mTORC1 signaling in isolated skeletal muscles (Hornberger et al. 2006).

More recently, a direct link between ROS/RNS, $[Ca^{2+}]_i$, mTOR signaling, and mechanically induced skeletal muscle hypertrophy was proposed by Ito

et al. (2013a, b). Specifically, evidence was obtained for a mechanism whereby the initiation of chronic mechanical loading of skeletal muscle induces a rapid and transient (within minutes) increase in nNOS-derived NO, which reacts with NADPH oxidase 4 (Nox4)-derived O_2^- to form $ONOO^-$. This mechanically-induced $ONOO^-$, in turn, induces an increase in $[Ca^{2+}]_i$ via the activation of sarcoplasmic reticulum–located transient receptor potential cation channel subfamily V (TrpV1) channels, which leads to the activation of mTORC1 signaling and a subsequent hypertrophic response over 7 days (Fig. 4) (Ito et al. 2013a, b). The results of this study open up an exciting new field of investigation in the pursuit of identifying the mechanism(s) that regulate the activation of mTORC1 signaling in response to mechanical overload or resistance exercise. However, further studies are required to confirm the many aspects of this proposed mechanism and, particularly, to determine how an increase in $[Ca^{2+}]_i$ activates mTORC1 signaling. Moreover, further work is required to reconcile this mechanism with previous work that suggests that Ca^{2+} does not play a role in the mechanically induced activation of mTORC1 signaling (Hornberger et al. 2006). Finally, it needs to be determined whether this mechanism is only relevant for the chronic overload model or whether it is also active during an acute bout of resistance exercise in rodents and humans.

In summary, there are currently several putative mechanisms that may be responsible for the activation of mTORC1 signaling by increased mechanical loading or resistance exercise (Figs. 2, 3, and 4). To date, significant evidence is mounting to suggest that the IGF-1/PI3K/Akt pathway does not play a significant role in the mechanically induced activation of mTORC1. Furthermore, very limited evidence also suggests that ERK1/2 signaling may not be involved in this process. Of the remaining potential mechanisms, a mechanically induced increase in PA has so far received the most experimental support, while limited, but promising, data suggests possible roles for mechanically induced increases in amino acids and TSC2 translocation–mediated increases in GTP-Rheb. Finally, the most recent hypothesis implicates mechanically induced increases in $ONOO^-$ and a subsequent Ca^{2+}-induced activation of mTORC1 signaling. Further research is required to determine whether one particular mechanism plays a dominant role under different mechanical loading conditions or whether there is a degree of synergism or redundancy between them.

5 Conclusion

Since the discovery of rapamycin and mTOR, there has been a huge expansion in our knowledge of the mechanisms that regulate cell growth, including muscle cell growth. Moreover, these advances have enabled the discovery of the important role that mTORC1 signaling plays in the regulation of protein synthesis and skeletal muscle mass in response to mechanical overload/resistance-type exercise. However, despite these advances, there are still many questions that need to be answered. For example, is mTORC1 signaling necessary for resistance training–

induced muscle hypertrophy in humans? Does mTORC1 signaling play a role in the acute responses and chronic adaptations to endurance or sprint exercise? To what extent, if any, does mTORC1 play a role in the regulation of basal/resting skeletal muscle protein synthesis and mass? What are the specific roles of 4E-BP1 and $p70^{S6K1}$, and their associated downstream targets, in mechanical stimuli–induced increases in protein synthesis and muscle mass? What are the mechanically sensitive upstream molecular mechanisms that regulate mTORC1 signaling? Based on these questions, it is clear that significantly more research is required to fully elucidate the many roles of mTORC1 signaling in skeletal muscle and, in particular, its role in the regulation of protein synthesis and skeletal muscle hypertrophy in response to different forms of mechanical stimuli. Ultimately, the answers to these questions will help in the development of more effective exercise programming and in the potential development of pharmacological interventions aimed at preventing/attenuating the loss of, or increasing, skeletal muscle mass.

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