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Review article

The functional role of polyamines in eukaryotic cells

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ABSTRACT

Polyamines, consisting of putrescine, spermidine and spermine are essential for normal cell growth and viability in eukaryotic cells. Since polyamines are cations, they interact with DNA, ATP, phospholipids, specific kinds of proteins, and especially with RNA. Consequently, the functions of these acidic compounds and some proteins are modified by polyamines. In this review, the functional modifications of these molecules by polyamines are presented. Structural change of specific mRNAs by polyamines causes the stimulation of the synthesis of several different proteins, which are important for cell growth and viability. eIF5 A, the only known protein containing a spermidine derivative, *i.e.* hypusine, also functions at the level of translation. Experimental results thus far obtained strongly suggest that the most important function of polyamines is at the level of translation.

1. Introduction

Polyamines consisting of putrescine [$NH_2(CH_2)_4NH_2$], spermidine [$NH_2(CH_2)_3NH(CH_2)_4NH_2$], and spermine [$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$] are strongly involved in cell growth and viability in mammalian cells (Cohen, 1998; Igarashi and Kashiwagi, 2010; Pegg, 2016; Tabor and Tabor, 1984), and it was shown that knockout of genes encoding either ornithine decarboxylase (ODC) or *S*-adenosylmethionine decarboxylase (AdoMetDC), rate limiting enzymes of polyamine biosynthesis, caused early embryonic lethality in mice development (Nishimura et al., 2002; Pendeville et al., 2001). Since polyamines are fully protonated under physiological conditions, they can interact with nucleic acids, especially with RNA, ATP, specific kinds of proteins, and phospholipids (Watanabe et al., 1991). In this review, the physiological functions of polyamines are discussed based on their interactions with the acidic compounds.

2. Polyamine distribution in mammalian cells

Polyamine concentration in cells is correlate with the cell proliferation rate, and polyamines interact with acidic substances like nucleic acids. Polyamine distribution was determined in rat liver by estimating the binding constants of polyamines to macromolecules (DNA, RNA and phospholipids) and nucleotide triphosphates and their concentrations (Watanabe et al., 1991). Since ATP is a major nucleotide triphosphate, the binding constants of polyamines to ATP were measured as a representative of four nucleotide triphosphates. Polyamines did not bind to cytoplasmic proteins significantly. The concentrations of K^+ and Mg^{2+} in rat liver were assumed to be 150 mM and 2 mM, respectively. The intracellular water space of rat liver was assumed to be 5.5 μl of cell volume /mg protein (Watanabe et al., 1991).

As shown in Table 1, the concentration of spermidine and spermine was 1.15 and 0.88 mM, respectively, and most polyamines exist as a polyamine-RNA complex. The amount of spermine bound to DNA, RNA, phospholipids and ATP was 0.19, 1.02, 0.05, and 0.97 mol/100 mol phosphates of macromolecules or ATP, and that of spermidine was 0.19, 1.22, 0.09 and 2.02 mol/100 mol phosphates of macromolecules or ATP, respectively. It has been reported that the $tRNA^{Phe}$ crystal structure includes two molecules of spermine (Quigley et al., 1978), which is close to our estimation for polyamine binding to RNA.

It is thought that the weaker binding of polyamines to DNA compared to RNA may be due to a difference in the B- and A-forms of double-stranded nucleotides, respectively. The average distance between two adjacent phosphates of B-form DNA (Protein Data Bank code ID2a) is 6.65 Å (6.16–7.44 Å) for the major groove and 7.35 Å (6.52–8.35 Å) for the minor groove, and that of A-form RNA (Protein Data Bank code 157D) is 6.35 Å (4.97–7.61 Å) for the major groove and

Abbreviations: AdoMetDC, *S*-adenosylmethionine decarboxylase; CR sequence, complementary sequence to 18S rRNA; DFMO, α-difluoromethylornithine; EGBG, ethylglyoxal (bis)guanylhydrazone; Kir, inward rectifier K^+ channel; MMP-9, matrix metalloproteinase-9; NMDA, *N*-methyl-D-aspartate; ODC, ornithine decarboxylase; PC-Acro, protein-conjugated acrolein; SSAT, spermidine/spermine N^1-acetyltransferase; 5′-UTR, 5′-untranslated region

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Table 1
Distribution of polyamines in rat liver.

Acidic substance (mM)		Spermidine mM (%)	Spermine mM (%)
	Total polyamine	1.15 (100)	0.88 (100)
	Free polyamine	0.08 (7.0)	0.02 (2.3)
DNA (28.4*)		0.05 (4.3)	0.05 (5.7)
RNA (73.8*)		0.90 (78.3)	0.75 (85.2)
Phospholipids (77.6*)		0.07 (6.1)	0.04 (4.5)
ATP (2.44)		0.05 (4.3)	0.02 (2.3)

* Phosphate concentration in DNA, RNA or phospholipids.

5.20 Å (4.20–5.98 Å) for the minor groove. On the other hand, the N-N distances of diaminobutane and diaminopropane moieties in all-trans-spermidine are 6.2 and 5.0 Å, respectively (Ouameur and Tajmir-Riahi, 2004). These results strongly suggest that a major part of the cellular function of polyamines may be explained through a structural change of RNA by polyamines.

3. Effect of polyamines on protein synthesis

The effects of polyamines on protein synthesis have been studied, since polyamines mainly exist as a polyamine-RNA complex as mentioned above. It was found that polyamines have not only a sparing effect on the Mg^{2+} requirement for poly(U)-dependent polyphenylalanine synthesis and globin mRNA-dependent globin synthesis, but also a stimulating effect, which cannot be fulfilled by any amount of Mg^{2+} in a rat liver cell-free system (Igarashi et al., 1974; Ogasawara et al., 1989). These results suggest that the structural change of RNA [poly(U) and globin mRNA] caused by polyamine binding is different from that caused by Mg^{2+} binding to RNA. Polyamines also caused the stimulation of rat liver Ile-tRNA formation (Igarashi et al., 1978). This was due to the stabilization of the accepter stem of $tRNA^{Ile}$ by polyamines, which contained the unstable G(5)-G(69) mismatch (Kusama-Eguchi et al., 1991). These results confirm that an unstable RNA structure is stabilized by polyamines.

In case of *Escherichia. coli*, synthesis of 20 different proeins has been identified as being stimulated by polyamines at the level of translation, and it was proposed that a set of genes whose expression is enhanced by polyamines at the level of translation can be classified as a “polyamine modulon” (Igarashi and Kashiwagi, 2015, 2018). Thus, a search was undertaken for proteins in eukaryotes that may be regulated by polyamines analogous to the polyamine modulon in *E. coli*. For this study, mouse mammary carcinoma FM3A or NIH3T3 cells were mainly used. A decrease in polyamine content was induced by an inhibitor of ODC, α-difluoromethylornithine (DFMO) (Mamont et al., 1978). The rate of cell growth decreased to approximately 25% of normal cell growth in the presence of DFMO for 72 h of cell culture. Under these conditions, the content of putrescine and spermidine becomes almost negligible, but that of spermine decreased to approximately 50% of control (Terui et al., 2015).

Thus far, eight proteins have been identified as proteins encoded by the polyamine modulon in eukaryotes (Imamura et al., 2016; Matsufuji et al., 1995; Nishimura et al., 2009; Terui et al., 2015; Uemura et al., 2009). Those are Cox4 (one of the subunits of cytochrome C) in yeast, Cct2 (T-complex protein, β-subunit), Hnrp L (heterogenous nuclear riboprotein L), Pgam1 (phosphoglycerate mutase 1), eEF1 A (elongation factor 1 A), p16 (a transcription factor), EXT2 (exostosin glycosyltransferase 2), and antizymes. Polyamines also stimulated the synthesis of thymidine kinase, DNA polymerase α and MINDY1 (ubiquitin

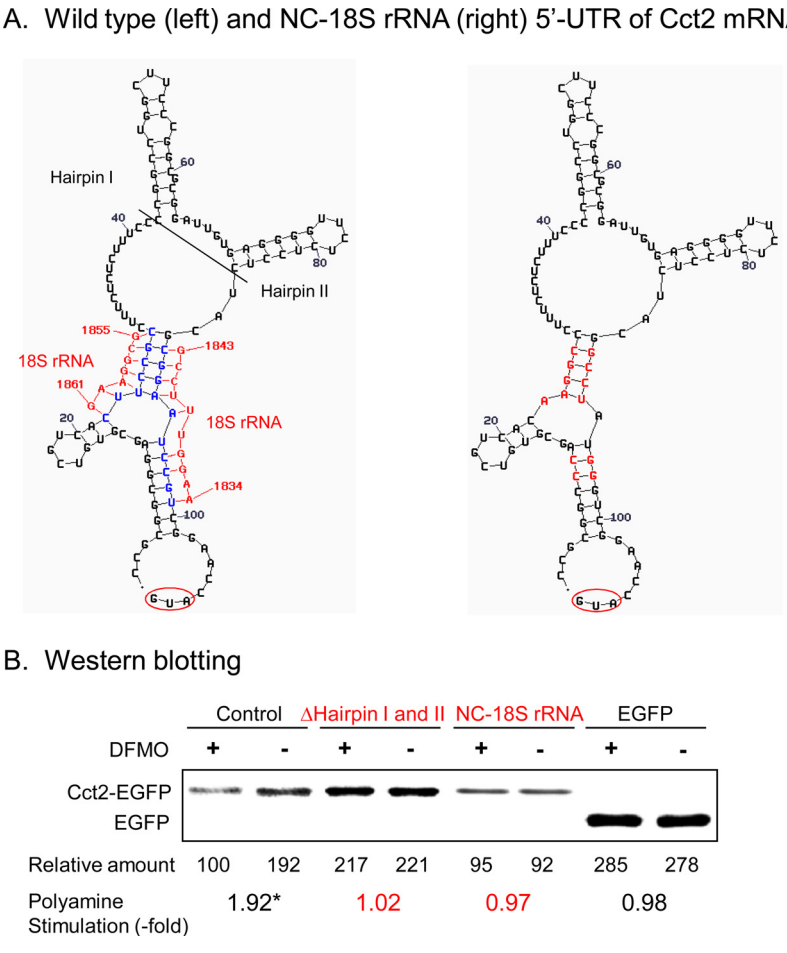

Fig. 1. Mechanism of polyamine stimulation of Cct2 synthesis. A. Candidates for take-off and landing sites of ribosome shunting, which are complementary in sequence to 18S rRNA, are shown in blue together with the sequence of 18S rRNA in red (left), and nucleotide sequence of modified 5′-UTR of Cct2 mRNA, which are not complementary to 18S-rRNA (NC-18S rRNA) are shown (right). B. The level of Cct2-EGFP synthesis was estimated by Western blotting. $*p < 0.05$.

carboxyterminal hydrolase 1, an inhibitor of ubiquitin-dependent protein degradation) (Igarashi and Morris, 1984; James et al., 2018). Those proteins are candidate proteins encoded by the polyamine modulon. It would be expected that at least 20 different proteins are encoded by the mammalian polyamine modulon, since a larger number of genes exist in mammalian cells than *E. coli*.

The mechanisms of polyamine stimulation of protein synthesis were then studied. Initiation of protein synthesis in eukaryotes occurs by the 5′-processive scanning of mRNAs by 40S ribosomal subunits from the m^7G-cap to the initiation codon AUG (Gingras et al., 1999). In contrast, polyamines stimulated Cct2 synthesis through stimulation of ribosome shunting of a stem-loop structure (hairpin structure) during scanning of the 5′-UTR of Cct2 mRNA (Chappell et al., 2006; Yueh and Schneider, 1996). In the 5′-UTR of Cct2 mRNA, there are two hairpin structures (Fig. 1A). When these two hairpin structures were removed from a Cct2-EGFP fusion mRNA, Cct2-EGFP synthesis was enhanced 2.17-fold in the polyamine-reduced (*i.e.* DFMO-treated) cells compared with wild type Cct2-EGFP mRNA, and polyamine stimulation became negligible (Fig. 1B). For ribosome shunting, take-off and landing sites, which are complementary in sequence to 18S rRNA, are necessary in the 5′-UTR of the mRNA (Yueh and Schneider, 1996). If these sequences were converted to non-complementary (NC-18S rRNA) sequences, polyamine stimulation of Cct2-EGFP synthesis disappeared (Fig. 1B). Polyamines also stimulated Cox4 synthesis in yeast through the ribosome shunting of the stem-loop structure in the 5′-UTR of Cox4 mRNA (Uemura et al., 2009).

There is another mechanism of polyamine stimulation of protein synthesis (Fig. 2A and B). It is thought that there is no SD (Shine-Dalgarno)-like sequences (Shine and Dalgarno, 1975) in eukaryotic mRNAs which exhibit complementarity to the nucleotide sequences at the 3′-end of 18S rRNA. However, complementary (CR) sequences, consisting of more than 5 nucleotides to the 3′-end of 18S rRNA, are present at -17 to -32 upstream from the initiation codon AUG in the 5′-UTR of 18 different mRNAs encoding translation factors except eEF1 A mRNA (Terui et al., 2015). Thus, the effects of the CR sequence in mRNAs and polyamines on protein synthesis were examined. Polyamines did not stimulate protein synthesis encoded by 18 different mRNAs possessing a CR sequence at the normal position. When the CR sequence was deleted, protein synthetic activities decreased to less than 70% of intact mRNAs. In eEF1 A mRNA, the CR sequence is located at -33 to -39 upstream from the initiation codon AUG, and polyamines stimulated eEF1 A synthesis about 3-fold. When the CR sequence was shifted to the normal position at -22 to -28 upstream from the AUG, eEF1 A synthesis increased in polyamine-reduced cells and the degree of polyamine stimulation decreased greatly (Fig. 2A and B) (Terui et al., 2015). The results indicate that a CR sequence exists in eukaryotic mRNAs, and location of the CR sequence in mRNAs influences polyamine stimulation of protein synthesis, like the SD sequence in prokaryotic mRNAs. Polyamines also stimulated p16 synthesis with the same mechanism.

The third mechanism of polyamine stimulation of protein synthesis was observed in EXT2 synthesis. It was found that a let-7b microRNA bound to the initiation region of the open reading frame of EXT2 mRNA, and suppressed EXT2 synthesis in DFMO-treated cells. Its suppression was reversed by polyamines through stimulation of the release of the let-7b microRNA from EXT2 mRNA (Imamura et al., 2016). This was confirmed using EXT2-EGFP mRNAs containing nucleotides with weak interaction (Mut 61–82) or strong interaction (Mut 67–71 or Mut 63–79) with let-7b microRNA, *i.e.*, polyamines did not stimulate EXT2-EGFP synthesis when these mutated mRNAs were used instead of wild type mRNA (Fig. 3).

Antizyme not only inhibits ODC activity but also stimulates its degradation (Heller et al., 1976; Murakami et al., 1992). It also inhibits polyamine transport (He et al., 1994; Mitchell et al., 1994). Synthesis of antizyme was stimulated by polyamines through +1 frameshifting at the termination codon existing in the open reading frame of antizyme mRNA (Matsufuji et al., 1995). This is the fourth mechanism of polyamine stimulation of protein synthesis. In Fig. 4, the mechanisms of polyamine stimulation of protein synthesis in eukaryotes are summarized.

It has been also reported that depletion of polyamines preferentially inhibits protein synthesis rather than the synthesis of DNA or RNA (Igarashi and Morris, 1984; Mandal et al., 2013).

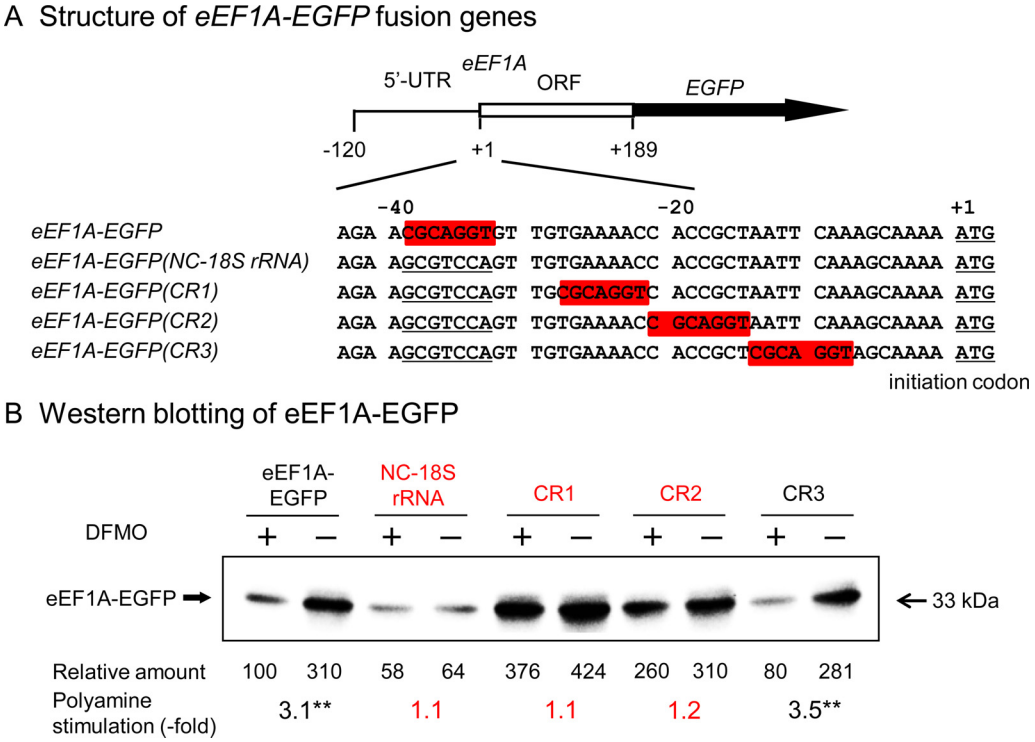

Fig. 2. Mechanism of polyamine stimulation of eEF1 A synthesis. Position of the CR sequence in various eEF1 A-EGFP mRNAs (A) and the level of eEF1 A-EGFP synthesis from these mRNAs (B) are shown. The CR sequence shown in red is a complementary sequence to the nucleotide sequence of 1851 to 1857 of 18S rRNA. ** $p < 0.01$.

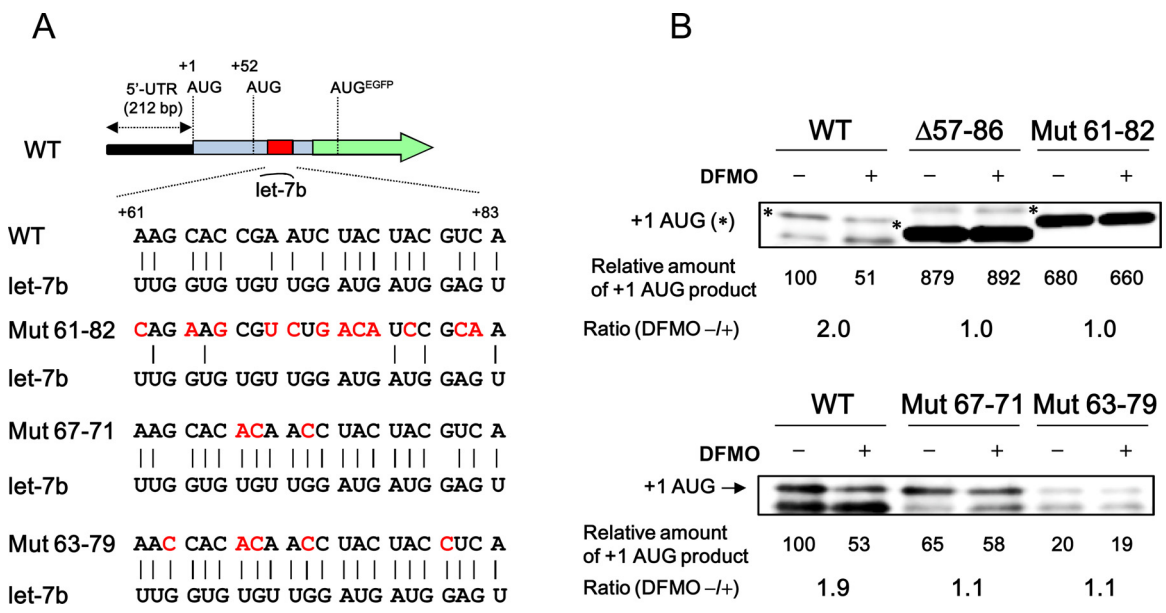

Fig. 3. Mechanism of polyamine stimulation of EXT2 synthesis. A. Nucleotide sequences of let-7b binding site in various EXT2-EGFP fusion genes are shown. B. The level of EXT2-EGFP fusion protein synthesis was measured by Western blotting.

4. Role of eIF5A on protein synthesis

Eukaryotic initiation factor 5 A (eIF5 A) is the only protein containing hypusine [*N*-(4-amino-2-hydroxybutyl)lysine] derived from spermidine at one specific lysine in eukaryotic cells (Lys-50 in human cells) (Park, 2006; Park and Wolff, 2018). Hypusine is formed by deoxypypusine synthase and deoxyhypusine hydroxylase. EIF5 A is essential for cell growth (Park et al., 1997) and the role of eIF5 A in cell proliferation is different from that of polyamines (Nishimura et al., 2005; Terui et al., 2015).

It was also reported that eIF5 A functions as a dimer and its molecular structure is similar to the monomer of its bacterial ortholog, EF-P in *E. coli* (Dias et al., 2013). EF-P is post-translationally modified to a hydroxylated β-lysyllysine at a specific lysine, and is essential for the synthesis of a subset of proteins containing proline stretches in bacterial cells (Doerfel et al., 2013). eIF5A also promoted translation of poly-proline-containing proteins in *S. cerevisiae* (Gutierrez et al., 2013). eIF5A is located near the E-site of the ribosomes with its hypusine residues adjacent to the acceptor stem of the P-site tRNA (Gutierrez et al., 2013). It was also shown that eIF5 A is involved in the cotranslational translocation of proteins into endoplasmic reticulum (ER) (Rossi et al., 2014). Recent data strongly suggest that eIF5 A stimulates translation elongation not only at polyproline stretches but also at many ribosome stalling sites and it also facilitates translation termination (Schuller et al., 2017). In addition, peptide bond formation of methionine with puromycin, an analogue of aminoacyl-tRNA, was also stimulated by eIF5 A (old name eIF4D) (Benne et al., 1978; Park, 1989).

It is also noted that two eIF5 A isoforms are generated from distinct genes (Caraglia et al., 2013; Mathews and Hershey, 2015). The major isoform, eIF5 A1, is considered constitutive and abundantly expressed in most cells. The second isoform, eIF5 A2, is expressed in a few normal tissues but is highly expressed in many cancers.

5. Modulation of DNA-related functions by polyamines

Polyamines weakly interact with DNA (Table 1). In rat liver, spermidine and spermine are estimated to bind to 0.18 mol each/100 mol phosphates of DNA, respectively. If polyamines recognize specific nucleotides preferentially, polyamines may have some effects at the DNA level. It has been reported that 50 μM spermidine or 3 μM spermine stimulated B to Z conversion of poly(dG-m^{5}dC)•poly(dG-m^{5}dC) in the absence of K^+ (or Na^+) and Mg^{2+} (Behe and Felsenfeld, 1981). In the presence of 150 mM NaCl, 50 μM to 1 mM spermine was necessary for B to Z conversion of poly(dG-m^{5}dC)•poly(dG-m^{5}dC) (Thomas et al., 1995). Thus, it is important to determine what concentration of spermidine or spermine is necessary to cause B to Z conversion of poly(dG-m^{5}dC)•poly(dG-m^{5}dC) in the presence of physiological concentrations of both Mg^{2+} and K^+ ions. If 1–2 mM Mg^{2+} exists together with 150 mM K^+, a higher concentration of spermidine or spermine is probably necessary.

As for the regulation of gene expression through B to Z conversion, there are several findings. One is that the binding of estrogen receptor to the upstream region of estrogen response genes was observed near the mid-point of the B-DNA to Z-DNA conversion (Thomas et al., 1995). The second is that Z-DNA formed in the upstream region of the human colony-stimulating factor 1 (CSF1) gene stimulated promoter activity of CSF1 gene (Liu et al., 2001). The third is that Z-DNA formed in the rat nucleolin (Ncl) gene in metabolically active, permeabilized nuclei inhibited the promoter activity of Ncl gene (Rothenburg et al., 2001). However, it remains to be clarified how polyamines are involved in B to Z conversion in these steps.

Polyamines also facilitate oligomerization of nucleosomal arrays *in vitro*, and polyamine mediated chromosome condensation is inhibited by histone hyper-acetylation (Pollard et al., 1999). These results suggest that polyamines are repressors of transcription *in vivo*, and that histone acetylation antagonizes the ability of polyamines to stabilize highly condensed states of chromosomal fibers. We recently found that genes encoding histone acetyltransferases (Gcn5 and Hat1) are members of the polyamine modulon (manuscript in preparation). In addition, it has been reported that polyamine depletion-induced Smads, transcription factors, are associated with a significant increase in transcription (Liu et al., 2003). However, it has been also reported that polyamines enhance the transcription of human polyamine-modulated factor-1, a transcriptional cofactor associated with the transcription factor Nrf-2, through the interaction with a polyamine response element in the promoter region (Wang et al., 1999). The polyamine-modulated factor-1 and Nrf-2 can, in turn enhance the transcription of several genes such as the gene encoding SSAT (spermidine/spermine N^1-acetyltransferase). Furthermore, it was recently shown that polyamines enhance E-cadherin transcription through c-Myc binding to an E-Pal box located at the proximal region of the E-cadherin promoter (Liu et al., 2009). These results suggest that the interaction of polyamines with a

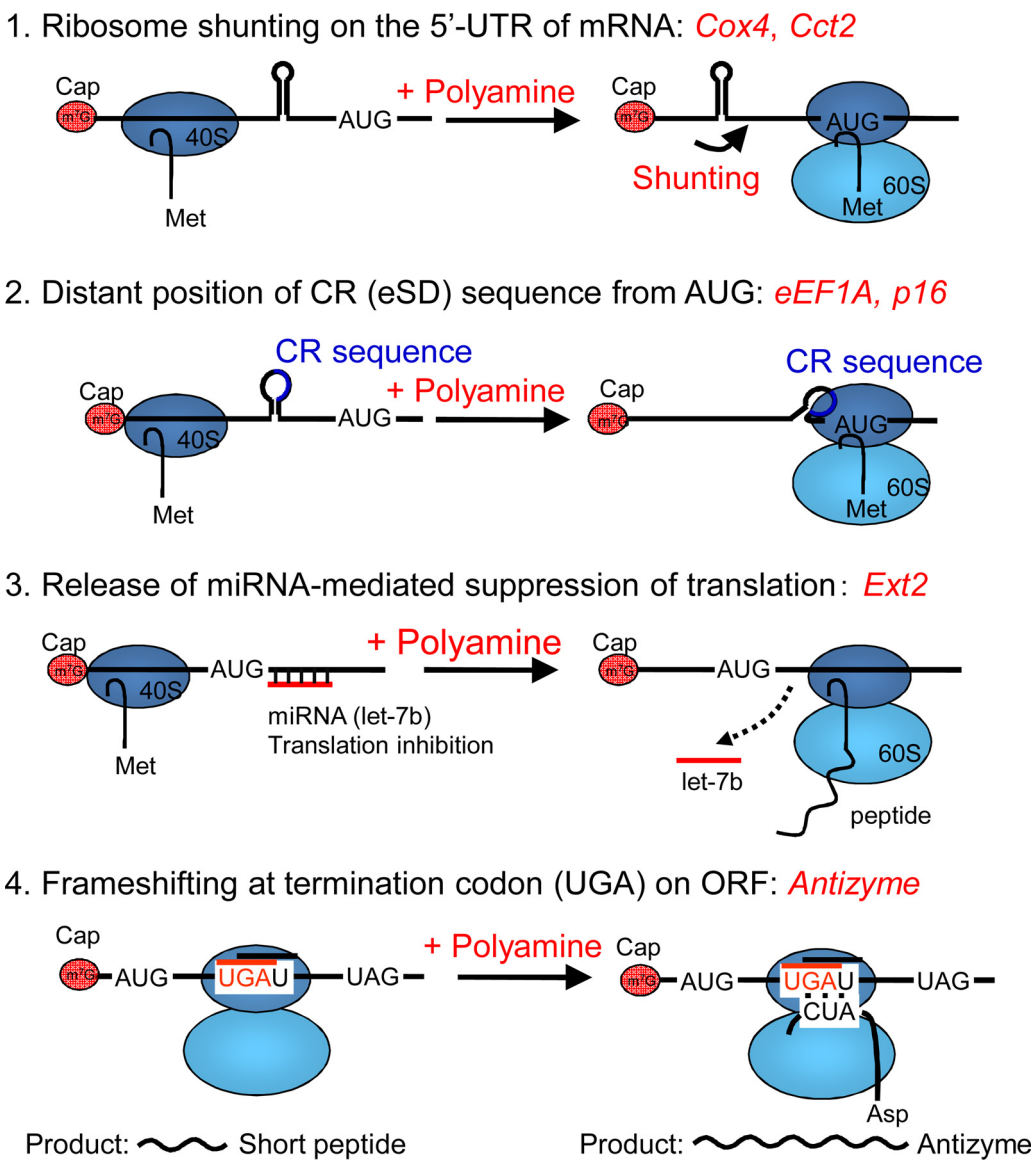

Fig. 4. Four mechanisms of polyamine stimulation of protein synthesis in eukaryotes.

specific region of DNA may influence the transcription of several genes.

6. Regulation of K^+ channel and NMDA receptors by polyamines

As mentioned above, polyamines do not bind to proteins significantly. However, specific interactions of polyamines, in particular spermine, with some types of ion channels have been reported (Williams, 1997). Intracellular spermine is responsible for intrinsic gating and rectification of strong inward rectifier K^+ (Kir) channels by direct plugging of the ion channel pore. These K^+ channels control the resting membrane potential in both excitable and non-excitable cells (Ficker et al., 1994; Lopatin et al., 1994). The spermine-binding site in Kir2.1 has been determined by measuring the effect of spermine blockade on the rate of MTSEA (2-amino ethylmethane thiosulfonate) modifications of cysteine residues strategically substituted in the pore of the channel. Spermine protected cysteines substituted at a deep location in the pore, between the "rectification controller" residue Asp-172 and the selectivity filter, against MTSEA modification. These data indicate that spermine stably binds at a deep site beyond the "rectification controller" residue Asp-172 located near the extracellular entrance to the channel (Kurata et al., 2006).

Activation of *N*-methyl-D-aspartate (NMDA) receptors, a subtype of glutamate receptors, is associated with the induction of various forms of synaptic plasticity, including some forms of long-term potentiation and long-term depression processes that may underlie learning and memory (Traynelis et al., 2010; Williams, 1997). Spermine has multiple effects at the NMDA receptors, including stimulation that increases NMDA receptor currents, and voltage-dependent block at a GluN2 subunit in a specific manner. The stimulation is observed in the NMDA receptor containing a GluN2B subunit, and the block containing GluN2 A or GluN2B (Fig. 5B) (Benveniste and Mayer, 1993; Rock and Macdonald, 1992; Williams et al., 1990). It has been hypothesized that these two effects of spermine involve at least two discrete spermine-binding sites on NMDA receptors (Williams, 1997). NMDA receptors are tetramers containing two molecule each of GluN1 and GluN2 subunit (Laube et al., 1997).

The structure of GluN1 and GluN2 are shown in Fig. 5A. For the activity of the NMDA receptor, both glycine and glutamate are necessary. The glycine binding site is located on the S domain of GluN1, and the glutamate binding site on the S domain of GluN2. Then the spermine binding site involved in the stimulation of the activity of NMDA receptors was searched for by comparison of amino acid sequences with PotD protein, a substrate binding protein of spermidine-preferential uptake system in *E. coli* (Kashiwagi et al., 1996; Sugiyama et al., 1996).

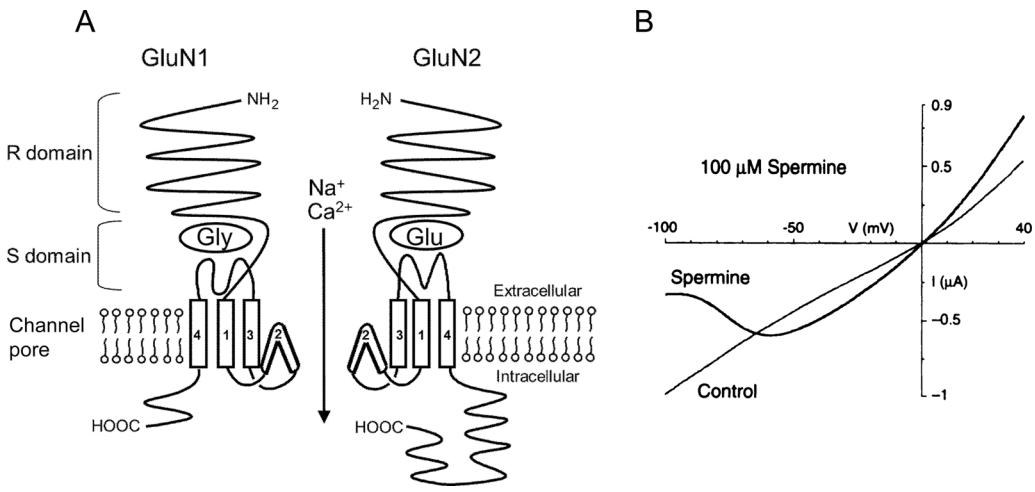

Fig. 5. Schematic showing the structure of NMDA receptor subunits (A) and effect of spermine on NMDA receptors (B). A. NMDA receptors are tetramer that contains combinations of GluN1 and GluN2 subunits. R domain, regulatory domain; S domain, agonist (substrate) binding domain. Portions of the M1, M3 and M4 domains also form part of the ion channel pore and vestibule. M; membrane spanning region. B. Schematized current-voltage (I–V) plots for NMDA (GluN1/GluN2B) receptors. Spermine (100 μM) potentiates NMDA receptor currents at depolarized membrane potential and inhibits them at hyperpolarized membrane potentials.

Several amino acid residues were identified that influence spermine stimulation of the activity of NMDA receptors by measuring the effect of spermine on the activity of amino acid substitution mutants of NMDA receptors expressed in *Xenopus laevis* oocytes. These amino acid residues were located in the regulatory (R) domain (the NH_2-terminal domain) (Fig. 5A) (Masuko et al., 1999). The strength of spermine binding to the purified R domain was in the order GluN1-R (Kd = 18.5 μM) > GluN2B-R (Kd = 32.5 μM) > GluN2 A-R (Kd = 140 μM) (Han et al., 2008).

Next, amino acid residues involved in spermine binding on the R domain were determined by measuring activity of the mutants at the region of R domain (Masuko et al., 1999), together with binding site of ifenprodil (Tomitori et al., 2012), which is an atypical NMDA receptor antagonist that selectively inhibits NMDA receptors containing GluN2B subunit (Williams, 1993). The *Kd* values for ifenprodil bound to GluN1-R and GluN2B-R were 0.18 and 0.21 μM, respectively (Han et al., 2008).

Then, models were constructed based on the published crystal structure of the GluN1 and GluN2B R domains, which form a heterodimer (Karakas et al., 2011). As shown in Fig. 6A, the spermine binding site was formed by the residues near the cleft between the R1 and R2 lobes of the GluN1 R domain (GluN1R) together with residues on the surface of the R2 (C-terminal side) lobe of the GluN2B R domain (GluN2BR). The ifenprodil binding included residues on the surface of the R1 lobe (C-terminal side) of GluN1R together with residues near the cleft between the R1 and R2 lobes of GluN2BR (Fig. 6B). The modeling suggests that an open space between the two R1 lobes of GluN1R and GluN2BR is promoted through spermine binding and that the R1 lobes of GluN1R and GluN2BR approach each other through ifenprodil binding – an effect opposite to that seen with the binding of spermine. Accordingly, these models can explain well the effects of spermine and ifenprodil on the NMDA receptor activity.

Spermine also functions as a voltage-dependent channel blocker (Fig. 5B). To investigate the site and mechanism of action of the voltage-dependent block by spermine, effect of spermine were compared at −20 mM and at −100 mV using GluN1 and GluN2B receptors with point mutations in the transmembrane and pore-forming regions. Block by spermine was predominantly affected by mutations in the M3 segment of GluN1 and especially in the M1 and M3 segments of GluN2B together with the M2 loop region of both subunits (Fig. 7) (Jin et al., 2008). These regions in M1 and M3 are in the outer vestibule of the channel pore and may contribute to a spermine-binding site. Mutations in different regions, predominantly the M3 segment and M2 loop of GluN1 and the M3 segment of GluN2B, influenced spermine stimulation, a surprising finding because spermine stimulation is thought to involve a spermine-binding site in the R domain. Substitution of the residues involved in spermine stimulation in the M1 and M3 segments of GluN1 (P557 G, T648 A, A649C, A653 T, V656 A and L657 A) also generates a constitutively open channel (Kashiwagi et al., 2002). The results suggest that the M3 segment of GluN1 is strongly involved in the channel opening together with the R domain. The results are consistent with the proposal that the relative position of the M1 and M3 transmembrane segments and M2 loop are staggered or asymmetrical in GluN1 and GluN2B subunits (Fig. 7) (Beck et al., 1999; Sobolevsky et al., 2007), and with the idea that stimulation and block by spermine involve separate binding sites and distinct mechanisms. The binding sites of anthraquinone spermidine and tribenzyl spermidine, inhibitors of NMDA receptors, are located at the region of M2 loop (Kashiwagi et al., 2004).

Lastly, it was tested whether polyamines can permeate NMDA receptors expressed in *Xenopus laevis* oocytes and HEK293 cells (Hirose et al., 2015b). It was found that polyamines, especially spermidine, can permeate NMDA channels expressed from GluN1/GluN2 A or GluN1/GluN2B (Fig. 8). Furthermore, spermidine and Ca^{2+} influx was observed in the presence of Mg^{2+}, although Na^+ influx was strongly inhibited by Mg^{2+}. The *Km* values for spermidine influx through GluN1/GluN2 A and GluN1/GluN2B were 2.2 mM and 2.7 mM, respectively, in the presence of isotonic extracellular ion solutions. Spermidine uptake by NMDA receptors was dependent on the presence of glycine and glutamate, and inhibited by Ca^{2+} and by mementine, an NMDA receptor channel blocker. The *Km* values for Ca^{2+} influx through GluN1/GluN2 A and GluN1/GluN2B were 4.6 mM and 3.3 mM, respectively, under the same ionic conditions. The results indicate that spermidine and Ca^{2+}, but not Na^+, can permeate NMDA receptors in the presence of Mg^{2+}. Spermidine, if released locally from presynaptic terminals, where its concentration is high in synaptosomes and synaptic vesicles (Masuko et al., 2003), can permeate NMDA receptors and play a role in synaptic plasticity mediated by NMDA receptors together with Ca^{2+}.

7. Modulation of ATP-and phospholipid-related functions by polyamines

Phosphorylation of proteins plays important roles in signal transduction in eukaryotic cells. It was found that polyamines, especially spermine, make a ternary complex with ATP-Mg^{2+} that can affect phosphorylation by protein kinases (Meksuriyen et al., 1998). Spermine predominantly interacts with the β- and γ-phosphates of ATP in the presence of Mg^{2+} (Fig. 9A). The activity of protein kinase A was stimulated about 2-fold by spermine in the presence of 1–10 mM Mg^{2+} and 30 mM KCl (Fig. 9B). Spermine caused a decrease in the *Km* value of ATP (from 0.2 mM to 0.12 mM), suggesting that a ternary complex of ATP-Mg^{2+}-spermine plays important roles in ATP-involved enzymatic reactions. It is also shown that phosphorylation of GST-EF-1δ, a translational elongation factor fused to GST, by EF-1δ kinase (casein kinase 2-type enzyme) and that of MCM4 (multichromosome maintenance

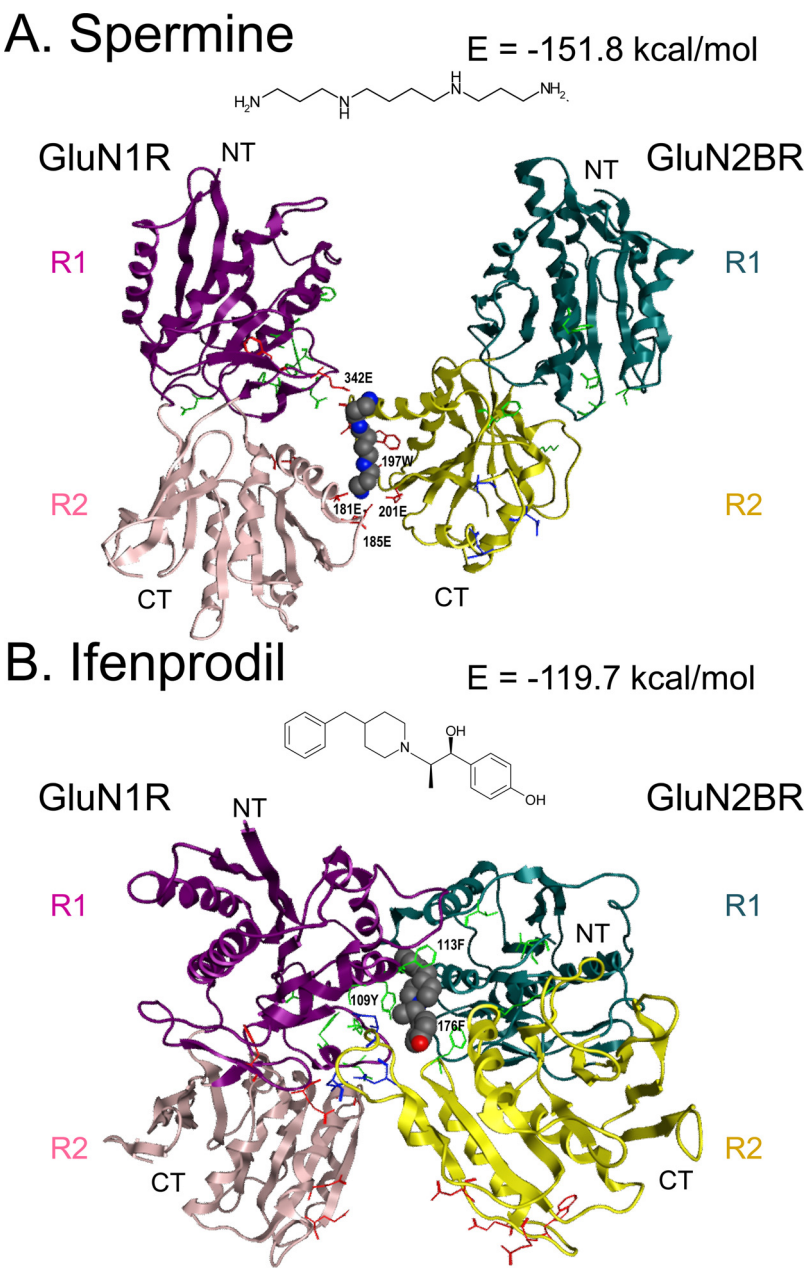

Fig. 6. Modeling of spermine (A) and ifenprodil (B) binding sites on GluN1R and GluN2BR heterodimers. Molecular modeling of GluN1R and GluN2BR, and docking simulation were performed as described previously (Tomitori et al., 2012). NT, N-terminus; CT, C-terminus; R1 and R2, R1 and R2 lobes, respectively. The models show the approximate orientation of GluN1R and GluN2BR. The interaction energy of these complexes is also shown.

protein 4) by Cdc7, which regulates initiation and progression of DNA replication, are enhanced by spermine 2- to 5-fold in the presence of Mg^{2+} without monovalent cations (Delalande et al., 1999; Kakusho et al., 2008). In these two reports, it was not mentioned whether the *Km* value of ATP is decreased by spermine. Thus, the possibility remains that spermine directly interacts with substrate protein or protein kinase. However, it has been recently reported that a cell permeable ATP-polyamine-biotin complex promoted biotin labeling of kinase substrates in living cells (Fouda and Pflum, 2015). These results may suggest that ATP-Mg^{2+} makes a ternary complex with spermine.

Polyamines also interact weakly with phospholipids (Table 1). It was shown that spermine inhibited lipid peroxide formation in liver microsomes (Kitada et al., 1979) and in vesicles prepared with mixed soy bean phospholipids (Tadolini et al., 1984). However, more careful experiments are necessary to clarify the *in vivo* interaction between spermine and phospholipids.

8. Modulation of cell cycle progression and apoptosis by polyamines

It is known that polyamine deficiency delays cell cycle progression with most cells at the G_1/S boundary - *i.e.*, the rate of DNA synthesis is decreased by polyamine deficiency. Thus, the role of polyamines at the G_1/S boundary and in the G_2/M phase of the cell cycle was studied using synchronized HeLa cells treated with thymidine or thymidine plus aphidicolin (Yamashita et al., 2013). Synchronized cells were cultured in the absence and presence of DFMO plus EGBG [ethylglyoxal bis (guanylhydrazone), an inhibitor of AdoMetDC]. When polyamine content was reduced by treatment with DFMO and EGBG, the transition from G_1 to S phase was delayed. In parallel, the level of $p27^{Kip1}$ and $p21^{Cip1/WAF1}$, inhibitors of cyclin-dependent protein kinases CDK2 and CDK4 (Nakayama and Nakayama, 1998), was greatly increased. So, the mechanism was studied in $p27^{Kip1}$ synthesis, because the level of

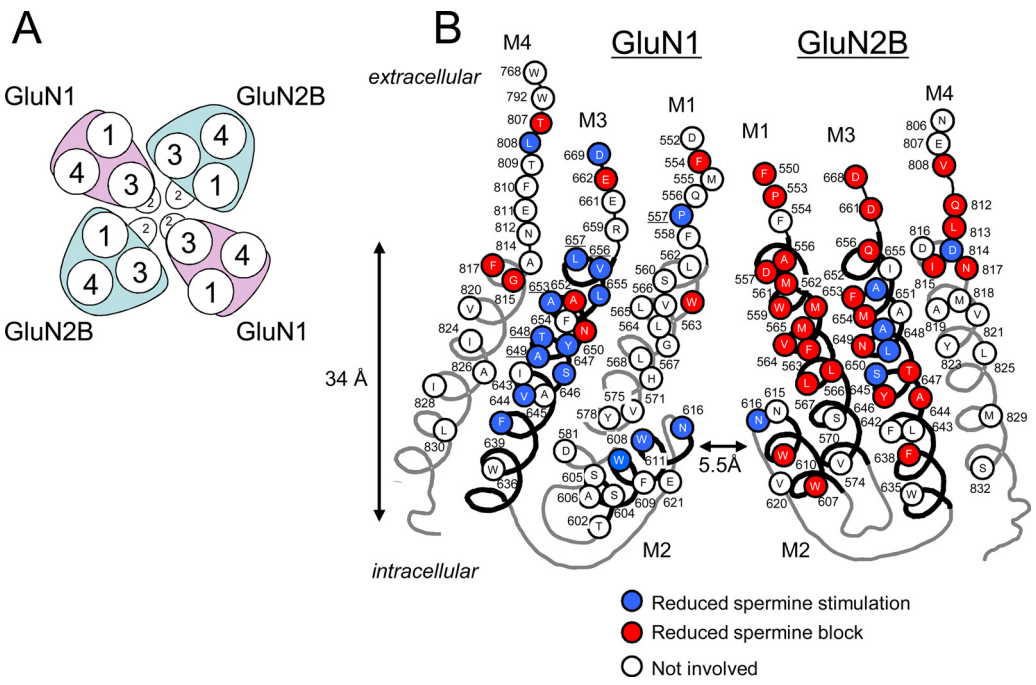

Fig. 7. Models to illustrate possible subunit arrangement (A) and residues that affect spermine stimulation and block in the channel pore and vestibule of GluN1/GluN2B receptors (B).

$p27^{Kip1}$ was more pronounced than that of $p21^{Cip1/WAF1}$ under the condition of polyamine deficiency. Synthesis of $p27^{Kip1}$ was stimulated at the level of translation by a decrease in polyamine levels, because of the presence of long double-stranded 5′-UTR in $p27^{Kip1}$ mRNA. Synthesis of ODC was also inhibited by polyamines due to the existence of long double-stranded 5′-UTR (Ito et al., 1990). Similarly, the transition from the G_2/M to G_1 phase was delayed by a reduction in polyamine levels. In parallel, the number of multinucleate cells increased by 3-fold. This was parallel with the inhibition of cytokinesis due to an unusual distribution of actin and α-tubulin at the M phase. One of the reasons for an unusual distribution of actin and α-tubulin may be due to the decrease in Cct2 protein (Fig. 1), because Cct2 is a chaperonin located in the cytoplasm and assists in the folding of actin, tubulin and several other proteins (Yokota et al., 1999). Since an association of polyamines with chromosomes was not observed by immunofluorescence microscopy at the M phase, polyamines may have a minor role in structural change of chromosomes at the M phase. In general, the involvement of polyamines at the G_2/M phase was smaller than that at the G_1/S boundary.

There are many reports that polyamines influence apoptosis, which finally leads to DNA fragmentation. Protective effects were observed in heat shock-induced cell death of rat thymocyte (Grassilli et al., 1995), tumor necrosis factor induced apoptosis of T cell hybridoma (Penning et al., 1998), epidermal growth factor induced apoptosis of breast cancer cells (Thomas et al., 1999), and B cell antigen receptor mediated apoptosis during B cell clonal deletion (Nitta et al., 2001).

B cell antigen receptor mediated apoptosis in a WEHI murine B cell line was mimicked by polyamine depletion caused by DFMO and EGBG, and addition of exogenous polyamine reversed the observed features of apoptosis (Nitta et al., 2002). Depletion of polyamines induced activation of caspase-3 and disruption of the mitochondrial membrane potential (Δφm). Overexpression of Bcl-XL, an anti-apoptotic Bcl-2 family protein, completely inhibited Δφm disruption, caspase activation, and cell death (Nitta et al., 2002). These results suggest that the depletion of polyamines triggers the mitochondria-mediated pathway for apoptosis, resulting in caspase activation. Similar results were obtained with HEK293 T cells with an adenovirus encoding a key polyamine catabolizing enzyme, spermidine/spermine N^1-acetyltransferase 1 (SSAT1) (Mandal et al., 2015).

On the contrary, polyamines enhanced apoptosis during interferon-independent antiviral response in Jurkat T cells (Grandvaux et al., 2005). In this case, polyamine content increased significantly due to the enhancement of arginase II activity by interferon regulatory factor 3. Since accumulated polyamines inhibit cell growth (He et al., 1993), apoptosis of Jurkat T cells may be enhanced by overaccumulation of polyamines. It has been also reported that apoptosis of 320.3 murine myeloid cells is induced by ODC, although polyamine levels were not shown (Packham and Cleveland, 1994).

9. Tissue damage by acrolein produced from spermine

It is well known that the addition of spermine or spermidine to culture medium containing ruminant serum inhibits cell growth (Higgins et al., 1969). This effect is caused by oxidation of polyamines by serum amine oxidase (Bachrach, 1970). Serum amine oxidase catalyzes the oxidative deamination of spermine and spermidine to produce hydrogen peroxide (H_2O_2) and aminoaldehyde, which is spontaneously converted to acrolein ($CH_2=CH\text{-}CHO$) (Tabor et al., 1964). The same products are also formed from spermine *in vivo*, when spermine is released from ribosomes. It was found that acrolein is more toxic than H_2O_2 (Sharmin et al., 2001).

Thus, it has been examined whether acrolein can serve as a biomarker for several diseases caused by tissue damage. Since polyamines have been suggested to be one of the uremic “toxins”, the level of each polyamine, its oxidized product, acrolein, and spermine oxidase in plasma of patients with renal failure was investigated. The level of putrescine increased, whereas the level of spermine decreased in plasma of patients with renal failure. These patients also had an increased level of spermine oxidase activity and free and protein-conjugated acrolein (PC-Acro). Acrolein levels were about 6-fold higher than in plasma of normal subjects, and the level of PC-Acro was well correlated with the severity of renal failure (Sakata et al., 2003). In the case of stroke, the size of infarct was nearly parallel with the multiplied value of PC-Acro and total polyamine oxidases (spermine oxidase plus acetylpolyamine oxidase) (Tomitori et al., 2005). Furthermore, silent brain infarction can be found with 84% sensitivity and specificity by

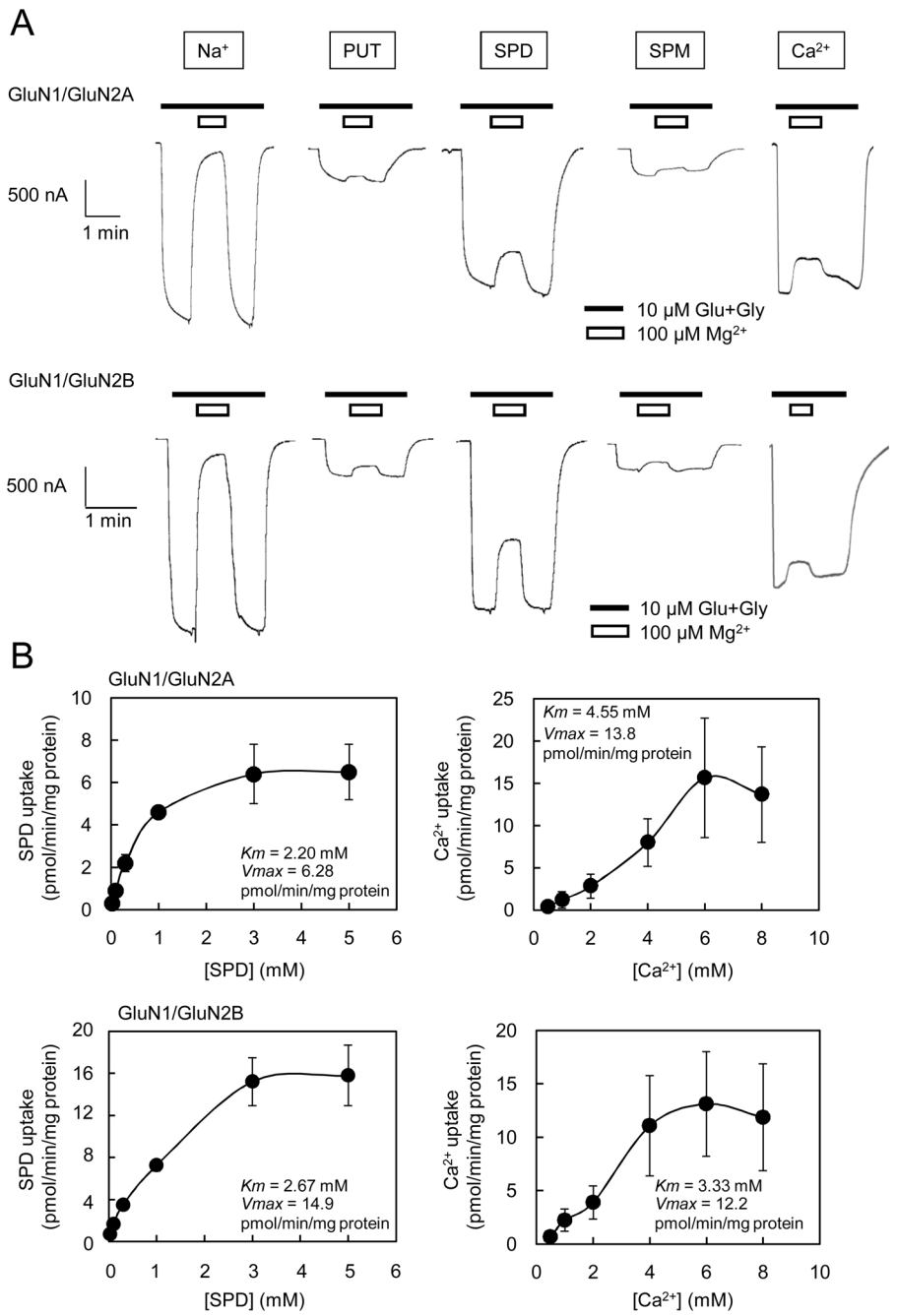

Fig. 8. Effect of Mg^{2+} on inward currents in oocytes expressing GluN1/GluN2 A or GluN1/GluN2B and voltage-clamped at −70 mV (A) and spermidine and Ca^{2+} uptake by GluN1/GluN2 A and GluN1/GluN2B expressed in *Xenopus* oocytes (B). B. The *Km* and *Vmax* values were measured according to the Lineweaver-Burk plot. Values are mean ± S.E.M. of fifth times experiments.

measuring PC-Acro together with interleukin-6 and C-reactive protein (Yoshida et al., 2010). Severity of dementia was also estimated by measuring PC-Acro together with amyloid-β $(Aβ)_{40/42}$ (Waragai et al., 2012). In addition, the altered recognition patterns of immunoglobulins due to acrolein conjugation are at least partially involved in autoimmune diseases (Hirose et al., 2015a). These results indicate that acrolein is strongly involved in tissue damage in elderly people.

Molecular mechanisms of acrolein toxicity and acrolein detoxification were then studied, with the followings findings.

1 Acrolein-conjugated low density lipoprotein (LDL) induced macrophage foam cell formation. Acro-LDL was preferentially taken up by macrophages *via* scavenger receptor class A type 1. Since apoB in Acro-LDL is conjugated with acrolein, it is not susceptible to protease activity in macrophages. Thus, macrophages were converted to foam cells, causing atherosclerosis (Watanabe et al., 2013).
2 Acrolein interacted with Cys-150 at the active site of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the activity was strongly inhibited, causing a decrease in ATP content. In addition, the inactivated GAPDH translocated to nucleus and caused apoptosis (Nakamura et al., 2013).
3 Matrix metalloproteinase-9 (MMP-9) activity was enhanced by acrolein conjugation with Cys-99 in the propeptide domain of MMP-9. Through this conjugation, the propeptide domain was released from the active site of MMP-9. Thus, MMP-9 activity increased (Uemura et al., 2017).

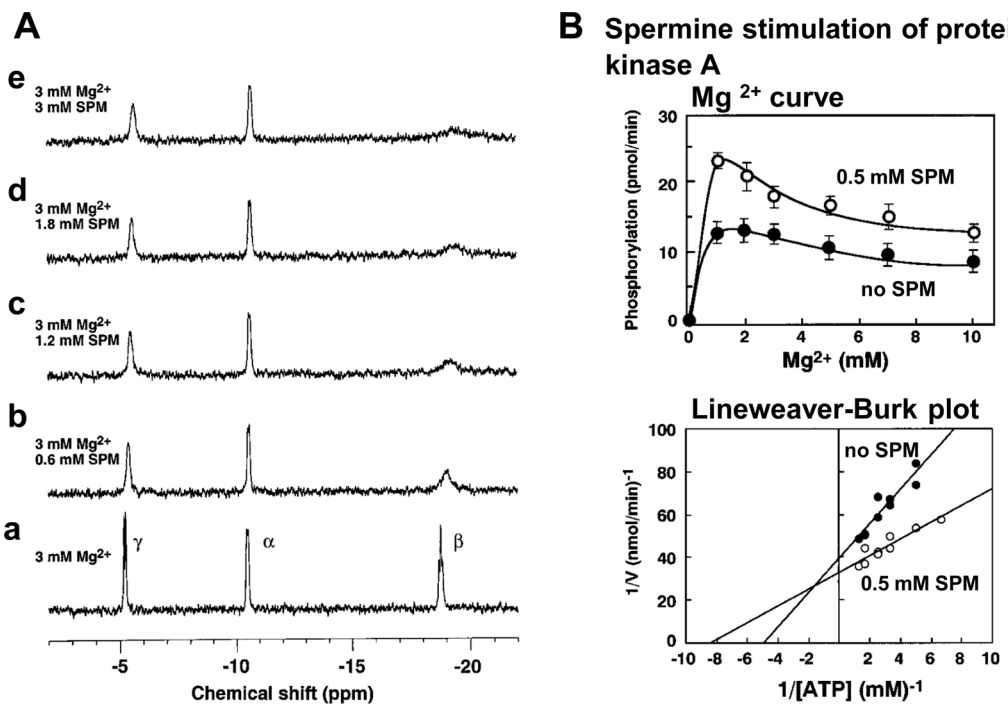

Fig. 9. Change of ^{31}P NMR spectra of ATP-Mg^{2+} at pH 7.8 during the titration of with spermine (A) and effect of spermine on protein kinase A activity (B). B. The reaction mixture containing various concentrations of ATP was incubated for 10 min in the absence (●) and presence (○) of 0.5 mM spermine with 1 mM Mg^{2+} and 30 mM K^+.

4 Acrolein in cells was detoxified by conjugation with glutathione (GSH) catalyzed by GSH *S*-transferase (Uemura et al., 2018). This is an important finding, which clearly show the physiological significance of GSH present in cells in the mM range. We believe that GSH *S*-transferase is more important than GSH peroxidase for acrolein detoxification in cells.

10. Future perspectives

Based on the idea that "there is no function without interaction with target molecules", physiological functions of polyamines were studied at the level of RNAs in our laboratory. In *E. coli*, we found 20 examples of the polyamine modulon (Igarashi and Kashiwagi, 2018). We thus far identified only 8 types of polyamine modulon, including antizyme in eukaryotic cells [6 proteins in Fig. 4, and Hnrpl & Pgam1 (Nishimura et al., 2009)]. In preliminary experiments, we identified 4 additional polyamine modulons. Since the total gene numbers are approximately 4- to 6-fold more in eukaryotic cells than *E. coli*, we believe that there are more than 20 members of the polyamine modulon. If the polyamine modulon is found to be larger in number, the physiological functions of polyamines will be clarified in more detail just like in *E. coli*.

In mammalian cells, spermine exists together with spermidine. So, interaction of spermine with other substances like proteins, ATP and DNA also contributes to the physiological functions of polyamines. Through these kinds of research, the physiological functions of polyamines in mammalian cells will be clarified in near future.

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