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Mammalian Polyamine Metabolism and Function

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Summary

Polyamines are ubiquitous small basic molecules that play multiple essential roles in mammalian physiology. Their cellular content is highly regulated and there is convincing evidence that altered metabolism is involvement in many disease states. Drugs altering polyamine levels may therefore have a variety of important targets. This review will summarize the current state of understanding of polyamine metabolism and function, the regulation of polyamine content, and heritable pathological conditions that may be derived from altered polyamine metabolism.

INTRODUCTION

Polyamines are ubiquitous small basic molecules (Figure 1). Polyamine research is a very active area with numerous publications covering genetic, biochemical and physiological studies using mammals, plants, protozoan parasites and many microorganisms, including thermophiles that have a wider variety of polyamines. Many of these elegant studies in non-mammalian systems have had a profound effect on the whole field but, in order to focus this article, only mammalian polyamine biochemistry will be covered with emphasis on the enzymology of polyamine synthesis and interconversion, the regulation of cellular polyamine content, the functions of polyamines, and the potential role of polyamines in heritable human disease. In general, only primary references to articles published in the last three years are included. Earlier work on polyamine metabolism, function and the investigations of the role of polyamines in normal and neoplastic growth is covered in multiple reviews (1–10) and in the cited articles.

POLYAMINE SYNTHESIS

The only polyamines synthesized in mammalian cells are putrescine, spermidine and spermine. Agmatine (decarboxylated arginine) is not produced by mammals (11) but is synthesized by plants and by many bacteria, including the intestinal flora. It is possible therefore that it may be derived from food or intestinal microorganisms, although there is an active mammalian agmatinase that degrades it (12). Agmatine has been shown to be active as a neurotransmitter but it is not established that it this is physiologically important and the function of agmatinase may be to limit any effect of dietary agmatine.

Ornithine and S-adenosylmethionine decarboxylases

Putrescine is formed by the action of ornithine decarboxylase (ODC, Figure 1) (13). This enzyme is therefore a critical step in maintaining polyamine levels and is exquisitely regulated as described below. ODC is frequently described as the rate-limiting step in polyamine synthesis but this is inaccurate. ODC is usually the rate-limiting factor in the

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production of putrescine but the supply of the aminopropyl donor; dcAdoMet by the action of S-adenosylmethionine decarboxylase (AdoMetDC, Figure 1) also influences the conversion of putrescine into the higher polyamines. Once converted to dcAdoMet, AdoMet is exclusively diverted to polyamine biosynthesis since methyl transferases do not use dcAdoMet as a substrate. The steady state level of dcAdoMet is therefore kept very low (c. 1–2% of the AdoMet content) and its supply limits the conversion of putrescine to the higher polyamines.

Mammalian ODC is a typical amino acid decarboxylase that requires PLP as a cofactor (14). In contrast, all known AdoMetDCs are members of a more limited class of decarboxylases that use a covalently bound pyruvate as the prosthetic group (15,16). Such decarboxylases have the advantage that their activity is not dependent on the availability of vitamin B6 but have two significant disadvantages. Firstly, protonation of the incorrect carbon atom in the Schiff base intermediate causes the conversion of the pyruvate to alanine and the total loss of enzyme activity (Figure 2). Such protonation, although rare, occurs with both PLP and pyruvoyl decarboxylases, but those dependent on PLP can be reactivated by replacing the cofactor. Secondly, the information for cofactor synthesis must be contained within the protein sequence itself. The pyruvate is formed from an internal serine residue in an autocatalytic reaction that converts a single proenzyme molecule (π) into two subunits (α and β) with the pyruvate covalently linked to the amino terminus of the α chain (Figure 2). Detailed studies of this reaction using crystallography, site-directed mutagenesis and biochemical techniques with AdoMetDC proenzymes from various sources have elucidated the mechanism for these reactions (17). These investigations show that there is only a small overall structural change between the unprocessed and the processed form and that some of the residues forming the active site pocket are also involved in the processing reaction. This provides obvious constraints on the structure of the active site and may account for the relatively small number of pyruvoyl-dependent decarboxylases.

Mammalian AdoMetDC is activated by putrescine in two ways (15,16). The processing reaction is stimulated by putrescine and the catalytic activity of the enzyme is enhanced (Figure 2). Recent structural and biochemical studies indicate that there is one putrescine-binding site per ($\alpha\beta$) unit and that this site is located at a significant distance from the active site. Putrescine binding to the $(\alpha\beta)_2$ dimer that makes up the enzyme is quite strongly cooperative and binding causes structural and electrostatic alterations at the active site (18). The physiological consequences of the activation of the enzyme by putrescine are that the supply of both substrates for spermidine synthase (Figure 1) are linked and putrescine is thus efficiently converted into spermidine. Mammalian cells and tissues contain much higher amounts of spermidine and spermine than putrescine, whereas in species that lack this putrescine activation, putrescine may be the predominant polyamine.

Aminopropyltransferases and 5′-methylthioadenosine phosphorylase

Synthesis of the higher polyamines is brought about by aminopropyltransferases termed spermidine synthase and spermine synthase (19) (Figures 1 and 3). Despite the close similarity of their reactions, human spermidine synthase and spermine synthase are distinct enzymes and show strict substrate specificity (20,21). The structures of their active sites are similar differing mainly in the restricted space for the amine substrate in spermidine synthase that ensures only putrescine can be used (Figure 3A) . Both structures contain two fully conserved key aspartic acid residues (Asp^{104} and Asp^{173} in spermidine synthase; Asp^{201} and Asp^{276} in spermine synthase) that play a key part in the catalytic mechanism (Figure 3C). Human spermine synthase, which is a dimer of two identical subunits, has a C-terminal domain that contains the active site and is similar in structure to spermidine synthase (21). It also has an N-terminal domain with remarkable structural similarity to AdoMetDC (Figure 3B). The function of this domain is not yet understood but it cannot

have AdoMetDC enzymatic activity since the serine that forms the pyruvate prosthetic group described above is not present. Its complete or partial deletion led to a total loss of spermine synthase activity and prevented dimerization indicating the dimeric form is required for activity. It is noteworthy that AdoMetDC in protozoal parasites can form a heterodimer in which one monomer is the processed αβ form containing pyruvate similar to the mammalian structure described above and the other monomer is an unprocessed homolog (22). Only the heterodimer has significant activity and the availability of the unprocessed homolog regulates dcAdoMet synthesis and thus controls spermidine levels (23). The possibility that interactions between spermine synthase and AdoMetDC affect the polyamine content of mammalian cells clearly requires further investigation.

Both aminopropyltransferases are inhibited by the 5′-methylthioadenosine (MTA) product with spermine synthase being more sensitive (19). The structures with bound MTA provide a plausible explanation for this since the hydrophobic surfaces involved in interaction with the adenine ring of MTA is larger in spermine synthase (21). This inhibition does not normally have great importance in limiting polyamine synthesis *in vivo* since MTA is normally rapidly degraded by MTA phosphorylase (2). The action of MTA phosphorylase starts a pathway by which the purine and methylthio- moieties of AdoMet that are not used for polyamines are recycled via salvage pathways. However, many tumors lack MTA phosphorylase due to either deletion or promoter hypermethylation of the *MTAP* gene, which is located close to the oncogenes $p16^{INK4a}$ and $p15^{INK4b}$, and are unable to bring about this recycling (24).

Interconversion and degradation

Several enzymes are involved in the reversal of the aminopropyltransferase reactions, which like the decarboxylases are effectively irreversible (Figure 1) (8,9,25). Spermine oxidase (SMO) converts spermine to spermidine plus 3-aminopropanaldehyde but does not act significantly on spermidine (9). It exists as multiple splice variants, some of which are present in the nucleus (9,26), but their discrete functions are unclear. Acetylpolyamine oxidase (APAO) converts N^1-acetylspermidine into putrescine plus *N*-acetyl-3-aminopropanaldehyde (27–30). APAO also acts on efficiently on N^1-acetylspermine to form spermidine. APAO was previously described as polyamine oxidase but it has negligible activity towards unacetylated polyamines *in vivo*. Both APAO and SMO are flavoproteins that generate reactive aldehydes and H_2O_2 and may cause oxidative damage. This may be a more serious problem with SMO since APAO is located in peroxisomes.

Products from both the SMO and APAO reactions, as well as acrolein, a highly toxic product of spermine oxidation formed after cell damage by extracellular copper-dependent oxidases (31), have been linked with renal failure (32) and stroke (33,34).

The substrates for the APAO reaction are synthesized by the action of spermidine/spermine-N^1-acetyltransferase (SSAT) (25). The net results of the SMO and APAO/SSAT reactions are to convert the higher polyamines to putrescine, which is more readily excreted from the cell and can also be degraded by diamine oxidase. The acetylated polyamine products of the SSAT reactions, which include N^1,N^{12}-diacetylspermine, are also exported. Therefore, activation of these pathways can reduce polyamine content. Studies with transgenic mice that overexpress SSAT and with mice in which the *Sat1* gene that encodes SSAT is inactivated have provided convincing demonstrations that this enzyme is an important regulatory step in a maintaining polyamine content (35–38). SSAT binds to a number of other proteins including α9β1 integrin (39) and HIF-1α (40) and may influence their activities (25).

REGULATION OF POLYAMINE CONTENT

With important exception of the activation of AdoMetDC by putrescine described above, polyamine content is controlled by changes in content of the key enzymes (Figure 1) rather than by alterations in their activities by post-translational modifications or by binding effector molecules of low molecular weight. Alterations in efflux and uptake mechanisms also play an important role in maintaining cellular polyamine content.

Regulation of ODC

ODC activity appears to be regulated solely via changes in the amount of ODC protein rather than modification of its catalytic activity. Regulation occurs at levels of mRNA synthesis, mRNA translation and stability, and protein degradation (Figure 4) (13,41). Degradation is controlled by an intricate pathway involving two proteins termed antizyme (AZ) and antizyme inhibitor (AZIn) [(13,42–45) and references therein]. ODC turns over very rapidly and degradation, which does not require ubiquitination, is accelerated by high levels of polyamines. ODC is a homodimer with two active sites formed at the dimer interface between the N-terminal domain of one subunit and the C-terminal domain of the other (14). Thus, only the dimer form is active but association between the two subunits is quite weak and the dimers are in rapid equilibrium with inactive monomers (13). AZ binds to the ODC monomer thus inactivating the protein but, more importantly, targeting it for degradation by the 26S proteasome. AZ synthesis is increased in response to high polyamine levels predominantly via increasing a +1 frameshifting mechanism, which is needed to allow read through of a stop codon that prevents AZ synthesis (43,46,47). A second protein termed AZIn binds to AZ more tightly than ODC and can displace it and thus prevent the degradation (Figure 4). The structure of AZIn is quite similar to that of the ODC monomer but it lacks catalytic activity and is monomeric (48). There is only one functional ODC gene but there at least four AZ and two AZIn genes that differ significantly in their sites of expression and slightly in properties (49,50). Recently, disruption of the gene encoding AZIn-1 has been shown to be lethal in mice, confirming the critical role of this protein in regulating polyamine levels (51). Moreover, infection with *Pneumocystis* increases levels of AZIn-1 in alveolar macrophages resulting in elevated polyamine content, which provides additional evidence of the regulatory importance of this protein (52). AZ and AZIn also turn over rapidly but require ubiquitination for proteasomal degradation (Figure 4). Degradation of AZ is inhibited by polyamines and AZIn is stabilized by binding to AZ (44). These changes would also help with maintaining polyamine homeostasis.

Recent studies in yeast have shown that prion [PSI(+)] increases AZ by modulating the +1 frameshifting required for its expression and in this way affects the cellular polyamine content and the cellular physiology (53). Although it is not yet known if similar alterations occur to AZ production in mammals these experiments suggest an exciting link between prion-derived pathophysiology and polyamines.

Many stimuli have been shown to increase the level of ODC mRNA [see reviews (1–4,6,7,9,10,13)]. The *Odc* gene promoter region contains sequences that allow response to hormones, growth factors and tumor promoters including a cAMP response element, CAAT and LSF motifs, AP-1 and AP-2 sites, GC-rich Sp1 binding sites and a TATA box. It is of particular interest that ODC mRNA synthesis is increased by the *c-Myc* oncogene. The *Odc* gene promoter contains two E-boxes that are occupied by the inactive Mnt/Max complex in quiescent cells and activated by the Myc/Max transcription factor when c-Myc levels are increased (54). ODC is also a target of the *Ras* oncogene via post-transcriptional mechanisms, which may involve altered mRNA stability (7,41).

Translational regulation of ODC synthesis is also an important factor contributing to ODC levels. ODC has a long 5′UTR, and both cap-dependent and internal ribosome entry site (IRES)-mediated translation have been reported (55,56). The translation factor eIF4E plays an important role in both cap and IRES-mediated ODC mRNA translation. High levels of eIF4E increase cap-dependent translation presumably by unwinding the 5′UTR structure. Dephosphorylation of eIF4E was reported to mediate the effect of *Ras* on IRES-mediated translation possibly via increasing the availability of specifictranslation initiation factors such as eIF4G and eIF4A (56).

Regulation of AdoMetDC

In addition to the activation of AdoMetDC proenzyme processing and enzyme activity by putrescine described above, the amount of AdoMetDC protein is highly regulated to maintain cellular polyamine levels at multiple steps including transcription, mRNA translation and protein turnover (Figure 2) (15,16). The amount of AdoMetDC is negatively regulated by increased spermidine/spermine content at all three of these steps and, conversely, drugs and other factors reducing their amount increase AdoMetDC (57).

Many physiological processes leading to increased growth including tissue regeneration, hormonal stimulation and differentiation, enhance AdoMetDC activity by elevating the level of AdoMetDC protein (15,16,58–60). The mechanism by which transcription is increased is not well understood although the gene contains a number of binding sites for key cell-regulatory transcription factors and may contain a spermidine-responsive element.

Degradation of AdoMetDC requires polyubiquitination (44,61). Reduction of polyamine content in response to various drugs causes a marked increase in the half-life of AdoMetDC protein (16,44). It is possible that the preferential degradation of the inactive transaminated form of the enzyme produced by incorrect catalytic turnover as described above accounts for the rapid turnover of the protein (61) (Figure 2).

The best understood regulatory step in the synthesis of AdoMetDC is the translational regulation. Elegant studies from the laboratories of Morris, using mammalian AdoMetDC mRNA (62), and Michael, using mRNA from plants (63), have shown that there is a highly conserved system in which the key element in the translation regulation is a small open reading frame (ORF) in the 5′UTR of the mRNA. In human cells, this encodes a peptide MAGDIS and acts as a cis-acting element imparting control by cellular polyamines on translation of this message. Increased polyamine content reduces synthesis of the MAGDIS peptide by stabilizing the nascent peptide-tRNA complex at the final codon. This ribosome pausing controls the access to the downstream reading frame encoding AdoMetDC (62,63).

Effect of nitric oxide

Both ODC and AdoMetDC have key active site cysteine residues that are involved in the protonation of the Schiff base intermediate. These residues, Cys^{360} in ODC and Cys^{82} in AdoMetDC, react readily with nitric oxide inactivating the enzymes. Nitric oxide is therefore a potent inhibitor of polyamine synthesis and this may mediate some of its antiproliferative actions (64).

Regulation of aminopropyltransferases

It is generally accepted that the formation of the products of these enzymes is determined by the availability of the substrates rather than by fluctuations in the levels of spermidine synthase or spermine synthase. Studies with CAG/SpmS transgenic mice in which spermine synthase levels are increased dramatically by expression from a strong ubiquitous promoter showed only a small increase in spermine levels (65). However, there are a number of

reports describing physiological alterations in spermidine synthase activity [reviewed (19)] and such changes may have been overlooked in many studies where only the decarboxylases forming the aminopropyltransferase substrate were measured. Recent studies suggest both aminopropyltransferase genes are targets for increased transcription by c-Myc (66,67) (Nilsson J, personal communication).

Regulation of SSAT, APAO and SMO

SSAT is an extremely inducible enzyme whose content is adjusted in response to alterations in polyamine content to maintain polyamine homeostasis and via a variety of other stimuli including toxins, hormones, cytokines, natural products, stress pathways, and ischemia-reperfusion injury [see (9,25,68–70) and references therein]. The *k-Ras* oncogene negatively regulates SSAT, which contributes to the increase in polyamines brought about by Ras activation (7). Increases in SSAT are initiated by alterations in *Sat1* gene transcription and amplified by alterations at the other regulatory steps including mRNA splicing, mRNA translation and protein turnover. These changes have been described in detail in a recent review (25) and are summarized in Figure 5.

Induction of SMO leading to decreased spermine occurs in response to polyamine analogs (9), TNF-α (71), *H. pylori* infection (72), prostatic neoplasia (73) and during cell differentiation (74). Flux through APAO activity is normally regulated by the availability of the substrates provided by SSAT rather than by changes in APAO activity.

Polyamine uptake and efflux

There are transport systems for both the uptake of polyamines and for their efflux. These are currently poorly understood at the biochemical level, but potent inhibitors have been produced, and these may prove to be useful drugs for cancer chemotherapy when administered together with inhibitors of the biosynthetic pathway (75). The polyamine transport system is not highly specific and can transport a number of related compounds including paraquat, mepacrine and synthetic drugs conjugated with a polyamine (76,77). Polyamine transport is blocked by AZ, which contributes to the ability of AZ to reduce polyamine levels (13), but the mechanism of this effect is unknown. Carrier-mediated uptake systems have been characterized from microorganisms and protozoal parasites but many attempts to isolate similar systems from mammals have not yet been successful. However, endocytic pathways for polyamine transport have been described in mammalian cells. Fluorescent polyamines known to be transported via an uptake system used by normal polyamines have been localized in discrete vesicles (78). Cell surface heparin sulfate proteoglycans have been implicated in polyamine transport, and uptake of polyamines was blocked by a single chain variable fragment anti-heparan sulfate antibody (79). Recently, a caveolin-regulated system has been shown to transport polyamines in colon cancer cells. Phosphorylation of caveolin-1 at Tyr^{14} increased the activity of this system (80). Such phosphorylation was stimulated by k-Ras providing another step at which this oncogene may increase cellular polyamines. Thus, polyamine transport can follow a dynamin-dependent and clathrin-independent endocytic uptake pathway. The extent to which this accounts for all polyamine transport is unclear.

Recent studies have identified SLC3A2, previously known as a glycosylated heavy chain of a cationic amino acid transporter, as a part of a putrescine and acetylpolyamine efflux system. SLC3A2 with its partner y+ LAT light chain was found to bring about arginine uptake and putrescine efflux (81). This efflux was coupled to arginine uptake suggesting that there is a putrescine/arginine exchange reaction. SSAT was co-localized with SLC3A2 and was co-immunoprecipitated by an anti-SLC3A2 antibody indicating that this efflux system may be closely linked to polyamine acetylation. The expression of SLC3A2 was negatively

regulated by k-Ras (81). Thus, this oncogene can increase polyamine content by affecting both influx and efflux. Whether the SLC3A2-dependent mechanism is the sole polyamine export system is not known.

FUNCTIONS OF POLYAMINES

The importance of polyamines for mammalian development is shown by studies with mouse knockouts of the ODC or AdoMetDC genes, which are lethal at very early embryonic stages. Although the study has not been reported, it is very likely that spermidine synthase knockouts would also be lethal since spermidine could not be made in the ODC or AdoMetDC knockouts and spermidine is known to be essential for viability in yeast (82). Spermine synthase knockouts are also not viable on the backgrounds commonly used for gene deletions studies but B6C3H mice with a deletion of this gene from the X chromosome have been bred from a female offspring of an irradiated mouse. These mice were termed gyro (Gy) based on a circling behavior pattern in affected males. The Gy mice have a greatly reduced size, sterility, deafness, neurological abnormalities, and a short life span. All of these changes are reversed when spermine synthase activity is restored (83,84). These results provide definitive proof of the critical importance of polyamines for normal physiology.

It should be noted that, because of the extensive nature of the interactions with cellular macromolecules, the concentration of free polyamines is much less than calculated based on total polyamine content. This free pool brings about the interactions described above, and rapid changes due to increased synthesis/uptake/efflux may have critical effects on cellular physiology even though total polyamine levels change very little. A major problem in the polyamine field is that there is no way to measure the free polyamine concentrations and methods using specific interacting dyes or other reporter molecules are critically needed.

Gene regulation

Polyamines are strongly positively charged at physiological pH and bind to acidic sites on cellular macromolecules including proteins, nucleic acids and phospholipid membranes (3,5,85). All of these interactions are likely to have some physiological effect. A vast number of papers have described these interactions and alterations in properties brought about by polyamines in *in vitro* experiments. It is difficult to assess the physiological significance of these results but it is clear that polyamines affect RNA and DNA structure, ribosome function, and the activity of many enzymes including kinases and phosphatases (Figure 6). Polyamine response elements have been identified in some genes (5,10) and the transcription of many genes appears to be influenced through the polyamine status (2–7). These genes include transcription factors such as c-Myc and c-Jun (86,87), which can lead to additional signaling pathways. Similarly, polyamines selectively influence the translation of many mRNAs; examples include the effects on AZ, AdoMetDC and SSAT synthesis described above (Figure 6).

There is a vast literature describing experiments indicating effects of polyamines on signaling pathways. Many of these papers use inhibitors, such as α-difluoromethylornithine (DFMO) that inactivates ODC, to alter polyamine content and then compare the signaling target to control cells and cells in which the inhibitor effect is abolished by provision of exogenous polyamines. In this way, effects on the levels and phosphorylation status of key regulatory proteins, such as importin-α1, Akt/protein kinase B, GSK-3β, Src, cdk-4, p21Cip1, p27Kip, p53, Mdm2 and EGFR have been observed (67,87–95).

It seems likely that, as in *E. coli* where a “polyamine modulon” of polyamine regulated genes important for cell growth has been defined (96,97), there is a large subset of genes

whose products are regulated by polyamine-mediated effects on transcription (Figure 6). These transcriptional effects may be direct or secondary to changes in the level of transcription factors due to polyamine effects on either their translation or on regulatory kinases/phosphatases (90,98). Translational effects may occur via direct interaction with the relevant mRNA at the ribosome or via changes in the activity of proteins selectively affecting translation.

Polyamine-related alterations in cell:cell interactions mediated via cadherins (99) or Toll-like receptors (100), effects on the cytoskeleton mediated by changes in the activity of G-proteins such as Rac1 and RhoA (101–103), and alterations in the microtubule network (104) are also possible sites of physiological polyamine action. The latter is also likely to be affected by certain polyamine analogs (10).

Hypusine

Spermidine has an essential role as the substrate for the hypusine [N^8-(4-amino-2-hydroxybutyl)lysine] modification of the eIF5A (105). This highly conserved protein with two isoforms in vertebrates requires the post-translational modification of an internal lysine to form hypusine for activity. The eIF5A precursor is first modified by deoxyhypusine synthase, which attaches the aminobutyl group of spermidine to a specific lysine residue. Deoxyhypusine hydroxylase then forms the complete hypusinated eIF5A. The mature form of eIF5A is essential for growth and protein synthesis in yeast (5,82,106) and it is very likely that this is also the case in mammals. Synthetic polyamine analogs are only able to rescue cells from polyamine depletion-induced growth inhibition if they are substrates for deoxyhypusine synthase (107). The exact function(s) of eIF5A are still not fully understood. Roles in ribosomal protein synthesis are relatively well established although not understood in detail (5,107–109), but other functions including effects on RNA transport and mRNA stability (110) and in viral replication (111) have also been suggested. Recently, eIF5A has been implicated in skeletal muscle stem cell differentiation (112), brain function (113), and response to heat stress (114).

EFFECTS OF POLYAMINES ON ION CHANNELS

Polyamines play important roles in the regulation of ion channels (Figure 6). Both glutamate receptor ion channels, which mediate excitatory synaptic transmission in the mammalian brain, and inwardly rectifying potassium channels (Kir), which control membrane potential and potassium homeostasis in many cell types, are affected as well as certain connexin-linked gap junctions and some other channels that affect intracellular calcium signaling or Na^+ transport [(115–118) and references therein].

Glutamate receptors

Polyamines influence glutamate receptors mediating slow voltage-dependent responses such as *N*-methyl-D-aspartate (NMDA) receptors, and those producing fast responses at excitatory synapses, such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainate receptors (115).

The NMDA receptors are involved in synaptic plasticity and may also play a role in seizure activity. Spermine (and, to a lesser extent, spermidine) has multiple effects on these receptors including stimulation and a weak voltage-dependent inhibition representing an open-channel block. Spermine stimulation, which occurs at μM concentrations via binding to the extracellular R domain, causes an enhancement of the current gated by glutamate and glycine. There is a “glycine-dependent stimulation”, which produces an increase in the affinity of the receptor for glycine. Binding of spermine can also produce a “glycine-independent stimulation” at saturating concentrations of glycine. At physiologic pH,

spermine stimulatory effects are seen at NR1/NR2B receptors but not at NR1/NR2A receptors (119). The spermine block occurs via interactions in the outer vestibule of the channel pore. Mutations affecting this interaction have been mapped (120).

The AMPA-type glutamate receptors are responsible for fast excitatory neurotransmission in the CNS. They are heteromeric ligand-gated channels composed of four possible subunits (GluR1–4) whose properties depend on the presence of GluR2. Those lacking the GluR2 subunit are permeable to Ca^{2+} ions, possess a high single-channel conductance, and are subject to a block by endogenous intracellular polyamines (predominantly spermine) that confers profound rectification on the responses and influences frequency-dependent facilitation at synapses expressing these receptors. Binding of spermine occurs within the pore region of the channel and is use and voltage dependent. Thus, polyamines may regulate the amount of Ca^{2+} flux and the excitability threshold at developing synapses. Repetitive activation of these receptors results in facilitation due to polyamine unblocking. Polyamines may also affect the activity via interactions with PKC (121). Stargazin, a transmembrane AMPA receptor regulatory protein greatly reduces this polyamine-mediated block (122).

Kainate receptors are formed from the GluR5-7 and KA1-2 subunits. They are similar to AMPA receptors but have unique roles in synaptic function in sensing pain, neuronal development and synaptogenesis and in supporting plasticity processes that are the cellular bases of learning and memory. Spermine potentiation of GluR6 is voltage independent, does not affect receptor desensitization, and only slightly shifts the agonist affinity of the receptor. Thus, similar to its action on NMDA receptors, spermine potentiates kainate receptors by relieving proton inhibition of the receptor (123).

Kir channels

The inward rectifier potassium channel gene family consists of 7 subfamilies (Kir1-7). These channels are tetramers of pore-forming subunits with two transmembrane domains (M1 and M2) separated by a P-region. The P-loop and a segment of the M2 domain form the transmembrane pore. There is also a cytoplasmic pore containing the binding site for the ligands and other regulators and controlling access to the transmembrane pore. Voltage-dependent block by intracellular polyamines is the common mechanism underlying the inward rectification in all the Kir channels. All of the natural polyamines can bind and have some effects in experimental conditions but the affinity increases from putrescine to spermidine to spermine (116,124). This block has been most intensively studied in some strongly inward rectifying channels such as Kir2.1 and Kir6.2 (125–127). Evidence for different states in these channels with low and high affinity blocks has been published (128). Two different regions with negatively charged micro-environments are critical for binding polyamines and determining inward rectification characteristics; one is in the cytoplasmic pore and the other in the transmembrane pore (124,126). Models in which pre-positioning of polyamines at the cytoplasmic pore then facilitates entry of polyamines into a deeper binding site located within the membrane pore, are consistent with the experimental data using polyamines and experimental analogs (126,127).

As mentioned above, Gy mice, which have no spermine and elevated spermidine levels, are totally deaf. These mice have an almost complete loss of endocochlear potential. This may be explained by effects of the polyamine imbalance on the cochlear lateral wall-specific Kir4.1 channel, which is known to play a critical role in the maintenance of this potential (129). It has been shown that SSAT binds to the α9β1 integrin and that this binding is needed for its function in cell migration (39). The mechanism for this effect appears to be via a local effect of altered polyamine concentrations on the Kir4.2 channel co-localized in focal adhesions at the leading edge of migrating cells (39).

Gap junctions

Gap junction proteins form aqueous pores between cells that are typically open under resting conditions and allow homeostatic electrical and chemical coupling between them. Polyamines (mainly spermine) may regulate conductance in connexin40, but not connexin43, gap junctions via interactions with two cytoplasmic amino-terminal domain glutamate residues. This presumably alters the conformational structure of the putative voltage sensor and spermine receptor of connexin40, without causing significant alteration of the electrostatic surface charge potentials that contribute to the ion selectivity of this gap junction channel (117).

POLYAMINES AND HERITABLE HUMAN DISEASE

Spermine synthase and Snyder-Robinson syndrome

The only inherited human disease that is definitively associated with deranged polyamine metabolism is Snyder-Robinson syndrome (SRS). This is an X-linked mental-retardation and developmental disease that is caused by an alteration in the *SpmS* gene that encodes spermine synthase and is located on the X chromosome at Xp22.1 (129–131). Other characteristic features of this condition include a marfanoid habitus, skeletal defects, osteoporosis and facial asymmetry as well as hypotonia and movement disorders. The first mutation found to cause this condition was a point mutation at the 5′-splice site of intron 4. This induces predominantly incorrect splicing forming an inactive truncated spermine synthase protein but a small amount of normal protein is also formed (130). This allows some spermine synthesis (as assayed using cultured lymphoblastoid and fibroblast lines from SRS patients) but there is a greatly increased spermidine:spermine ratio. More recently, three other point mutations leading to SRS have been identified in the *SpmS* gene (129,131). These are all miscoding point mutations (Gly^{56} to Ser, Val^{132} to Gly and Ile^{150} to Thr). These positions are all highly conserved and the alterations lead to a large loss of spermine synthase activity. The sites of alteration are not close to the active site in the spermine synthase crystal structure, and it is likely that they affect dimerization, which was shown to be needed for activity (21). Studies with lymphoblastoid lines from these patients also show a very low spermine synthase content and activity and a high spermidine:spermine ratio. The phenotype of the SRS patients is not closely related to that of the Gy mice, although the mice also have neurological defects. However, it should be noted that the mice are totally deficient in spermine synthase and completely lack spermine, whereas the human patients do have some synthase activity, which is presumably able to maintain some spermine content even though there is a drastic increase in the spermidine:spermine ratio. The molecular basis for the phenotypic alterations caused by the SRS alteration is not yet established but changes in critical ion channel activity or possibly transcription/translational effects on key regulatory proteins are likely explanations.

SSAT/APAO-linked conditions

It is possible that a second human disease produced by altered polyamine metabolism is keratosis follicularis spinulosa decalvans (KFSD). A patient with KFSD was found to have a duplication of a region of the X chromosome that included *Sat1* (132). Cultured fibroblasts from the patient had a 3-fold increase in SSAT activity and a consistent reduction in spermidine and increase in putrescine levels. Studies of further patients are clearly needed, but it is possible that gain of function mutations in the *Sat1* gene are a cause of KFSD. Transgenic overexpression of SSAT in mice does give rise to striking skin changes (133).

Alterations in SSAT expression in transgenic mice have a wide variety of pleiotropic effects attributable to an altered metabolic flux through the polyamine pathway and concomitant changes in acetyl CoA and ATP levels (36–38). It is possible that genetic or environmental/

dietary conditions that reduce SSAT might be related to obesity and diabetes. Although it has been suggested that this may be relevant to human disease, clinical studies to examine this have not yet been carried out.

Low SSAT gene expression due to reduced levels of SSAT mRNA that correlated with the C allele of the *Sat1* 342A/C polymorphism, which is located in the vicinity of the PRE region of the *Sat1* gene promoter, has been linked to depression and a propensity to suicide (134,135). The mechanism by which SSAT is linked to behavioral effects is not understood but may be linked to alterations in kainate receptor activity (6).

Cells from patients with Niemann-Pick type C disease were reported to be more sensitive to the toxic effects of 3-aminopropanaldehyde, which is formed by the action of APAO on N^1-acetylated polyamines produced by SSAT (136). These findings suggest that this neurodegenerative lysosomal storage disease may be related to damage due to the prolonged exposure of the organelle to toxic products of the SSAT/APAO pathway.

ODC and susceptibility to carcinogenesis

A single nucleotide polymorphism in the human *Odc* gene occurs in intron 1, where there is an A/G variation at position +317 relative to the transcription start site (137,138). This position is located between the two E-boxes and may affect Myc/Max binding and hence ODC transcription. The ODC promoter with the minor A allele (c. 24% in Caucasians) was more active than that containing the major G allele (c. 76%); this may cause individual differences in response to Myc-inducing stimuli. Tests for the association between these ODC genotypes and the occurrence of new colorectal adenomas were negative, but aspirin treatment reduced adenoma risk predominantly in subjects with at least one A allele (137,139). Similarly, the presence of this allele appeared to be protective against breast cancer (140). Studies with mice also suggest that quite modest reductions in ODC activity may have an effect on the propensity to tumor formation in some tissues. Although *Odc* heterozygosity did not influence induction of neuroblastomas by N-Myc (94), lymphoma development in response to an activated c-Myc was markedly delayed in $Odc^{(+/-)}$ transgenic mice (67), and substantially fewer skin papillomas developed in these mice when subjected to a two stage carcinogenesis protocol (141). Similarly, transgenic expression of AZ, producing a moderate reduction of ODC, blocked skin carcinogenesis in response to two stage carcinogenesis protocols (142), UV radiation (143), or overexpression of the MEK oncogene (144) and reduced tumor incidence in both *N*-nitrosomethylbenzylamine-induced forestomach tumor (145) and nitroquinoline-*N*-oxide-induced esophageal tumor models (145)(D. J. Feith, unpublished observations). These experiments provide a reasonable explanation for the remarkable efficacy of low doses of the ODC inhibitor DFMO in a recent human chemoprevention trial. Daily oral doses of DFMO plus sulindac produced a substantial reduction in colorectal adenoma recurrence in this three-year trial (146,147).

CONCLUSIONS AND FUTURE DIRECTIONS

The biochemistry of the polyamine biosynthetic pathway is now well understood. Additional details and sites of regulatory input will no doubt be found in the future, but there is a good broad understanding of the exquisite regulation of the highly inducible, controlling enzymes ODC, AdoMetDC and SSAT. It is possible that SMO may also be an important regulatory step and studies of the role of this enzyme in controlling polyamine levels and in contributing to pathophysiology by leading to oxidative damage are an exciting new area. Polyamine transport and efflux pathways in mammals are still only partially characterized but recent progress in this field suggests that a much better understanding of the mechanism and the importance of the transporters allowing polyamine movement across membranes will soon be available.

Polyamine functions are so varied and wide-ranging that it is hard to provide a comprehensive overview that covers all aspects. Polyamines are an essential part of the cellular milieu and influence virtually all metabolic reactions either directly or indirectly. It is clear that close regulation of the free polyamine content is essential for normal growth, development and mammalian physiology. The general concept of a polyamine-responsive modulon as developed in bacteria clearly also applies to mammalian cells. Studies of the number of polyamine responsive genes and the extent to which they are changed by physiological alterations in polyamine content are both feasible and likely to be informative. It is important to stress that such studies cannot be carried out using gene array techniques alone. The expression of many polyamine responsive genes is likely to be controlled post-transcriptionally; predominantly, but not exclusively, at the level of translation. Thus, the more laborious proteomic approaches are essential. A better understanding of the polyamine-responsive steps both downstream and possibly upstream of signaling cascades mediated via oncogenes will not only provide new therapeutic opportunities but also increase knowledge of the initiation and maintenance of neoplastic growth.

Effects of polyamines on ion channels are well established as a result of *in vitro* experiments using isolated cell preparations. Elegant biochemical and molecular biological studies have provided mechanisms for these effects and led to the synthesis of agonists and antagonists. Studies using intact animals that clearly demonstrate the physiological importance of these changes are much less advanced, but these are needed urgently, since it is likely that effects of polyamines on ion channels are critical for normal function in mammals. It is also a potential concern with the use of drugs altering polyamine levels that unwanted effects on ion channels could have both short term and long term side effects.

The possibility that genetic variations in proteins controlling cell polyamine levels affect susceptibility to disease including cancer deserves more intensive investigation. The proof of principle for such studies is already available in the work on SRS and on the polymorphisms in the promoter region of the ODC gene. The severe but rare phenotype in SRS is related to a more than 90% reduction in spermine synthase activity. More subtle changes in either spermidine or spermine synthase may lead to alterations in the spermidine:spermine ratio that could affect ion channel activity leading to significant effects on the many critical effects of the Kir channels and glutamate receptors.

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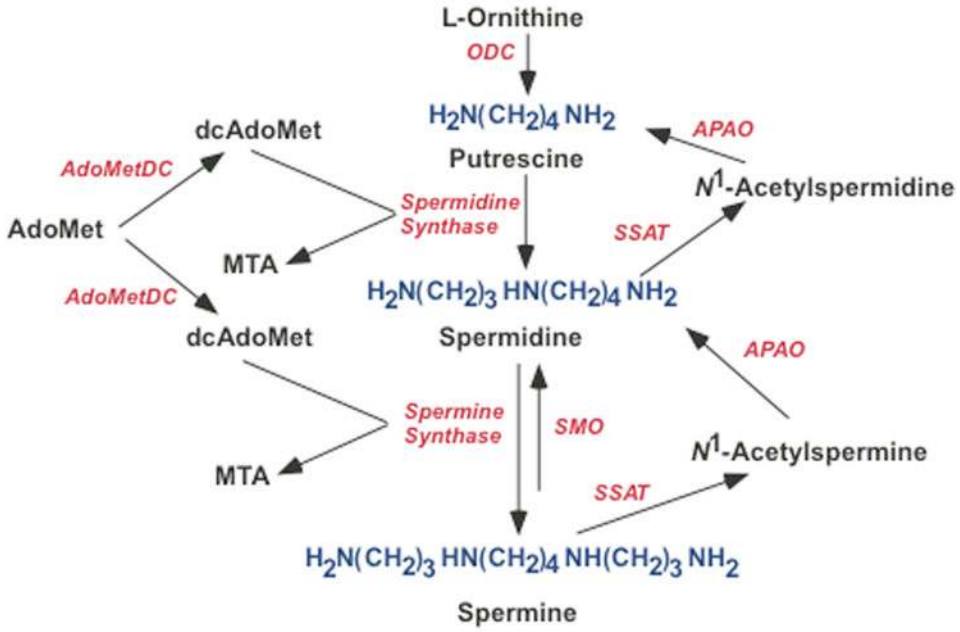

Figure 1.
Polyamine structures, biosynthesis and interconversion. Polyamine structures are shown in blue. Enzymes are shown in red italic fonts (ODC, L-ornithine decarboxylase; AdoMetDC, S-adenosylmethionine decarboxylase; SMO, spermine oxidase; SSAT, spermidine/spermine-N^1-acetyltransferase; APAO, acetylpolyamine oxidase).

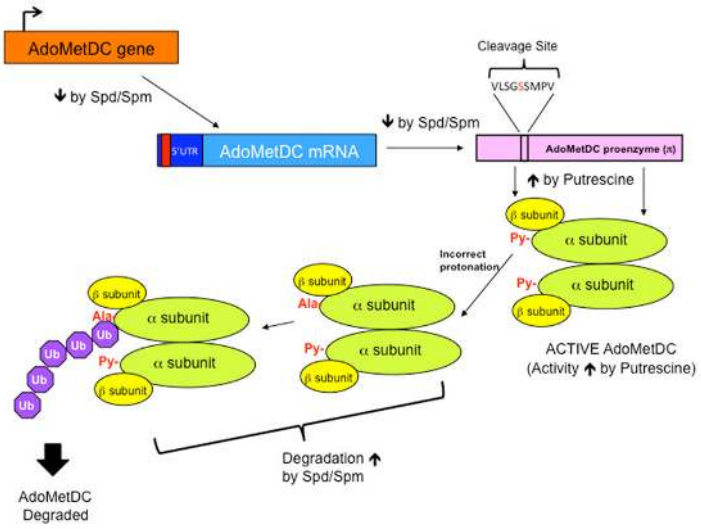

Figure 2.

Regulation of AdoMetDC. The transcription of the AdoMetDC gene and the translation of its message are negatively regulated by spermidine and spermine (Spd/Spm). The translational regulation involves the 5′UTR(shown in dark blue) where a small upstream ORF encoding the peptide MAGDIS (shown in red) is located. The mRNA encodes a proenzyme (π subunit) that undergoes a spontaneous processing reaction generating the pyruvoyl (shown as Py in red) prosthetic group at the amino terminus of the α subunit and a small β subunit. This processing and the activity of the final $(\alpha\beta)_2$ enzyme are activated by putrescine. Incorrect protonation of the pyruvate group during enzymatic reaction can lead to its transamination and conversion to Ala permanently inactivating the enzyme. Degradation of the enzyme by the proteasome requires polyubiquitination and is increased by Spd/Spm. It is possible that transamination precedes ubiquitination as shown, but details are not yet known.

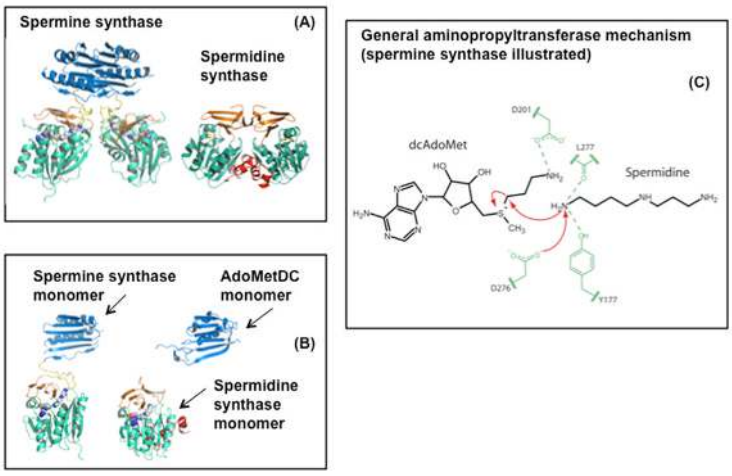

Figure 3.

Structure and mechanism of aminopropyltransferases. Panel A shows the homodimeric forms of spermine synthase and spermidine synthase. Panel B shows the similarity between the N-terminal domain of spermine synthase monomer and the AdoMetDC proenzyme, and the similarity between the structure formed by central- and C-terminal domains of spermine synthase and the spermidine synthase monomer. Panel C shows the general mechanism for aminopropyl transfer illustrated with spermine synthase.

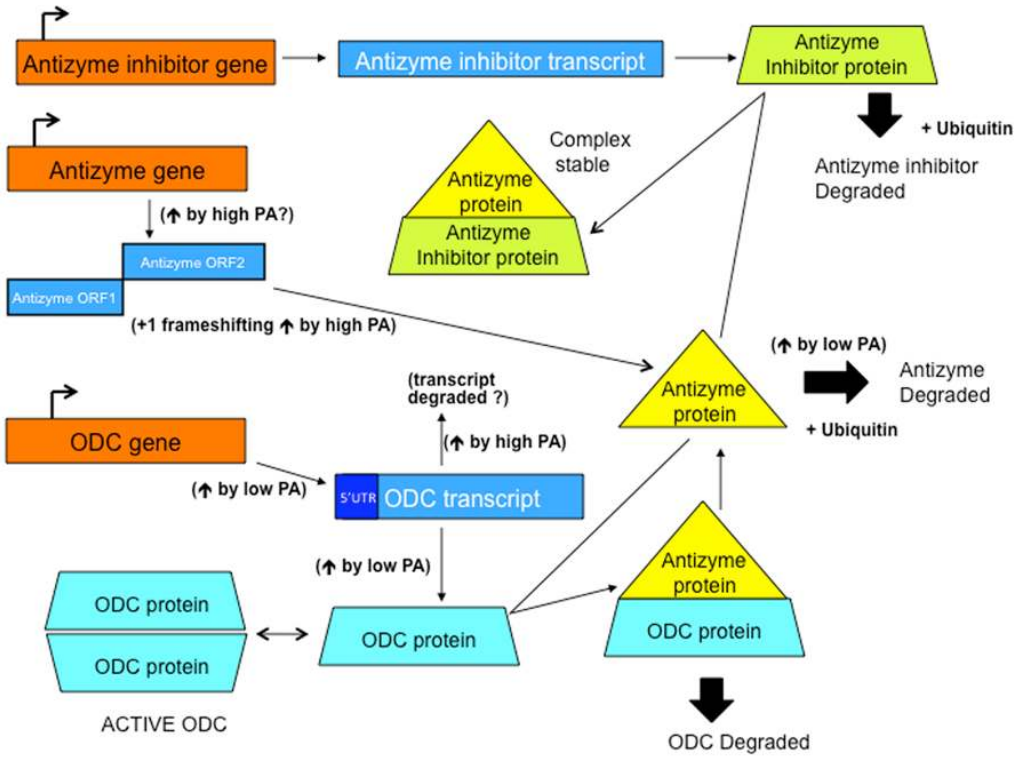

Figure 4.

Regulation of ODC. ODC is regulated by polyamines (PA) at the levels of transcription, translation and protein stability and probably also by transcript stability as shown. ODC translation is regulated by the 5′UTR (shown in dark blue). Only the dimeric form of ODC is active. Antizyme protein (yellow) plays a critical role in the stability of ODC since it binds to the ODC monomer and targets it for proteasomal degradation without need for ubiquitination. Antizyme is released and can recycle to produce further ODC degradation. Antizyme synthesis is increased by high polyamine levels by stimulating the frameshifting needed for correct translation past the internal stop codon, and also possibly by increased transcription. Antizyme is degraded by the proteasome after polyubiquitination and degradation is increased by low polyamine levels. Antizyme inhibitor is an inactive ODC paralog, which is monomeric and is degraded after polyubiquitination. It binds tightly to antizyme forming a stable complex and preventing ODC degradation.

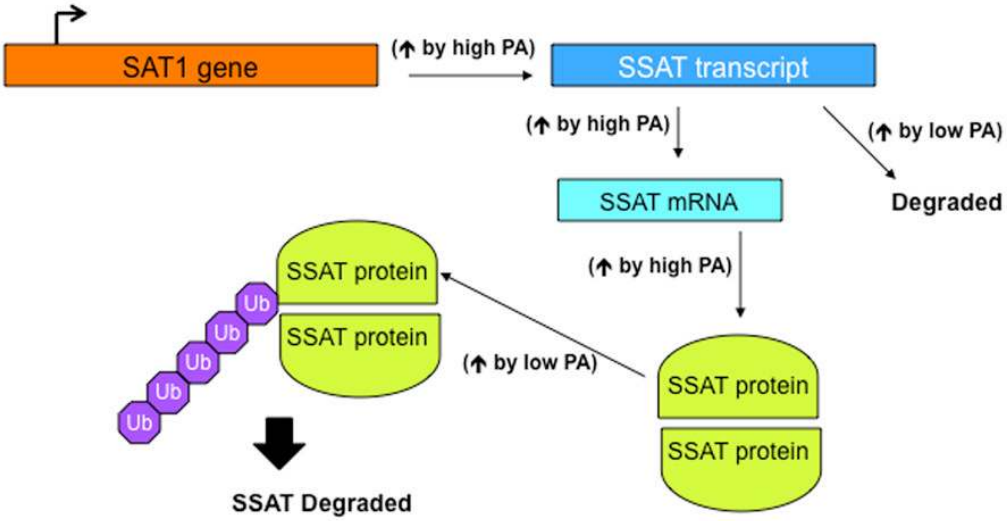

Figure 5.
Regulation of SSAT. Transcription of the *Sat1* gene encoding SSAT, correct processing of the initial transcript and translation of its mRNA are increased by polyamines (PA). The pre-mRNA transcript can also be processed incorrectly and degraded when polyamine levels are low. The active SSAT is a homodimer and it is rapidly ubiquitinated and degraded. Polyamines inhibit this degradation.

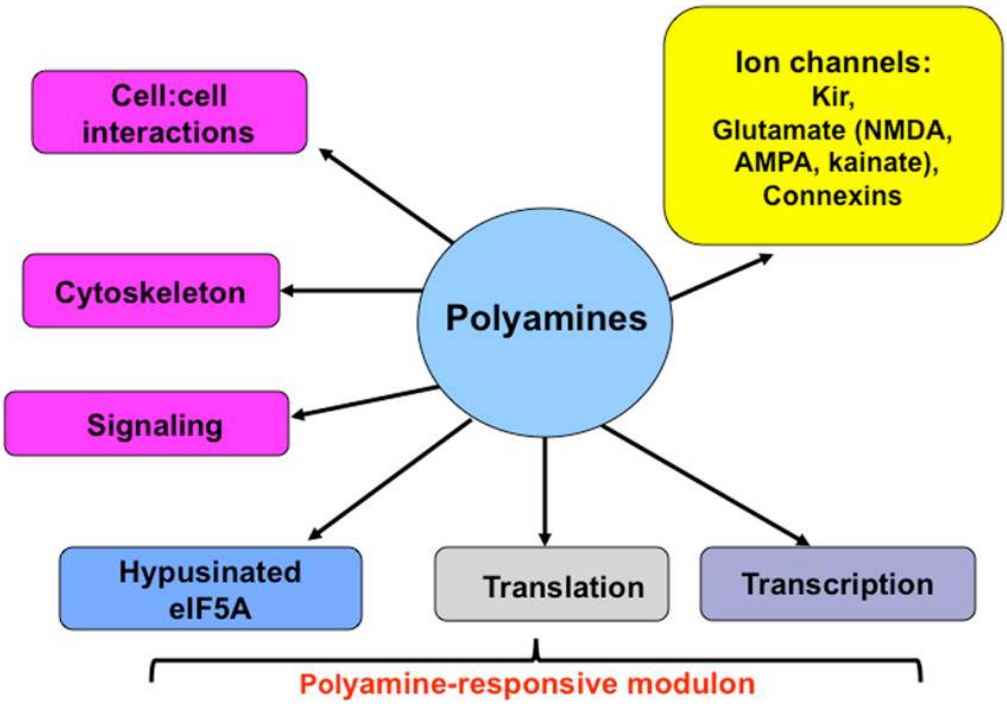

Figure 6.
Functions of polyamines. Polyamine levels affect ion channels, cell-cell interactions, the cytoskeleton, signaling via phosphorylation and other mechanisms, activity of eIF5A via the role of spermidine as a precursor for its hypusination, transcription and mRNA translation. The effects on transcription and translation (both direct and indirect) alter the cellular levels of many proteins making up the polyamine-responsive modulon as described by Igarashi and colleagues (96,97).