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Regulation of Ca²⁺ channel and phosphatase activities by polyamines in intestinal and vascular smooth muscle – implications for cellular growth and contractility

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ABSTRACT

Polyamines added extracellularly to intestinal and vascular smooth muscle cells cause relaxation through inhibition of Ca²⁺ channel activity. Intracellularly applied polyamines also affect Ca²⁺ channel properties. Polyamines do not readily pass over the plasma membrane because of their positive charges but in permeabilized smooth muscle preparations they have free access to the cytoplasm. In this system they increase sensitivity of the contractile machinery to Ca²⁺ through inhibition of myosin phosphatase activity. The magnitude of Ca²⁺ channel and phosphatase inhibition depends on the number of positive charges on the polyamine molecule. Polyamines have an obligatory, but yet undefined, role in regulation of cell growth and proliferation. Several groups of protein kinases, such as tyrosine and mitogen activated protein (MAP)-kinases transmit the growth signal from the plasma membrane to the cell nucleus where mitosis and protein synthesis are initiated. The data reviewed here show that polyamines may affect such signal transmission via inhibition of phosphatase activity.

Keywords calcium, ion-channels, polyamines, protein phosphatases, putrescine, smooth muscle, spermidine, spermine.

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The polyamines putrescine (PUT), spermidine (SPD) and spermine (SP) are a group of aliphatic polycations (2, 3 and 4 positive charges, respectively, at physiological pH) that occur in all living cells (Pegg 1986). Although the physiological function of polyamines is still not well understood, there is general agreement that these amines are required for cell growth and proliferation. Polyamine concentrations may vary during the cell cycle and increased levels have been found after trophic stimuli and in association with development of proliferative diseases such as cancer (Russell & Durie 1978, Fredlund *et al.* 1995). If cells are depleted of polyamines by the use of drugs that block key enzymes in polyamine metabolism, their growth is arrested (Pegg 1986, 1988). Unfortunately, the anti-cancer effect of polyamine synthesis inhibitors has been disappointing because of escape from cellular polyamine deprivation by uptake from the extracellular environment (Pegg 1988). Recent studies suggest that polyamine uptake depends on cell surface proteoglycans and that combined treatment with inhibitors of heparan

sulphate proteoglycan assembly (xylose) and polyamine synthesis [difluoromethylornithine (DFMO)] prevents tumour formation (Belting *et al.* 1999, 2002).

The mechanism by which polyamines affect cellular growth and proliferation has not been identified. High concentrations (mM) of polyamines are found not only in tissues with high cell-turnover but also in quiescent tissues, such as muscle and neuronal tissues, suggesting that polyamines are important also for other processes than growth. Polyamines have been reported to affect both excitable membrane properties and second messenger systems (Tadolini & Varani 1986, Schuber 1989, Yoshida *et al.* 2001). Specific interactions of polyamines with different ion channels have also been reported (Scott *et al.* 1993, Williams 1997). The effects on ion channel properties are observed at concentrations varying from nM to mM, and have been reported for polyamines applied intracellularly or extracellularly, and sometimes by either route.

Recent findings show that polyamines increase Ca²⁺-activated force in intestinal and vascular smooth muscle through inhibition of myosin phosphatase

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activity, causing increased phosphorylation of the 20 kDa myosin light chains, which is the predominant mechanism for regulation of contraction in smooth muscle. In other systems, modulation of polyamine levels have been shown to influence tyrosine kinase and mitogen activated protein kinase (MAPK) activities as well as the cytoskeleton. Polyamines also inhibit Ca^{2+} channels in vascular and intestinal smooth muscle, suggesting that they might serve as endogenous regulators of intracellular $[Ca^{2+}]$. A direct effect of polyamines on ion channel properties has been demonstrated in K^+ channels, where inward rectifier properties depend on high-affinity binding of polyamines to the channel pore (Ficker *et al.* 1994, Lopatin *et al.* 1994). Polyamines modify the properties of physiological regulatory systems by affecting ligand binding to charged molecules and surfaces, thus explaining the diversity of their effects. Often the order of potency of the different polyamines in eliciting any given effect follows their amount of positive charge, consistent with electrostatic interaction as the dominant mechanism of action.

POLYAMINE METABOLISM

Putrescine is formed by a decarboxylation of the amino acid ornithine, and SPD and SP are then formed by a sequential addition of first one and then a second aminopropyl group (Fig. 1). The decarboxylation of ornithine, which is a key step in polyamine formation, is catalysed by ornithine decarboxylase (ODC). This enzyme is blocked by the inhibitor α-difluoromethylornithine (DFMO; Metcalf *et al.* 1978). Another key step is the decarboxylation of S-adenosylmethionine to decarboxylated S-adenosylmethionine. Decarboxylated S-adenosylmethionine serves as the aminopropyl donor

in the synthesis of SPD and SP (Fig. 1). The decarboxylation of S-adenosylmethionine is catalysed by adenosyl/methionine decarboxylase (AdoMetDC). This reaction is blocked by the potent and selective AdoMetDC inhibitor CGP48664 (Stanek *et al.* 1993, Regenass *et al.* 1994). Cellular polyamine concentrations are tightly regulated. The cellular levels of the higher polyamines SPD and SP are prevented from increasing by the presence of an interconversion pathway, where SPD and SP are acetylated (catalysed by SPD/SP N^1-acetyltransferase, SSAT) and oxidized (catalysed by polyamine oxidase) back to PUT (Pegg 1986, 1988). Degradation of PUT involves an oxidative deamination catalysed by diamine oxidase yielding the intermediate metabolites 4-aminobutyric acid (GABA) and pyrrolidin (Seiler 1992). The rate-limiting enzymes in polyamine biosynthesis and catabolism, i.e. ODC, AdoMetDC and SSAT, have extremely short half-lives and are therefore able to respond rapidly to appropriate stimuli or altered polyamine levels by adjusting their concentrations. Because of their positive charges the polyamines do not readily pass the plasma membrane. Under normal circumstances cells depend primarily on *de novo* synthesis for their supply of polyamines. However, when cellular polyamine synthesis is inhibited the uptake of polyamines is increased, compensating for the loss of endogenous synthesis (Seiler & Dezeure 1990).

POLYAMINE CONTENTS IN ORGANS AND TISSUES

The concentrations of polyamines vary between different organs and tissues (Bachrach 1973, Russell & Durie 1978). In general, tissues with high rates of proliferation and protein synthesis, such as the large and small intestine, liver, prostate and pancreas, contain
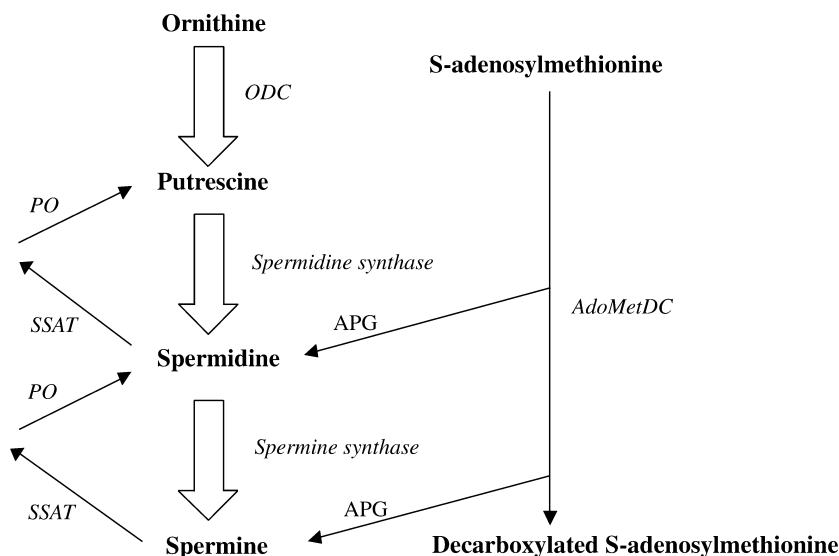
Figure 1 Polyamine formation pathways. The enzymes involved in polyamine biosynthesis are: ornithine decarboxylase (ODC), adenosylmethionine decarboxylase (AdoMetDC), spermidine synthase and spermine synthase. The enzymes involved in the interconversion pathway are: Spermidine/spermine-N^1-acetyltransferase (SSAT) and polyamine oxidase (PO). APG stands for aminopropylgroup.

higher levels of polyamines than more quiescent tissues such as brain, heart and skeletal muscle. Based on measurements of polyamine concentrations in intestinal smooth muscle (Swärd *et al.* 1994), we estimated the total intracellular SP concentration to be about 0.4 mM, while the SPD and PUT concentrations were lower (0.2 and 0.02 mM, respectively). At these concentrations exogenously added polyamines affect contractile activity, Ca^{2+} channel activity and Ca^{2+} sensitivity of the contractile machinery in intestinal and vascular smooth muscle (De Meis 1967, Nilsson & Hellstrand 1993, Swärd *et al.* 1994, 1995, Gomez & Hellstrand 1995, 1999, Nilsson *et al.* 1995). It has been argued that a substantial fraction of the total pool of polyamines is bound to adenosine triphosphate (ATP) to phospholipids and to cellular macromolecules, such as DNA and RNA, or is located in a separate compartment. Thus, only the free intracellular concentration, which is considerably lower (in the μM range; Watanabe *et al.* 1991), would be of physiological interest. This should be kept in mind in situations where polyamines are administered in buffers of extracellular composition containing no high energy phosphates. On the other hand, in buffers of intracellular composition, where high energy phosphates are added to physiological concentrations, the concentration of free polyamines will be considerably lower than the total concentration, approximating the physiological situation.

EFFECTS OF POLYAMINES ON INTACT SMOOTH MUSCLE

Contractility and intracellular [Ca²⁺]

Polyamines have been shown to attenuate electrical and mechanical activity in uterine smooth muscle (Hashimoto *et al.* 1973, Maruta *et al.* 1985). Addition of polyamines to intestinal smooth muscle causes relaxation of spontaneous as well as agonist-evoked contractions and decreases intracellular $[Ca^{2+}]$ (Nilsson & Hellstrand 1993, Swärd *et al.* 1994). Spermine is more potent than SPD, while PUT has no effect. At 1 mM SP totally inhibits spontaneous contractions in taenia coli and decreases force and intracellular $[Ca^{2+}]$ in smooth muscle activated with 20 mM K⁺ (Figs 2 and 3). In vascular smooth muscle from rat portal veins polyamines inhibit spontaneous as well as agonist

(phenylephrine) stimulated contractions (Nilsson *et al.* 1995).

Ca²⁺ channel activity

The inhibiting effect of SP and SPD on contractile activity and intracellular $[Ca^{2+}]$ can be explained by inhibition of inward current through voltage-sensitive Ca^{2+} channels (Gomez & Hellstrand 1995). In agreement with results from microelectrode and force experiments, SP is more potent than SPD, while PUT has no effect on Ca^{2+} channel activity. Exogenously applied polyamines are able to inhibit L-type Ca^{2+} channels from the outside as well as from the inside of the plasma membrane. In Figure 4 the effect of SP on single channel activity in an inside-out patch is shown. Inhibition of SPD and SP synthesis in intestinal smooth
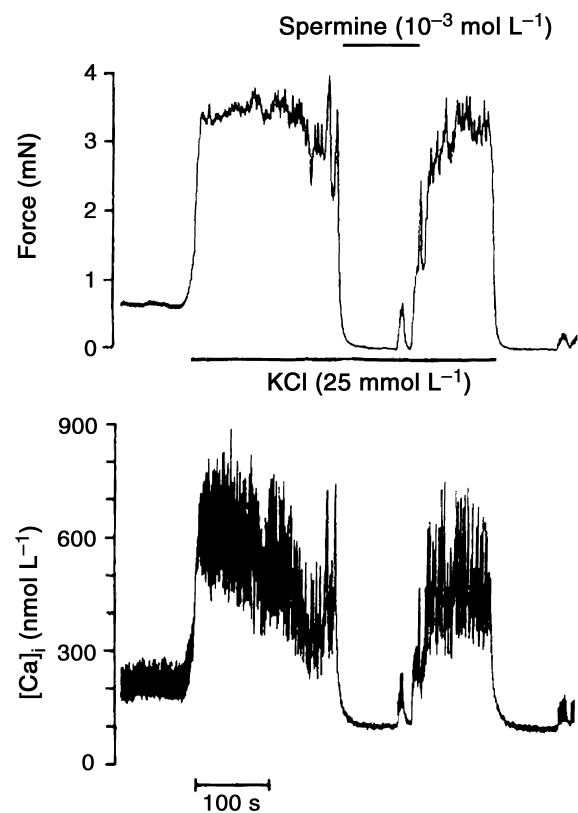
**Figure 3** Spermine (1 mM) added during depolarization with 25 mM K⁺ inhibits force and decrease $[Ca^{2+}]_i$ to basal level in rat taenia coli. From Nilsson & Hellstrand (1993) (© Acta Physiologica Scandinavica 1993) by permission.

Figure 2 Spermine (1 mM) totally abolishes spontaneous contractions in rat taenia coli. From Nilsson & Hellstrand (1993) (© Acta Physiologica Scandinavica 1993) by permission.

(a) Control

(b) +Sp (1mM)

2 pA

100 ms
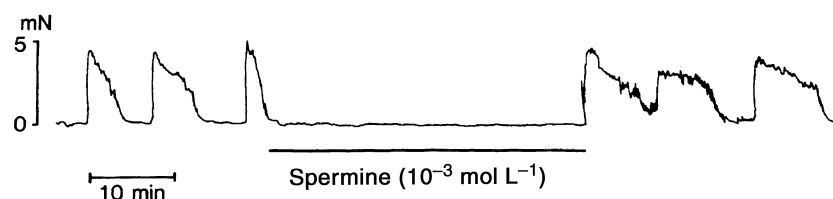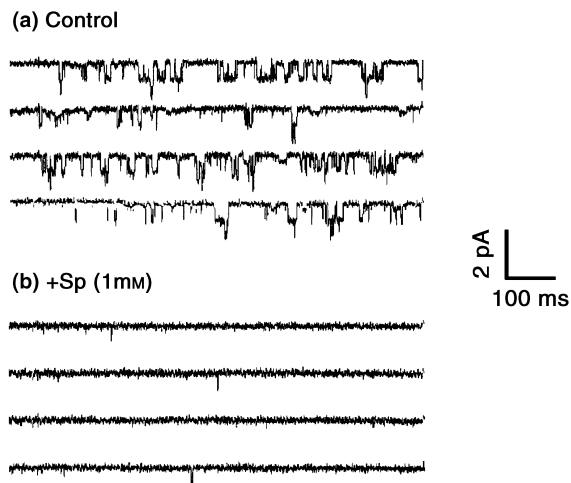
Figure 4 Spermine inhibits single channel activity in inside-out patches from smooth muscle cells of guinea-pig ileum. Current traces from the same patch in response to depolarizing pulses from –70 to 0 mV recorded in the absence (a, control) or in the presence (b) of 1 mM spermine (SP). From Gomez & Hellstrand (1999) (© Springer-Verlag 1999) by permission.

(a)

Control

CGP 48664

50 pA

50 ms

(b)
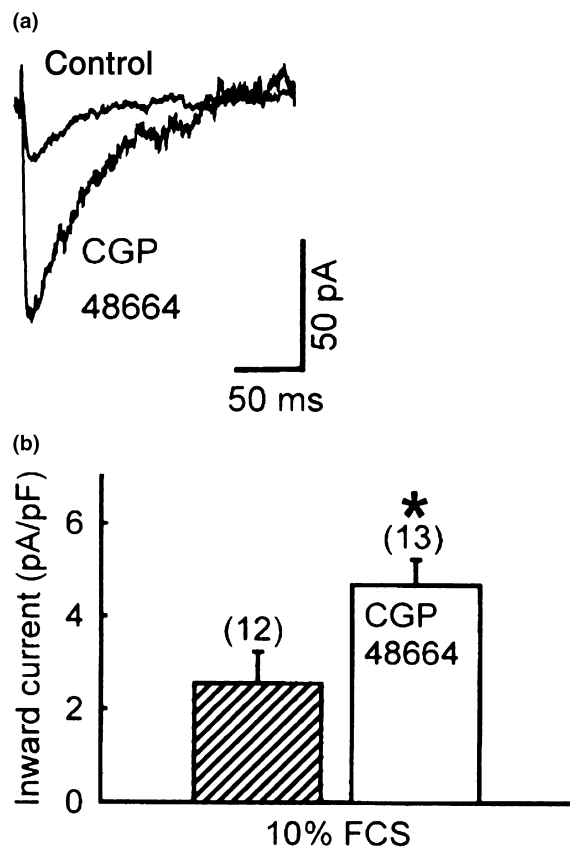
Figure 5 Whole-cell currents recorded from perforated patches from ileal smooth muscle cells cultured in the absence or in the presence of the polyamine synthesis inhibitor CGP 48664. The CGP reduces the levels of endogenous polyamines and thereby the inward currents are increased. Panel a shows representative original recordings and b summarized data. From Gomez & Hellstrand (1999) (© Springer-Verlag 1999) by permission.

muscle by culture in the presence of CGP 48664 causes enhanced Ca^{2+} channel activity, consistent with the acute effects of SPD and SP (Fig. 5, Gomez & Hellstrand 1999). Also, depletion of polyamines by synthesis inhibition using DFMO in insulin-secreting RINm5F cells is paralleled by increased voltage-activated Ca^{2+} currents (Sjöholm *et al.* 1993). Thus, it is possible to affect Ca^{2+} channel activity through alterations in polyamine levels by influencing the synthetic machinery of the cell itself.

K^+ channel activity

Pharmacological modification of intracellular polyamine levels has also been found to affect the K^+ inward rectifier (KIR) channel and the large conductance Ca^{2+} dependent K^+ channel (Bianchi *et al.* 1996, Shyng *et al.* 1996, Snetkov *et al.* 1996). Our results on Ca^{2+} channels do not exclude the possibility that modulation of endogenous polyamine levels also influences rectification of K^+ channels. However, modulation of Ca^{2+} channels cannot be considered a consequence of any effect on K^+ channels, as all path-clamp experiments were designed to exclude K^+ currents. In addition, the fact that spontaneous activity is enhanced in polyamine-depleted intestinal smooth muscle, demonstrates that the dominant effect in the present experiments is an increased inward current rather than increased outward current through K^+ channels, which would lead to reduced membrane excitability.

EFFECTS OF POLYAMINES ON PERMEABILIZED PREPARATIONS

Ca^{2+} sensitivity

Extracellularly applied polyamines do not readily pass over the plasma membrane because of their positive charges. In intestinal and vascular smooth muscle permeabilized with the detergents Triton X-100 or β-escin, the polyamines diffuse over the plasma membrane and extra-/intracellular steady-state concentrations are achieved. Addition of SP to permeabilized intestinal smooth muscle increases force and phosphorylation of myosin 20-kDa light chains at clamped $[Ca^{2+}]$ (Fig. 6). Also SPD and PUT increase the sensitivity of the contractile machinery to Ca^{2+} but their effects are smaller than that of SP (Swärd *et al.* 1994). Similarly, in permeabilized vascular smooth muscle polyamines increase the Ca^{2+} sensitivity of the contractile machinery (Nilsson *et al.* 1995). The polyamine-induced increase in myosin light chain phosphorylation may be the result of enhanced myosin light chain kinase or alternatively decreased myosin phosphatase activity. Detailed analysis of *in situ* myosin light chain kinase and
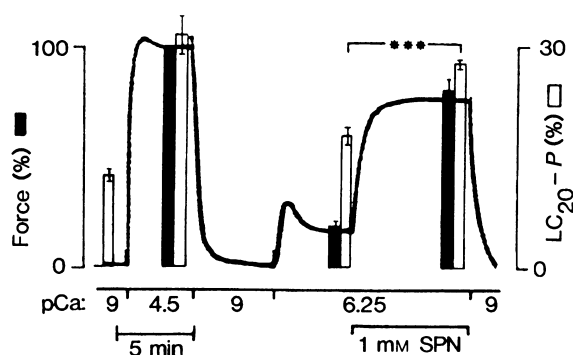
Figure 6 Addition of 1 mM spermine (SP) to permeabilized ileum smooth muscle increase force (shaded columns) and myosin light chain (LC 20) phosphorylation (open columns) at submaximal [Ca^{2+}] (pCa 6.25). One representative force record is superimposed. From Swärd *et al.* (1995) (© American Physiological Society 1995) by permission.

phosphatase activities revealed that SP acts by inhibiting the myosin phosphatase activity (Swärd *et al.* 1995).

Ingebritsen & Cohen (1983) have characterized protein phosphatases into four groups named PP-1, PP-2A, PP-2B and PP-2C. The catalytic subunit of PP-1 (PP-1c) forms complexes with more than 50 regulatory subunits with distinct substrate specificity (Cohen 2002). Most of the myosin phosphatase activity in smooth muscle is accounted for by the phosphatase PP-1M (Alessi *et al.* 1992, Hartshorne *et al.* 1998). This enzyme is composed of three subunits: a type 1 catalytic subunit; a 20-kDa subunit of unknown function; and a large 110–133 kDa subunit that targets the complex to myosin (Hartshorne *et al.* 1998). The targeting subunit is readily degraded, often to a 58-kDa fragment, explaining past difficulties in purifying this enzyme. Pato and coworkers purified a type 1 phosphatase consisting of the 58 kDa myosin binding subunit and the catalytic subunit (Pato & Adelstein 1983a, b, Pato & Kerc 1985, Tulloch & Pato 1991). This enzyme is readily inhibited by polyamines (Swärd *et al.* 1995). Spermine causes a more pronounced inhibition compared with SPD, which in turn has more effect than PUT, correlating with the number of positive charges on the polyamine molecule. It has to be taken into consideration that the effect of polyamines on purified smooth muscle phosphatase might be substrate-dependent. Also in cardiac muscle the charge distribution rather than the size of the polyamine molecule seems to be important for the interaction between polyamines and myofilament proteins recently reported by Harris *et al.* (2000). However, in this tissue polyamines cause decrease in force, but increase rate of force development, rather than Ca^{2+} sensitization, presumably because the mechanism of force regulation in this tissue is not to the same extent dependent on myosin light chain phosphorylation.

Some studies have been presented showing that polyamines affect protein phosphorylation via inhibition of phosphatases also in other systems than smooth muscle. Oetken *et al.* (1992) showed a reduced tyrosine phosphorylation in polyamine-starved LSTRA cells. They also presented evidence that polyamines reduce the activity of tyrosine phosphatases. In insulin secreting RINm5F cells a dose-dependent suppression of PP-1-like protein phosphatase activity by polyamines (SP > SPD > PUT) has been demonstrated (Sjöholm & Honkanen 2000).

Intracellular Ca^{2+} release and uptake

Spermine (1 mM) inhibits the intracellular Ca^{2+} release evoked by carbachol in guinea-pig ileum smooth muscle, where the cell membrane has been made permeable by treatment with β-escin, while receptor function is retained (Swärd *et al.* 1994). The observation that SP inhibits Ca^{2+} release also after G-protein activation by guanosine-SO-(3-thiotriphosphate (GTPγs)) shows that SP interacts with a step distal to the muscarinic receptor. On the other hand, the inositoltriphospite (IP$_3$) mediated release of Ca^{2+} from the sarcoplasmic reticulum, which is the most distal step in the signalling cascade between activation of the muscarinic receptor and Ca^{2+} release, is not affected by SP. Taken together these results implicate that SP affects intracellular Ca^{2+} release at a step between G-protein activation and the formation of IP$_3$. Spermine has been shown to increase Ca^{2+} uptake in heart, liver and brain mitochondria, suggesting that SP affects cytosolic [Ca^{2+}] also via this mechanism (Lenzen & Rustenbeck 1991, Lenzen *et al.* 1992, Rustenbeck *et al.* 1993, 1998a, b).

MODULATION OF ENDOGENOUS POLYAMINE LEVELS IN SMOOTH MUSCLE

Modulation of endogenous polyamine levels *in vivo* has not proven easy to accomplish because of escape phenomena, such as uptake of polyamines from the gastrointestinal tract. To avoid these problems and yet work with intact tissue as opposed to isolated cells, we treated organ-cultured smooth muscle with the AdoMetDC inhibitor CGP 48664 (Swärd *et al.* 1997). Ileal smooth muscle strips were cultured for 5 days in the absence or presence of CGP 48664 under growth-stimulated conditions (10% foetal calf serum added). The CGP 48664 (1 and 10 μM) caused a marked decrease in both SPD and SP levels and a decrease in the sensitivity to extracellular Ca^{2+} (Figs 7 and 8). When polyamine synthesis is inhibited, uptake of polyamines is stimulated to compensate for the loss of endogenous
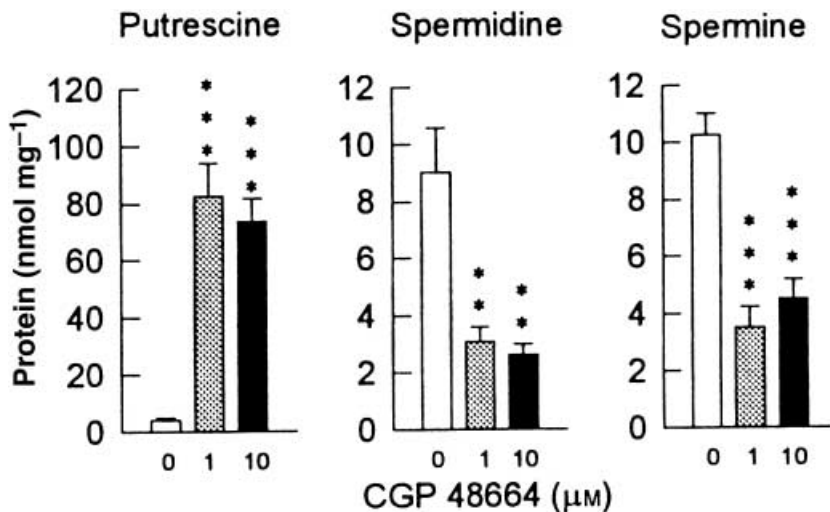
Figure 7 Putrescine, spermidine and spermine levels in ileal smooth muscle strips cultured for 5 days in the presence of the AdoMetDC inhibitor CGP 48664. The CGP 48664 causes a marked decrease in spermidine and spermine concentrations. Putrescine is increased after treatment with CGP 48664 because of loss of spermidine/spermine feedback inhibition of ODC. $**P < 0.01$, $***P < 0.001$ compared with controls. From Swärd *et al.* (1997) (© American Physiological Society 1997) by permission.
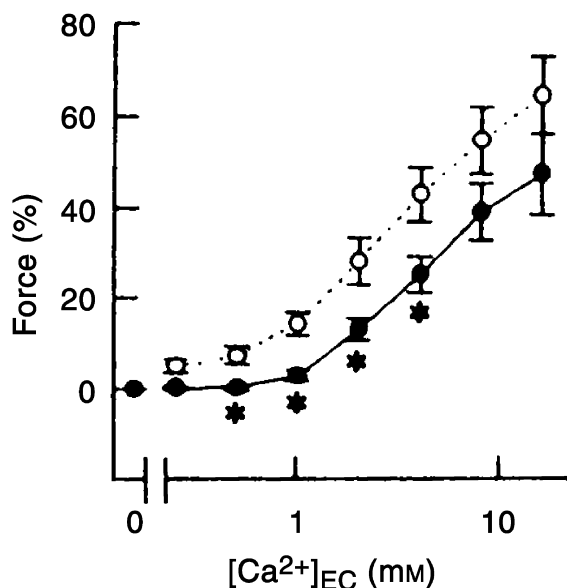
Figure 8 Force as a function of extracellular $[Ca^{2+}]$ in ileal smooth muscle strips cultured in the presence (filled circles) or in the absence (open circles) of CGP 48664. $*P < 0.05$ compared with control. From Swärd *et al.* (1997) (© American Physiological Society 1997) by permission.

production (Pegg 1986). The decrease in Ca^{2+} sensitivity was reversed when SPD and SP (50 μM each) were added to the culture. These experiments were performed in the presence of the diamine oxidase inhibitor aminoguanidine to prevent degradation of exogenously added polyamines to toxic aldehydes. Recently Belting *et al.* (1999, 2002) have shown that uptake of polyamines depends on cell surface proteoglycans and that combined treatment with inhibitors of heparan sulphate proteoglycan assembly (xylose) and polyamine synthesis (DFMO) prevents uptake of polyamines. These authors also showed attenuation of

tumour growth *in vivo*. Hence, this new concept might be a way of accomplishing reduction in cellular levels of polyamines *in vivo*, which up to now has been a major obstacle.

EFFECTS OF POLYAMINES ON MAPK ACTIVITY

Peptide hormones, growth factors and neurotransmittors stimulate growth through activation of plasma membrane receptors. Growth signalling from the plasma membrane towards the nucleus involves sequential steps of protein phosphorylation via tyrosine kinases and different MAPKs (p38, p42/44). Cellular polyamine deficiency has been shown to reduce intracellular microfilaments and the binding of the activated epiderm growth factor (EGF)-receptor to actin (McCormack *et al.* 1998). Hence, the kinase activity of the EGF receptor is reduced by 50%. Accordingly, ODC overexpressing cells show an increase in EGF receptor tyrosine kinase and MAPK activities (Moshier *et al.* 1995, Manni *et al.* 1998). Lowering of cellular polyamines in cultured leukaemia L1210 cells by DFMO treatment causes an attenuation of p42/44 MAPK phosporylation compared with non-treated control cells (Flamigni *et al.* 1999). Addition of SP to cultured mouse osteoblastic cells enhances the phosphorylation of both p38 and p42/44 MAPKs (Yamaguchi *et al.* 2000). These data suggest that polyamines regulate the phosphorylation state of tyrosine kinase and MAPKs, and that these effects might be associated with interactions with the cytoskeleton.

POLYAMINES AND CYTOSKELETON

Filamentous actin is formed through polymerization, and the actin filaments are organized as stress fibres

within the cell. Both actin polymerization and formation of stress fibres seem to require polyamines (Oriol-Audit 1978, Parkkinen *et al.* 1997, Yuan *et al.* 2000). Stress fibres are part of the cellular cytoskeleton, which is a complex meshwork of cytoskeletal proteins that attach through focal adhesion to the extracellular matrix. The cytoskeleton can both sense and respond to extracellular stimuli such as mechanical forces, hormones and neurotransmittors. This is true for both smooth muscle and non-muscle cells. It has consequently become apparent that formation of stress fibres is analogous to smooth muscle contraction, although distinctive differences are to be expected because of differences in the concentration and localization of cytoskeletal and regulatory proteins. The cytoskeleton affects different important steps in the growth process such as mitosis and cell migration.

The Ras homology A (RhoA) protein belongs to the group of small GTPases. Expression of constitutively active forms of RhoA is sufficient for stress fibre formation and toxins that inactivate RhoA block the formation of stress fibres (Narumiya *et al.* 1997). In parallel an important role of RhoA in regulation of smooth muscle contraction has become evident (Gong *et al.* 1996, Otto *et al.* 1996). RhoA acivates Rho-associated kinase, which in its turn increases the phosphorylation of the regulatory myosin light chains. In smooth muscle, myosin activation by Rho-associated kinase primarily depends on inhibition of the myosin phosphatase PP-1M (Swärd *et al.* 2000). Depletion of cellular polyamines by DFMO reduces RhoA protein and cell migration (Rao *et al.* 2001). These effects are reversed by addition of exogenous SPD. Thus, it is an interesting possibility that polyamines affect the cytoskeleton through interaction with RhoA expression.

SPD and SP inhibit myosin phosphatase activity as delineated above (Swärd *et al.* 1995), which in turn may promote the formation of stress fibres. The SP-mediated inhibition of myosin dephosphorylation leads to increased myosin phosphorylation and rapid contraction of smooth muscle and accordingly, depression of cellular SPD and SP levels impairs contractility (Swärd *et al.* 1995, 1997). It is an intriguing possibility that the effects of polyamines on smooth muscle are, at least partly, the result of interaction with the cytoskeleton and that this effect is mediated via RhoA.

CONCLUSIONS

The inhibition of phosphatase activity by polyamines may explain many, but not all, of their acute physiological actions. As growth and proliferation are regulated largely by phosphorylation-dependent signals, part of the polyamine effects on these processes are also associated with inhibited phosphatase activity. However, as polyamines by virtue of their positive charges have a high affinity for negatively charged molecules and surfaces, multiple effects arise because of direct binding of polyamines to ion channels, regulatory proteins and nucleic acids. Thus, this class of ubiquitous molecules constitutes a component of the intracellular environment that profoundly affects cell homeostasis. Changes in polyamine contents, such as in normal and malignant growth, alter this homeostatic function, explaining why modulation of polyamine contents is a potent mechanism for regulating both cellular functions and growth responses.

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