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REVIEW ARTICLE

Influence of polyamines on membrane functions

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Introduction

Polyamines (putrescine, spermidine and spermine) are ubiquitous polycationic metabolites in prokaryotic and eukaryotic cells. Their roles in the regulation of major functions in cell growth, cell differentiation and their intricate and exquisitely regulated biosynthetic pathways have attracted considerable attention during the last few decades. Authoritative reviews and books have dealt with these aspects, including the design of enzyme inhibitors which by depleting cells of their normal polyamine contents provoke cell growth arrest and other pharmacologically important phenomena (see, e.g., Heby, 1981; Pegg & McCann, 1982; Tabor & Tabor, 1984; Pegg, 1986, 1988; McCann *et al.*, 1987). Although the functions attributed to the polyamines are numerous, their modes of action at a molecular level remain a matter of speculation. Because of their protonated amino groups (at physiological pH values), polyamines are known to bind to negatively charged cellular macromolecules such as nucleic acids. This has been amply documented and studied, and it is thought to explain many of the polyamine-related effects at the cellular level (Marton & Morris, 1987). In agreement with that notion, interactions of polyamines with components of membranes, such as acidic (phospho)lipids or negatively charged residues of membrane-bound proteins, are to be expected which could affect some properties of biological membranes. Because of their cationic nature, polyamines present many characteristics of inorganic cations such as Mg^{2+} and/or Ca^{2+}. However, fundamental differences should be noted; because of their polycationic structure, i.e. the positive charges are distributed at fixed lengths along a conformationally flexible carbon chain, polyamines are not point-localized charges and are able to bridge critical distances [the distal ammonium charges in spermidine and spermine are separated, in the extended conformation, by 1.1 and 1.6 nm respectively (Liquori *et al.*, 1967)]. These features allow specific interactions and functions, which are not shared by the metal cations.

Effects of polyamines on membranes were among the earliest documented in the polyamine field (e.g. the stabilizing action of spermine on bacterial membranes; Mager, 1959; Tabor, 1960). Since then, numerous publications have indicated that polyamines might influence, if not modulate, membrane properties and functions. The purpose of this review is to summarize observations where polyamines were shown to be of importance in membrane functions. It is not meant to be exhaustive but will rather focus on selected areas where the activity of the polyamines might be of physiological significance. Reviews on the earlier work on the effect of polyamines on membrane structure and functions can be found in Tabor & Tabor (1964, 1972), Cohen (1971) and Bachrach (1973).

Interaction of polyamines with membrane components

Electrostatic interaction of the polycationic polyamines with anionic components of natural membranes and with negatively charged phospholipid head-groups in artificial systems (e.g. liposomes) has attracted attention for some time. The formation of complexes, by bridging and shielding the surface charges, is expected to reduce the repulsive forces between negatively charged membrane components and between membrane particles (leading to aggregation) without affecting the cohesive factors such as van der Waal's interaction forces. Here we will examine the consequences of such interactions from a phenomenological point of view; the physicochemical aspects and the binding studies will be addressed in more detail below.

Submillimolar concentrations of exogenously applied polyamines have been shown early on to stabilize protoplasts or spheroplasts, isolated from microorganisms, against osmotic shocks (Mager, 1959; Tabor, 1962; Harold, 1964). This stabilization was recently studied, with *Escherichia coli* in the exponential phase of growth, using a fluorescence polarization technique (Souzu, 1986). A dose-dependent lipid immobilization was observed with spermine and it was conjectured this might be due to some bridging between integral proteins and membrane lipid binding sites and/or between lipid head-groups. Similar stabilizations were observed with mitochondria (Tabor, 1960) and lysosomes (Powel & Reidenberg, 1982), and polyamines were also found to protect plant protoplasts against lysis and to retard their senescence (Altman *et al.*, 1977; for reviews see Slocum *et al.*, 1984; Smith, 1985). Polyamines bind to several subcellular elements such as plasma membranes and microsomes (Harold, 1964; Igarashi *et al.*, 1982); they also induce the aggregation of microsomes and other subcellular organelles (Tabor, 1960; Jamdar, 1979; Tadolini, 1980; Byczkowski *et al.*, 1982) and of acidic phospholipid vesicles (Hong *et al.*, 1983*a*; Schuber *et al.*, 1983; Tadolini *et al.*, 1985*a*). Interactions of spermine and spermidine with brain subcellular structures (e.g., myelin-rich, synaptosomes), and their significance for the functions of nerve cells, have been reviewed by Seiler & Deckardt (1976) and Seiler (1982). A possible modulator role of polyamines in synaptic transmission was also inferred from their influence on neurotransmitter uptake

Abbreviations used: Ins(1,4)P_2, inositol 1,4-bisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; PtdIns, phosphatidylinositol; PtdInsP_n, polyphosphoinositides; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; ODC, ornithine decarboxylase; PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine.

by synaptosomes (Law *et al.*, 1984). Up to now, for obvious reasons, we in general do not know to which structures polyamines are bound in a living cell. The meaning of results obtained *in vitro* by adding polyamines to membrane preparations are therefore somewhat obscured, and it seems difficult to delineate an effect relevant to an *in vivo* situation from an effect that might be circumstantially due to ionic effects.

An apparently important consequence of the binding of polyamines to the polar head-groups of the membranes is their protective effect against lipid peroxidation (Kitada *et al.*, 1979; Tadolini *et al.*, 1985*b*). In model systems, in order to observe an inhibition of fatty acid peroxidation induced by Fenton's reagent, the formation of a complex between the polyamines and the phosphate head-group of acidic unsaturated phospholipids is of paramount importance (Tadolini *et al.*, 1984; Tadolini, 1988). It was proposed that polyamines could take part in the passive cellular defence mechanism against the oxidative damage caused by Fe^{2+}/Fe^{3+} (Tadolini, 1988). Radical (i.e. superoxide radical anion and hydroxyl radicals) scavenging properties of polyamines in solution were also documented (Drolet *et al.*, 1986); however, in contrast with the studies of the group of Tadolini, who observed marked effects at submicromolar concentrations of spermine, these results were obtained only at much higher polyamine concentrations. Related to this aspect, it is known that amines can yield derivatives of phospholipid hydroperoxides in the presence of haem (Iio & Yoden, 1988). At a cellular level, the anti-peroxidative properties of polyamines were correlated with their protective effect against gastric lesions induced by acidified ethanol (Mizui *et al.*, 1987).

In erythrocytes, the lateral mobilities of fluorescein-labelled transmembrane glycoproteins (Schindler *et al.*, 1980) and Band 3 (Tsuji & Ohnishi, 1986) were found to be modulated by low concentrations of polyamines that interact with the cytoplasmic side of the cells. Spermine decreased dramatically their lateral diffusion independently from variations in lipid microviscosity and rotational motions. In the case of Band 3, which is associated to the cytoskeleton network via ankyrin, this effect was correlated with the polyamine-induced spectrin association state, i.e. spermine and spermidine favour polymerization of spectrin (Tsuji & Ohnishi, 1986; Farmer *et al.*, 1985). Such results could be explained if polyamines were bridging proteins and lipids in the membrane, increasing protein–protein interactions in the cytoskeletal network and bridging skeletal and membrane-bound proteins. A recent study with human erythrocyte membranes, employing e.s.r. techniques, is consistent with such assumptions (Wyse & Butterfield, 1988). Related to these findings is the work of Ballas *et al.* (1983) on the stabilization of erythrocyte membranes by polyamines. Intracellular physiological concentrations of spermine and spermidine were found to decrease the deformability and to increase the mechanical stability of resealed ghosts. These effects were specific to polyamines; Ca^{2+} and Mg^{2+} had an opposite effect. In some disorders which yield rheologically abnormal erythrocytes, e.g. sickle cell anaemia and Duchenne muscular dystrophy, these cells contain abnormally elevated polyamine levels (Ballas *et al.*, 1983). Finally, an increasing number of cytoskeletal proteins were found to interact non-covalently with acidic phospholipids, e.g. protein 4.1 with phosphatidylserine (Cohen *et al.*, 1988); these proteins are important in transmembrane signalling and in cytoskeleton–membrane interactions (reviewed by Burn, 1988). It is predictable that such interactions could also be modulated by polyamines. Related to this is the observation by Pohjanpelto *et al.* (1981) that polyamine starvation in polyamine-auxotrophic CHO cells causes disappearance of actin filaments.

Action of polyamines on membrane-bound enzymes

Modulation of the surface charge by polyamines could play an important role in the regulation of membrane-bound enzymes dealing with charged substrates (Wojtczak & Nalecz, 1979). Alternatively, complexation of polyamines to surface binding sites, by changing membrane surface accessibility and the phospholipid/medium interface, could affect the activity of enzymes acting on membrane components such as (phospho)lipids. Accordingly, Sechi *et al.* (1978) have reported that the hydrolytic activity of soluble phospholipases A_2 and C on phospholipid vesicles or mitochondrial membranes are impaired by polyamines (particularly by spermine), by a Ca^{2+}-unrelated mechanism. Polyamines when added to microsomal preparations were also found to affect the synthesis of prostaglandins in various tissues (Igarashi *et al.*, 1981). The inhibitory effect of spermine on prostaglandin E_2 synthesis was phospholipid-dependent.

Related to the senescence-delaying effect of polyamines on plant cells is their interesting antagonism with ethylene, the senescence-promoting hormone (Slocum *et al.*, 1984; Smith, 1985). Polyamines and ethylene, both deriving from *S*-adenosylmethionine, have in plants interrelated biosynthetic pathways. Exogenously applied polyamines have been reported to inhibit the biosynthesis of ethylene (Apelbaum *et al.* 1981; Suttle, 1981) by blocking its formation from 1-aminocyclopropane-1-carboxylic acid (Fuhrer *et al.*, 1982). This reaction is catalysed by an ill-defined constitutive membrane-associated enzyme system (Yang & Hoffman, 1984). The polyamine effect is somewhat antagonized by Ca^{2+} and it was suggested that through ionic interactions with membranes and changes in membrane microviscosity polyamines could modulate this latter activity (Ben-Arie *et al.*, 1982; Fuhrer *et al.*, 1982). The transformation of 1-aminocyclopropane-1-carboxylate into ethylene can also be induced by radicals (e.g. superoxide radical anion) generated from plant membranes (Yang & Hoffman, 1984), and polyamines acting as scavengers (see above) were found to strongly interfere with such a reaction (Drolet *et al.*, 1986). The specificity and the physiological meaning of these observations are difficult to assess at present, since we do not know whether senescence induced by ethylene can be regulated by signals which affect, *in vivo*, polyamine biosynthesis in plants.

Polyamines were found to modulate the activity of several enzymes involved in the biosynthesis of glycerolipids and phospholipids. Microsomal (rat adipose tissue and liver) *sn*-glycerol 3-phosphate acyltransferase and 1,2-diacyl-*sn*-glycerol acyltransferase were markedly and specifically stimulated by physiological concentrations of spermine independently from Mg^{2+} (Jamdar, 1977, 1979; Bates & Saggerson, 1981); however this action might be due to some extent to the formation of a complex between spermine and the acyl donor, e.g. palmitoyl-CoA, and/or the inhibition of the

hydrolysis of this latter compound (Jamdar, 1979). A similar effect was observed with the *sn*-glycerol 3-phosphate acyltransferase from *E. coli*; in this case spermidine caused a dramatic decrease of the K_m for glycerol 3-phosphate and a stabilization of the native conformation of the palmitoyl–acyl carrier protein (Vallari & Rock, 1982). Polyamines were also found to potentiate the activity of microsomal Mg^{2+}-dependent phosphatidate phosphohydrolase in the presence of optimal divalent ion concentrations (Jamdar & Osborne, 1983). This enzyme in rat adipose tissue is an ambiquitous enzyme, i.e. an enzyme which by changing its subcellular distribution (membrane-bound versus soluble) participates in the regulation of metabolic pathways (Wilson, 1980). It can be translocated by spermine from its cytosolic form to a membrane-bound form (spermidine and putrescine had no significant effect) and is thus activated 4-fold (Moller & Hough, 1982; Martin-Sanz *et al.*, 1985; Jamdar *et al.*, 1987). Polyamines could therefore modulate triacylglycerol synthesis. A similar observation was made with CTP:phosphocholine cytidylyltransferase (Jamdar *et al.*, 1987). In view of these observations, it was suggested that polyamines could modulate glycerolipid biosynthesis needed for membrane production during cell growth (Jamdar *et al.*, 1987). In support of this assumption is the observation that polyamines can also activate choline kinase in rat liver (Fukuyama & Yamashita, 1976).

The activities of microsomal mixed-function oxidases, which act either on xenobiotics (e.g. drugs; Chapman, 1976) or on physiological substrates (e.g. oestrogens; Jellinck & Perry, 1967), were markedly affected by polyamines. This observation is interesting since compounds known as inducers of cytochrome *P*-450 in liver stimulate polyamine biosynthesis (Costa *et al.*, 1976). Based on a thorough study of microsomal mixed-function oxidases and a reconstituted system, Dalet *et al.* (1983) concluded that the locus of polyamine action is cytochrome *P*-450 itself and that the stimulation could result either from an increased stability of the oxyferrous intermediate of cytochrome *P*-450 or from an acceleration of the second electron transfer from the NADPH–cytochrome *P*-450 reductase to cytochrome *P*-450.

Membrane-bound enzymes using UDP-galactose as cosubstrate were also found to be affected by polyamines. The 1,3-β-glucan synthase associated with plasma membranes in soyabean cells was dramatically activated by submicromolar concentrations of spermine (Fink *et al.*, 1987). This effect, which is synergistic with Ca^{2+}, is important in the regulation of callose formation and deposition during plant growth or after cell wall injury, e.g. by pathogens. Mammalian galactosyltransferases involved in the synthesis of glycoproteins are activated by polyamines (Baker & Hillgass, 1974; Navaratnam *et al.*, 1986). However, the physiological significance of this latter effect is questionable because of the cellular topology of these enzymes and the lack of specificity versus metal ions or other cationic species.

The activities of Na^+,K^+-ATPases from different origins were found to be modulated by polyamines, mainly by spermine (Nag & Ghosh, 1973; Heinrich-Hirch *et al.*, 1977). Spermine activated the enzyme at low K^+ and ATP concentrations, whereas an inhibition was observed at higher ATP and K^+ concentrations (Tashima *et al.*, 1977, 1978, 1981). Although it has been proposed that polyamines might regulate this enzyme, the results rather indicate a lack of a specific interacting site for spermine and a competition with monovalent activating cations (Quarfoth *et al.*, 1978). Polyamine effects on Na^+,K^+-ATPase were claimed to explain the hyperpolarizing activity of spermine on atrial muscle preparations (Kecskemeti *et al.*, 1987). Spermine inhibited also Mg^{2+}-ATPase from pig liver, whereas complex behaviour was found for the Ca^{2+}-ATPase from human erythrocytes (Peter *et al.*, 1973). In contrast, Mg^{2+}-ATPase from renal brush-border membranes was somewhat stimulated by polyamines (Elgavish *et al.*, 1984). With respect to these observations one should not overlook the formation of complexes between polyamines and anionic metabolites (Yip & Balis, 1980). The association constant of spermine and ATP is of the same order of magnitude as that of Mg^{2+} (Nakai & Glinsmann, 1977). It is therefore possible that spermine may have a regulatory role in nucleotide-dependent reactions by influencing intracellular levels of free and Mg^{2+}-bound nucleotides.

Finally, the effects of polyamines on membrane-bound acetylcholinesterase, and their possible regulatory significance, have been discussed by Kossorotow *et al.* (1974). Association of polyamines with membranes can also lead to changes in physical parameters of enzymes; examples of integral enzymes are known where spermidine and spermine can affect the Arrhenius plot discontinuities (e.g. glucose-6-phosphatase bound to hepatic endoplasmic reticulum; Johnson & Nordlie, 1980).

Polyamines and transport

The interaction of polyamines with membrane binding sites was found to affect transport of ions and metabolites (the effect of polyamines on M^{n+}-ATPases was treated in the section on membrane-bound enzymes). The meanings of such effects are difficult to assess and in most cases could be ascribed to nonspecific ionic interactions and membrane integrity stabilization. For example, in yeast, spermine potentiates the action of negatively charged membrane-disturbing agents and provides a protection against positively charged ones (Elferink, 1975). In plant cells polyamines limit the efflux of intracellular components, e.g. betacyanin from beet root disks (Naik & Srivastava, 1978), but Ca^{2+} has a similar effect. In maize root segments, exogenous polyamines caused an inhibition of K^+ uptake and H^+ extrusion through the plasma membrane (De Agazio *et al.*, 1988); kinetic studies indicated a competition of polyamines with respect to K^+.

Polyamines, when given exogenously, were able to modulate several functions of mitochondria. Byczkowski *et al.* (1982) indicated that polyamines, in the millimolar concentration range, altered State-4 respiration in mitochondria isolated from rat liver. It was hypothesized that polyamines could interfere with cation exchanges across the membrane, particularly that of K^+, by binding to superficial nonspecific anionic sites present at the inner membrane of the organelle. At micromolar concentrations spermine specifically prevents loss of respiratory control in ageing mitochondria and promotes the restoration of oxidative phosphorylation in aged mitochondria (Phillips & Chaffe, 1982). It was also found that spermine, by binding to submitochondrial particles (from bovine heart), activated, at micromolar

concentrations, the oligomycin-sensitive ATPase by increasing its $V_{max.}$ (Solaini & Tadoloni, 1984). This effect, which was specific for spermine, is abolished by proteolytic treatment of the membranes. Toninello *et al.* (1985, 1986) presented evidence of a transport of spermine into energized rat liver mitochondria, coupled to an enhanced uptake of P_i by this organelle. This transport is in competition with that of Mg^{2+}. Polyamines at micromolar levels activated several-fold the ADP/ATP translocator from mitochondrial inner membranes. This effect, which was shared by other cationic species, showed some structural specificity and was observed on the carrier reconstituted in liposomes or with mitoplasts (Krämer *et al.*, 1986). The polyamines could act, e.g., by binding to the cardiolipins which are tightly associated to the transporter.

Spermine and spermidine at physiological concentrations stimulate the transport *in vitro* of the precursor of ornithine carbamoyltransferase into rat liver mitochondria by an unknown mechanism (Gonzalez-Bosch *et al.*, 1987). The stimulatory effect of the polyamines, which is concentration-dependent, is inhibited by Mg^{2+}, another activator of the transport.

Submillimolar concentrations of spermine and spermidine, when administered, *in vitro*, from the secretory side of bullfrog gastric mucosa, inhibited histamine-stimulated acid secretion (Ray *et al.*, 1982). It was suggested that the polyamines, which did not affect the H^+,K^+-ATPase activity, acted by uncoupling the system, i.e. they decreased the ATPase-mediated uptake of H^+. In isolated renal brush-border membrane vesicles, polyamines stimulated the transport of D-glucose. This effect was rather specific, since L-glucose diffusional uptake was unaffected, and the polycationic species had an opposite action (Elgavish *et al.*, 1984). The transport (efflux) of lysosomal (rat liver) cysteine by a specific carrier was found to be stimulated by submillimolar concentrations of spermidine and, in a non-additive fashion, by divalent cations (Jonas *et al.*, 1987). Interestingly, in this case spermine was less effective than spermidine and the polyamines were thought to regulate the transport by interacting either with the carrier or the lysosomal membrane.

Polyamines and Ca^{2+} homeostasis

During recent years the importance of Ca^{2+} as a universal cellular messenger has been demonstrated (Rasmunssen & Barrett, 1984). Hormone-induced second messengers such as Ins(1,4,5)P_3 were shown to release Ca^{2+}, presumably from endo(sarco)plasmic reticulum (Berridge, 1987) or 'calciosome' pools (Volpe *et al.*, 1988). In this respect the properties of the cellular Ca^{2+} buffers and of the Ca^{2+}-transporting systems are of prime interest. The functions of polyamines in the maintenance of Ca^{2+} homeostasis were examined. Spermine and spermidine were found to play an important role in the regulation of Ca^{2+} transport in mitochondria isolated from rat liver (Nicchitta & Williamson, 1984) or brain (Jensen *et al.*, 1987) or in permeabilized hepatocytes (Nicchitta & Williamson, 1984). Mitochondria, besides a Na^+/Ca^{2+} exchanger, can also transport Ca^{2+} by a transmembrane-potential-driven mechanism; in this case Ca^{2+} is assumed to saturate anionic binding sites (e.g. cardiolipin) inside mitochondria (Carafoli, 1987). Spermine and spermidine, at submillimolar concentrations and in the presence of physiological free Mg^{2+} concentrations, were found to stimulate Ca^{2+} uptake by mitochondria (Nicchitta & Williamson, 1984; Jensen *et al.*, 1987); this was accomplished through an increase of the apparent affinity of the electrophoretic uniporter for Ca^{2+} (Lenzen *et al.*, 1986). Interestingly, Ruthenium Red, another polycationic species, in contrast to polyamines, completely blocks the uniporter-dependent uptake (Moore, 1971). Polyamines also affect mitochondrial Ca^{2+} efflux, through the Ruthenium Red-independent pathway, by decreasing the apparent K_m for efflux (Nicchitta & Williamson, 1984). No similar effect, however, was noted with the Ca^{2+} movement in endoplasmic reticulum (Nicchitta & Williamson, 1984). With regard to this latter point it is important that no strong binding was observed between polyamines and Ins(1,4,5)P_3 (Tadolini & Varani, 1986); this observation is, however, at odds with the strong binding of spermine to polyphosphoinositides and other polyphosphates such as 5-phosphoribosyl-1-pyrophosphate (Yip & Balis, 1980) and nucleotides (Nakai & Glinsmann, 1977). Micromolar concentrations of spermine and spermidine were also found to block efficiently a Ins(1,4,5)P_3-independent unidirectional Ca^{2+} efflux induced by Ca^{2+}-releasing drugs in sarcoplasmic reticulum subfractions (Palade, 1987). The physiological significance of these effects is presently difficult to assess since, e.g., mitochondria seem to play a relatively minor role in the cytosolic homeostasis of Ca^{2+} (Carafoli, 1987). Polyamines could therefore have a permissive role in cellular Ca^{2+} homeostasis rather than a messenger function in the regulation of Ca^{2+} fluxes.

In contrast with this interpretation are recent provocative results indicating a very rapid mobilization of polyamines and intracellular Ca^{2+} mediated by several cellular stimuli. Koenig *et al.* (1983*a*) showed that testosterone-induced processes in kidney cortex (e.g. Ca^{2+} fluxes across the plasma membrane and efflux from intracellular storage organelles, endocytosis, hexose and amino acid transport) were seemingly mediated by a rapid (< 2 min) and sustained increase in cellular polyamines. This is provoked by a rapid (< 1 min) and transient increase in ODC activity. Similar effects were found with the β-adrenergic agonist isoproterenol (Koenig *et al.*, 1983*b*), tri-iodothyrone (Koenig *et al.*, 1984), insulin (Goldstone *et al.*, 1985, 1986) and in K^+-depolarized synaptosomes (Iqbal & Koenig, 1985). The authors suggest that the polyamines serve here as intracellular messengers (or signals) which provoke an increase in cytoplasmic Ca^{2+} concentration. The signal would involve the binding of the polyamines to anionic sites with the consequent opening-up of Ca^{2+} channels; this latter point however has obtained no experimental support. In agreement with this hypothesis of the role of polyamines as messengers in Ca^{2+} fluxes is the finding that α-difluoromethylornithine, an enzyme-activated inhibitor of ODC, blocked the isoproterenol-evoked effects in isolated rat ventricular myocytes (Koenig *et al.*, 1988) and the release of neurotransmitters (γ-aminobutyrate, noradrenaline) from K^+-depolarized synaptosomes (Ibqal & Koenig, 1985). In sharp contrast with the other studies on ODC induction, which imply dependence on protein synthesis *de novo*, the hypothesis of Koenig and coworkers implies a receptor-mediated activation of a pre-existing latent form of ODC, presumably associated with the plasma membrane. Another group working on the mode of action of mitogens

in human T lymphocytes reached similar conclusions. Treatment of the cells with mitogenic monoclonal antibodies or lectins induces a rapid (< 1 min) elevation of ODC activity which reaches a plateau within 5–10 min (Scott *et al.*, 1985*a*,*b*; Mustelin *et al.*, 1986*a*,*b*). This activation of ODC was also found to require an increase in free cytosolic calcium concentrations (Scott *et al.*, 1985*a*). In connection with these increases of ODC activity triggered by cell stimuli, Mustelin *et al.* (1987) first suggested the occurrence in T lymphocytes of an ODC form covalently bound to the cytoplasmic surface of the plasma membrane via phosphoinositides, this form being releasable upon stimulation. However, this finding could not be confirmed (Mustelin *et al.*, 1989), and the rapid activation of ODC seems rather to involve G-proteins.

Polyamines and polyphosphoinositide metabolism

Polyamines have been shown to affect the formation and the metabolism of diacylglycerol and Ins(1,4,5)P_3, which are generated by a Ca^{2+}-dependent phosphoinositide-specific phospholipase C-catalysed hydrolysis of PtdIns(4,5)P_2, a phospholipid located in the inner monolayer of plasma membranes. These intracellular second messengers belong to the major pathway of signal transduction during the response of cells to external receptor ligands (for a review see Berridge, 1987). Diacylglycerol is an endogenous activator, at the plasma membrane level, of protein kinase C, whereas Ins(1,4,5)P_3, which diffuses through the cytoplasm, causes the release of Ca^{2+} from nonmitochondrial intracellular stores. Spermine has been shown to bind tightly to PtdInsP_n, especially to PtdIns(4,5)P_2 (Chung *et al.*, 1985; Meers *et al.*, 1986; Tadolini & Varani, 1986; Toner *et al.*, 1988). A strong complexation of spermine to PtdIns(4,5)P_2 could be observed even in the presence of physiological concentrations of Mg^{2+} (Toner *et al.*, 1988); such an interaction by interfering with the recognition step of this phospholipid by the phosphoinositidase could in principle modulate its turnover. This was indeed found to be the case, at least *in vitro*. It was shown that physiological concentrations of spermine and spermidine inhibited a phospholipase C-dependent hydrolysis of phosphoinositides in rat brain (Eichberg *et al.*, 1981) and in platelets (Nahas & Graff, 1982); in the latter case the inhibition was non-competitive with respect to Ca^{2+} and competitive with respect to PtdIns. On another hand, Sagawa *et al.* (1983) have indicated that the effects of polyamines on the phosphatidylinositol-specific phospholipase C is dependent upon the effective concentrations of substrate and Ca^{2+}; at low concentrations the enzyme is inhibited by spermine whereas at higher concentrations an opposite effect, i.e. stimulation, is observed. A subtle modulation of this enzyme activity by polyamines depending on the polyamine/Ca^{2+} ratio levels could therefore be predicted. However, the significance of these results *in vitro*, e.g. with regard to the formation of Ins(1,4,5)P_3 in cells, is somewhat blurred by the multiplicity of phosphoinositide-specific phospholipase C isoenzymes found in mammalian cells whose activity can be differentially affected by ligands (see, e.g., Ryu *et al.*, 1987). At a cellular level, inhibition of thrombin-induced platelet aggregation by spermine was correlated with a decreased production of inositol phosphates; the meaning of such experiments is however obscured by the very high concentrations of polyamines used (Israels *et al.*, 1986). More recently, Das *et al.* (1987) have described a strong inhibition by low concentrations of exogenous spermidine (IC_{50} 4.2 μM) of the inositol phosphates accumulation triggered, in human neutrophils, by the chemotactic peptide formyl-methionyleucylphenylalanine (fMet-Leu-Phe). Considering the results obtained *in vitro*, a direct effect of the polyamine on the phosphoinositidase seems however excluded. In indirect experiments, Vergara *et al.* (1985) concluded that spermine was able to induce a blockage of the Ca^{2+} signals in electrically stimulated muscle fibres by interfering with the formation of Ins(1,4,5)P_3.

Ins(1,4,5)P_3 5-phosphatase is a key enzyme which metabolizes the second messenger into the inactive Ins(1,4)P_2 and thus terminates its action on Ca^{2+} mobilization. This enzyme, which is predominantly particulate and associated with the plasma membrane, was found blocked by spermine at near physiological concentrations (Seyfred *et al.*, 1984). Spermidine and putrescine did not affect the activity of this Mg^{2+}-activated enzyme. The mechanism of inhibition remains to be clarified, since the polyamine could in principle compete with the divalent ion for a binding site on the enzyme or could complex Ins(1,4,5)P_3 (see the section on polyamines and Ca^{2+} homeostasis above).

During stimulation of polyphosphoinositide turnover, the activities of kinases are regulated in order to replenish the pools of PtdIns(4)P and PtdIns(4,5)P_2 from PtdIns. The activity of the two kinases involved in this biosynthesis were found to be affected by physiological concentrations of polyamines (Schacht, 1976). The PtdIns 4-kinase activity, in plasma membranes from A431 cells, is stimulated 8-fold by spermine in the presence of divalent cations (Mn^{2+} or Mg^{2+}) (Vogel & Hoppe, 1986). Similar observations were made recently with *Xenopus laevis* oocyte membranes (Gatica *et al.*, 1987). Lundberg *et al.* (1987), however, found only a modest effect of spermine on the same enzyme associated with rat liver membranes. The reasons for this discrepancy are unknown, but here again caution should be exercised since several types of this kinase can occur in a tissue (see, e.g., Endemann *et al.*, 1987). The formation of PtdIns(4,5)P_2, catalysed by the PtdIns(4)P 5-kinase, is also activated several-fold by polyamines in rat brain or liver plasma membranes (Lundberg *et al.*, 1986, 1987). Interestingly, work with a partially purified rat brain kinase indicated that maximal stimulation by spermine was observed at the approximate intracellular concentration of Mg^{2+} (Lundberg *et al.*, 1986; Cochet & Chambaz, 1986). The mechanisms of these polyamine effects and their relevance to situations *in vivo* remain to be established. In contrast to polyamines, 5′-deoxy-5′-methylthioadenosine, a metabolite formed in stoichiometric amounts with spermidine and spermine during the aminopropyl transfer steps, was found to be a good competitive inhibitor of the PtdIns kinase of human polymorphonuclear leukocytes (Pike & DeMeester, 1988). This observation, however, might not be relevant to cellular conditions, since in most systems this nucleoside is rapidly cleaved by a phosphorylase (Pegg, 1986).

Polyamines and protein kinase C

Protein phosphorylation and dephosphorylation represent fundamental regulatory mechanisms of cellular activity. Recently the essential role of protein kinase C (Ca^{2+}/phospholipid-dependent protein kinase)

(Nishizuka, 1984, 1986) has emerged; the intracellular distribution of this important enzyme can fluctuate rapidly between a soluble (inactive with most substrates) form, and a membrane-bound form (active). The physical translocation of protein kinase C implies a specific interaction between the enzyme, membrane phospholipids (phosphatidylserine) and Ca^{2+}; in this form protein kinase C can be activated by diacylglycerol, the second messenger released in membranes by a signal-induced hydrolysis of PtdInsP_{II}. According to a model (Bell, 1986), the docking of the kinase occurs on a cluster formed by PS and Ca^{2+} ions, the binding of diacylglycerol to this complex causing a drastic decrease in Ca^{2+} requirement for enzyme activation (Nishizuka, 1984, 1986). The enzyme, in various tissues, was found to be inhibited by spermine, non-competitively with respect to Ca^{2+} and PS (Qi *et al.*,1983). The activation of protein kinase C, from mouse pancreatic islets, by a phorbol ester [tetradecanoylphorbol acetate (TPA)], PS and Ca^{2+}, was also inhibited by spermine or spermidine (Thams *et al.*, 1986). This effect, which was correlated at the cellular level with a decreased insulin secretion, was attributed to a competition between polyamines and Ca^{2+} for the PS domain in the membrane which binds the kinase. From studies on liposomes, it is known that polyamines, especially spermine, can compete with Ca^{2+} for binding to PS (Meers *et al.*, 1986; Tadolini & Hakim, 1989). It is therefore conceivable that spermine, e.g. by competing for the Ca^{2+} binding sites, could interfere with the association of protein kinase C with membranes and consequently modulate its ability to respond to activation by diacylglycerol and to phosphorylate certain substrates. This was borne out in model systems. Moruzzi *et al.* (1987), using protein kinase C purified from rat brain and inside-out human erythrocyte membrane vesicles, were able to show that micromolar concentrations of spermine (spermidine was much less potent) greatly interfered with the formation of the active membrane-associated enzyme complex. That this might not be the only explanation for the inhibitory action of polyamines on protein kinase C activity was verified by the same group, which showed that spermine at millimolar concentrations interacted also with the catalytic domain of the enzyme and strongly inhibits its phosphorylation activity (Mezzetti *et al.*, 1988). In apparent conflict with the foregoing conclusions it was observed that spermine and spermidine greatly increased, *in vitro*, the phosphorylation of proteins associated with membranes in oocyte or cultivated nerve cells (Gatica *et al.*, 1987). In this work, however, the protein kinase(s) were not identified. Moreover, it is now appreciated that protein kinases C represent a family of functional homologous enzymes (Hanks *et al.*, 1988; Nishizuka, 1988).

Binding of polyamines to phospholipids

At first sight the interaction between polyamines and acidic phospholipids could be considered to be essentially electrostatic and follow the counter-ion condensation theory (Wilson & Bloomfield, 1979), i.e. the affinity increases with the number of positive charges (spermine > spermidine > putrescine) and the net negative charge of phospholipids. However, a simple polyelectrolyte effect is an oversimplification since structural elements should also be considered which can modulate these interactions and introduce some specificity, e.g. the binding strength appears to be related to the type of the acidic component of the vesicle (Hong *et al.*, 1983*a*; Schuber *et al.*, 1983). Tadolini *et al.* (1985*a*) found that the order of binding strength of spermine is related to the nature of the acidic head-group: PA > PS > PI > cardiolipin and on their density on the vesicles. The zwitterionic phospholipids, e.g. phosphatidylcholine, show no interaction with the polyamines. The affinity of spermine for polyphosphoinositides depends on the number of phosphate substituents: PtdIns(4,5)P_2 > PtdIns(4)P > PtdIns (Tadolini & Varani, 1986; Meers *et al.*, 1986; Toner *et al.*, 1988). Two landmark publications have tackled the physicochemical aspects of polyamine–phospholipid interactions (Chung *et al.*, 1985; Meers *et al.*, 1986). Binding of spermine to PS vesicles was measured by a microelectrophoretic mobility method (Chung *et al.*, 1985). The interaction could be explained by the Gouy–Chapman–Stern theory, i.e. the negative electrostatic potential in the aqueous diffuse double layer adjacent to the membrane surface, generated by the fixed negative charges of PS, is decreased by a specific adsorption of the polyamine to the membrane, thus changing the charge density (as opposed to a classical screening by a delocalized binding in the double layer). This is an important result, since it predicts structurally related effects involving polyamines and phospholipid head-groups. In vesicles of low PS concentrations a 1:1 complex with spermine was observed, whereas at higher PS concentrations higher-order (2:1) complexes occurred. These latter data suggest that spermine can lay parallel to the membrane surface, interacting with more than one negative charge. A consequence would be that the association of, e.g., spermine with a negatively charged membrane is sensitive to its charge density and to surface area (i.e. lipid molecular packing) occupied by the acidic head-group. Equilibrium dialysis studies revealed a preferential binding of spermine to PA over PS in unilamellar vesicles; i.e. the intrinsic association constants show a 10-fold difference (Meers *et al.*, 1986). Importantly, when on increasing spermine concentrations the bilayers come into close apposition (vesicle aggregation), the binding increases dramatically especially to PA liposomes. This indicates the emergence of new binding sites for spermine, with high affinity, in apposed PA membranes; the polyamine could possibly bridge the vesicles in a multivalent complex. Taken together these studies suggest a direct complexation of polyamines by the acidic groups of phospholipids in membranes. Some consequences are: (i) the complexes are sensitive to the bulk of the head-groups and effects due to steric hinderance are anticipated; (ii) the polyamines could cluster, and possibly phase-separate, acidic phospholipids in a membrane; (iii) polyamines, especially spermine, could bridge acidic phospholipid domains of closely apposed membranes. Some other consequences will be discussed in more detail in the section on polyamines and membrane fusion below.

Binding studies of exogenous polyamines to erythrocytes indicated a strong interaction with the membrane, resulting in a relocalization of the surface charge density distribution; the apparent binding affinity was higher in normal than in sickling cells (Chun *et al.*, 1977). Electrophoretic mobility was more affected by polyamines in normal red blood cells (Chun *et al.*, 1976, 1977). These results were ascribed to the differences in polyamine content and in surface charge between normal and sickling red blood cells.

Studies on membrane model systems, using 1-anilino-8-naphthalenesulphonate, have established that polyamines, by shielding the negative charges of acidic vesicles, promote the interaction of this fluorescent reporter molecule with the membranes (Yung & Green, 1986). Fluorescence polarization determinations indicate that if polyamines affect the environmental rigidity of the phospholipid head-groups, they do not seem to perturb the inner organization of the membranes (Yung & Green, 1986). Similar conclusions were reached some years ago with isolated beef heart mitochondria by Spisni *et al.* (1976*a*,*b*). A surface-rigidifying effect of physiological polyamine concentrations was also found, with a similar method, in microsomes isolated from bean leaves or in liposomes composed of the lipids extracted from this material (Roberts *et al.*, 1986). Taken together, these results indicate that polyamines can rigidify the membrane surface and this must be taken into account in order to explain their effect on membrane functions.

Polyamines and membrane fusion

Membrane fusion is an essential event taking part in many cellular processes such as membrane flow during cell growth, exocytosis (secretion, neurotransmission), endocytosis and cell division (Düzgünes, 1985). It has been extensively studied with model systems e.g., liposomes composed of acidic phospholipids and fusogenic lipids such as PE (Wilschut & Hoekstra, 1986). In most cases fusion was induced by divalent cations (reviewed by Düzgünes *et al.*, 1987) or proteins (Hong *et al.*, 1987). The mechanism of membrane fusion in such systems involved essentially two distinct steps: (i) aggregation of the vesicles leading to the apposition of the phospholipid bilayers, (ii) intermembrane dehydration, induced e.g. by Ca^{2+}, and momentary loss of bilayer structural integrity followed by local membrane coalescence and fusion (Nir *et al.*, 1983).

In model systems, polyamines were found to be modulators of Ca^{2+}-triggered fusion and could even induce fusion of certain membranes in the absence of divalent cations; they were effective (spermine > spermidine) at micromolar concentrations, i.e. at levels much lower than threshold concentrations (0.1–1 mM) of fusogenic divalent cations (Hong *et al.*, 1983*a*; Schuber *et al.*, 1983; Meers *et al.*, 1984, 1986; Ohki & Duax, 1986). As was already mentioned, polyamines by binding to acidic phospholipids decrease the surface charge density and surface potential of the membranes. The consequent reduction of the mutual electrostatic repulsion induces vesicle aggregation as the result of the increased contribution of van der Waals' attractive forces. A barrier to close contact, needed for fusion, however exists due to repulsive hydration forces; this occurs when the bilayers are separated by 2 to 3 nm. The reduction, by polyethylene glycol, of the spermine concentration needed for PA vesicle aggregation illustrates this point (Tadolini *et al.*, 1986). Interestingly, the kinetics of aggregation exhibit thresholds whose concentrations are remarkably dependent on the vesicle composition, i.e., as already noted, polyamines exhibit a binding specificity for certain phospholipid head-groups (Schuber *et al.*, 1983; Tadolini *et al.*, 1985*a*). Moreover this apparent co-operativity, shown in aggregation kinetics, indicates that modest shifts in polyamine concentrations may have pronounced effects on the ensuing fusion process. Spermine caused a dramatic increase in the observed rates of Ca^{2+}-induced fusion of PA vesicles and a large decrease in the threshold concentration of Ca^{2+} required for fusion. Spectacular effects were obtained with vesicles which were allowed to preaggregate in the presence of the polyamine alone, i.e. subsequent addition of Ca^{2+} led to an almost instantaneous fusion (Schuber *et al.*, 1983). The effect of polyamines on PS vesicle fusion was much less pronounced; this phospholipid specificity was explained by the formation, in the spermine-aggregated PA vesicles, of new Ca^{2+}-binding sites of high affinity, presumably by intervesicle chelation (*trans*-complex) (Meers *et al.*, 1986). Spermine has also a striking effect on the aggregation and overall rates of Ca^{2+}-induced fusion of vesicles containing PtdIns(4,5)P_2, thus confirming the strong interaction of polyamines with phosphomonoesters on the surface of membranes (Meers *et al.*, 1986). In contrast to the aforementioned examples, where polyamines are aggregation promoters and do not cause fusion by themselves, liposomes containing high proportions of PE, in addition to acidic phospholipids, undergo fusion in the presence of spermine alone (Schuber *et al.*, 1983). The lag phase occurring in this type of fusion indicates that the aggregation induced by spermine, by interaction with the negatively charged components of the vesicles, could be followed by a local phase separation of PE. Due to the fusogenic nature of PE after aggregation ($L\alpha$-H_{II} phase transition), close apposition of two PE microdomains could lead to vesicle fusion. Taken together these studies on model systems have demonstrated that polyamines do not simply mimic divalent cations such as Ca^{2+} and Mg^{2+}; their effects on fusion, which cannot be explained solely by charge neutralization, are based on specific polyamine–phospholipid interactions.

The relevance of these model studies to situations occurring *in vivo* is open to conjecture. Fusion of biological membranes is a complex process which involves several mechanisms, e.g. constitutive (second-messenger-independent) and signal-regulated exocytosis (Ca^{2+}-dependent) (Düzgünes, 1985; Kelly, 1985; Wilschut & Hoekstra, 1986). In secretory cells, stimulus-triggered exocytosis is correlated with 5–10-fold increases of free intracellular Ca^{2+} concentration, which can reach the millimolar range. Protein-free membrane contact regions are thought to be involved in the fusion of membranes; the extent of protein clearing is controversial but relatively small fusogenic domains (about 50 head-groups) might be sufficient and could be formed, e.g., by a Ca^{2+}-induced lateral phase separation of acidic phospholipids. Model studies with polyamines are obviously relevant to this aspect of this fusion mechanism. In addition, involvement of specific integral and/or soluble fusogenic Ca^{2+}-dependent proteins has also been documented, e.g. in secretion (Burgoyne, 1987, 1988), which could be in part responsible for the interaction specificity and regulation of the fusion process. Polyamines could participate in membrane fusion, e.g., in bridging intracellular vesicles or in the preassociation of exocytic vesicles to the cytoplasmic side of the plasma membrane, thus forming prefusion complexes which show a high affinity for Ca^{2+}. Since the polyamine–membrane interactions show a specificity for the phospholipid composition of microdomains and a strong dependence on polyamine concentrations, it is conceivable that modulation of fusion could be achieved by polyamine concentration changes occurring, e.g.,

during cell growth. Several observations appear to link intracellular polyamine levels to such a property, which certainly also involves the cytoskeleton. It was observed that Chinese hamster ovary cells, whose division was arrested by an ornithine decarboxylase inhibitor accumulate cytoplasmic vacuoles which disappear when the cells resume dividing upon addition of exogenous polyamines (Harada *et al.*, 1981). One can speculate that the lack of cellular polyamines could block membrane biogenesis, by altering normal membrane flow which involves fusion between plasma membrane and Golgi-derived vesicles (Morré, 1977). Inhibition of polyamine biosynthesis also blocks urinary secretion of β-glucuronidase from mouse kidney (Laitinen & Pajunen, 1983) and affects the phagocytic capacity of resident mouse peritoneal macrophages (Kierszenbaum *et al.*, 1987). Furthermore, high polyamine levels have been correlated with secretory activity, e.g. in rat ventral prostate cells (Fuller *et al.*, 1975; Piik *et al.*, 1977; Danzin *et al.*, 1979). As already mentioned, Koenig *et al.* (1983*a*,*b*, 1988) have found a correlation between rapid increases of polyamine levels in rat heart myocytes, triggered by isoproterenol, and an increased endocytosis. Related to this field are the observations on the effect of polyamines on coated vesicles and clathrin. Spermine inhibits the uncoating of coated vesicles (Di Cerbo *et al.*, 1984) and promotes the polymerization of clathrin into basket type structures (Nandi *et al.*, 1981); such effects are shared by basic molecules of widely different structures and are not specific for polyamines. In model systems it was found that clathrin interaction with PS-containing liposomes is inhibited by polyamines (Hong *et al.*, 1983*b*).

Conclusion

Polyamines exert an important role in major biological membrane functions; in many aspects this adds a new and rapidly expanding dimension to their structural and regulatory properties. Since almost invariably the effectiveness of polyamines is dependent on their number of positive charges, electrostatic interactions seem to be a major parameter in their mechanism of action. However, the binding specificity of polyamines to membrane components, which is not duplicated by inorganic ions, is commensurate with their exquisitely regulated intracellular concentrations. The fact that polyamines seem to affect such a large number of membrane functions is in agreement with their now accepted multifunctional effects.

Nevertheless, there remains the perdurable challenge to validate, at cellular level, the observations made with *in vitro* (or model) systems. This holds true also for the physiological responses elicited, in whole cells or tissues, by exogenously added polyamines. In this respect, the manipulation of endogenous concentrations of polyamines by use of specific inhibitors, or utilization of polyamine-auxotrophic cells, should be of some benefit.

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