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Inhibitory action of polyamines on protein kinase C association to membranes

Mariastella MORUZZI,* Bruno BARBIROLI,* Maria G. MONTI,* Bruna TADOLINI,† Gabriele HAKIM† and Gabriele MEZZETTI*‡

*Istituto di Chimica Biologica, Università di Modena, Via Campi 287, 41100 Modena, Italy, and †Dipartimento di Biochimica, Università di Bologna, Via Irnerio 48, 40126 Bologna, Italy

Physiological activation of protein kinase C requires the interaction of this enzyme with cellular membranes [Nishizuka (1986) Science **233**, 305–312]. In the present work a reconstituted system of protein kinase C and human inside-out erythrocyte vesicles was utilized to study the effect *in vitro* of naturally occurring polyamines on the activation process of protein kinase C. The active membrane-associated complex was conveniently determined by its ability to bind radioactive phorbol ester with an exact 1:1 stoichiometry. The association reaction of the enzyme to membrane was rapid, being complete within 1 min at 25 °C. The addition of polyamines, particularly spermine, greatly decreased in a dose-dependent manner the amount of protein kinase C bound to membranes (i.e. in the activated form). The effect observed was quite specific, since it was dependent on the chemical structure of the polyamine and it was manifest at micromolar concentrations of the polycation; the order of potency was spermine > spermidine > putrescine. A characterization of this effect is presented and possible physiological implications are discussed.

INTRODUCTION

Reversible protein phosphorylation represents a fundamental and ubiquitous mechanism in the regulation of cellular activities (Rubin & Rosen, 1975; Krebs, 1985). Increasing evidence suggests for Ca^{2+}-sensitive and phospholipid-dependent protein kinase (protein kinase C) a crucial role in signal transduction for a variety of extracellular messengers such as growth factors, hormones and other agents which induce phosphatidylinositol turnover, with transient production of diacylglycerol (Berridge, 1984; Nishizuka, 1986). This substance has been postulated to be the second messenger able to activate protein kinase C without a change in cytosolic Ca^{2+} concentration. In addition, phorbol esters, which usually act as tumour promoters, can substitute for diacylglycerol in activation of protein kinase C (Ashendel, 1985). Indeed, this enzyme appears to be the cellular phorbol ester receptor (Kikkawa *et al.*, 1983; Blumberg *et al.*, 1984), and it has been involved in the control of cell proliferation and differentiation (Schwantke *et al.*, 1985).

Protein kinase C exists in different compartments of the cell, and its intracellular distribution may vary rapidly and reversibly between soluble and membrane-bound forms (Kraft & Anderson, 1983; Hirota *et al.*, 1985). The translocation process is closely related to the activation of the enzyme itself, since its physiological regulation requires, as a prerequisite, a specific interaction with membrane phospholipids (Nishizuka, 1986; Bell, 1986).

Aliphatic polyamines (e.g. putrescine, spermidine and spermine) are normal cellular constituents and have been implicated in the control of major cellular functions (Moruzzi *et al.*, 1968; Tabor & Tabor, 1984). It has been reported that these polycations affect the translocation of other enzymes such as hexokinase and phosphatidate phosphohydrolase (Kurokawa *et al.*, 1983; Hopewell *et al.*, 1985). Furthermore, polyamines are thought to exert, at least in part, their biological action through an effect on selective protein-phosphorylation systems (Hara *et al.*, 1982; Cochet & Chambaz, 1983; Mezzetti *et al.*, 1986). In this light, Qi *et al.* (1983) and Thams *et al.* (1986) have shown that these polycations can inhibit protein kinase C activity *in vitro*; however, the intimate mechanism by which polyamines exert this action has not yet been clarified.

Since defining the molecular mechanism underlying the modulation of protein kinase C is essential to the understanding of its biological role in the regulation of a variety of cellular processes, we have undertaken the present new investigation into the action of polyamines on the activation of this enzyme, using a reconstituted system of inside-out human erythrocyte membrane vesicles. In the present paper we show that micromolar concentrations of polyamines greatly interfere with the protein kinase C activation process by inhibiting the formation of the active membrane-associated enzyme complex. In addition, a correlation with physiological conditions *in vivo* is reported.

EXPERIMENTAL

Materials

2-Mercaptoethanol, dithiothreitol, EDTA, EGTA, ATP, fatty-acid-free bovine serum albumin, leupeptin, lysine-rich histone (type III-S; histone H1), phosphatidylserine, 1,2-diolein, putrescine, spermidine, spermine, PDBu and phenylmethanesulphonyl fluoride were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. DEAE-cellulose DE-52 and phosphocellulose P11 were

Abbreviation used: PDBu, phorbol 12,13-dibutyrate.

‡ To whom all correspondence should be addressed.

obtained from Whatman, Maidstone, Kent, U.K. [γ-^{32}P]ATP (sp. radioactivity 2900 Ci/mmol), [^{3}H]PDBu (12.5 Ci/mmol), [^{14}C]spermine (118 mCi/mmol) and D-[U-^{14}C]glucose (200 mCi/mmol) were from New England Nuclear Chemicals, Frankfurt, Germany. BTS-450 was given by Beckman Instruments, Irvine, CA, U.S.A. All other reagents were of commercial origin, of the highest grade of purity.

Preparation of inside-out human erythrocyte membrane vesicles

Inside-out vesicles from human erythrocytes were prepared by the method of Steck (1974) as modified by Sarkadi *et al.* (1980). Freshly drawn erythrocytes were washed with 10 mM-Tris/HCl, pH 7.4, containing 150 mM-NaCl, and haemolysed with 10 mM-Tris/HCl, pH 7.4. Washed ghosts were incubated in 0.5 mM-Tris/HCl, pH 8.5, containing 50 μM-2-mercaptoethanol and 20 μM-EDTA, for 1 h at 4 °C and for 15 min at 37 °C. The sedimented pellet was homogenized by a 27-gauge needle and washed in 10 mM-Tris/HCl, pH 7.4.

Purification of rat brain protein kinase C

Protein kinase C isolated from Sprague–Dawley rat brain was routinely purified by the method of Walsh *et al.* (1984) by Ca^{2+}-dependent hydrophobic-interaction chromatography of the cytosol preparation, followed by ion-exchange chromatography on DEAE-cellulose DE-52. The enzyme preparation was further purified by chromatography on phosphocellulose as described in detail by Mezzetti *et al.* (1985*a*). In certain experiments the enzyme was further purified to near homogeneity by affinity chromatography on polyacrylamide (Girard *et al.*, 1985). During purification, protein kinase C activity was assayed by the method of Kikkawa *et al.* (1982), as described in detail by Walsh *et al.* (1984), with histone III-S as the substrate.

Association of protein kinase C with inside-out vesicles

Association of protein kinase C with the erythrocyte membranes was performed essentially as described by Wolf *et al.* (1985*b*), in 250 μl of a reaction mixture containing 20 mM-Tris/HCl, pH 7.5, 1 mM-dithiothreitol, 30 μg of bovine serum albumin/ml, 100 μM-EGTA, 50–200 μg of membrane protein and 25–100 μl of protein kinase C (8–10 nmol of ^{32}P incorporated into histone H1/min per ml). Protein concentration was about 0.4 mg/ml. $CaCl_2$ was added to give 1 μM free Ca^{2+} concentration, and when present, the indicated concentrations of polyamines were added to the mixture. The suspension was incubated at 25 °C for 10 min and then centrifuged in an Eppendorf Airfuge at 40000 g for 5 min at 4 °C. The pellet was resuspended in 100 μl of 20 mM-Tris/HCl, pH 7.5, and utilized immediately for binding studies.

[^{3}H]PDBu-binding assay

The amount of protein kinase C associated with the membranes was studied by measuring its [^{3}H]PDBu-binding activity (Wolf *et al.*, 1985*b*). Unless stated otherwise, 50 μl of resuspended membrane preparation were incubated with 50 μl of a reaction mixture containing 20 mM-Tris/HCl, pH 7.5, 2 mM-dithiothreitol, 10 mM-$MgCl_2$, 100 μM-$CaCl_2$, 10 μg of bovine serum albumin and 40 nM-[^{3}H]PDBu for 20 min at 25 °C in the absence or presence of 500-fold excess of radioinert PDBu. At 25 °C the binding reactions reached equilibrium within 1 min, and remained stable for an additional 30 min. Bound [^{3}H]PDBu was separated from free [^{3}H]-PDBu by filtration through Whatman GF/C glass-fibre filters. The filters were dried and counted for radioactivity in a toluene-based scintillation cocktail (Mezzetti *et al.*, 1980). The determination was performed in triplicate, and specific binding was calculated as the difference between total binding and non-specific binding (i.e. in the presence of excess of radioinert PDBu). Analysis of specific-binding data was performed by the method of Scatchard (1949), and regression analysis was used for curve fitting.

Studies on spermine binding to membranes

Both the aggregation and binding experiments were conducted under the standard conditions used for the association of protein kinase C to membranes.

The aggregation of the membrane suspension in the presence of various amounts of spermine was monitored by the turbidity changes of the solution at 500 nm. The A_{500} change obtained 5 min after spermine addition was plotted as a function of the polyamine concentration (Tadolini *et al.*, 1986).

Spermine binding to the membrane was measured by incubating the membrane suspension with increasing concentrations of [^{14}C]spermine (50 d.p.m./pmol). After incubation for 10 min at 25 °C, the reaction mixtures were centrifuged for 15 min at 40000 g in a Spinco ultracentrifuge. The radioactivity content of the pellets was determined after digestion with 0.5 ml of BTS-450. Non-specific trapping of spermine, inferred by determining the percentage of [^{14}C]glucose (2×10^6 d.p.m.) trapped at the different spermine concentrations, was subtracted as a blank.

The concentration of protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS

Protein kinase C is normally present in the inactive form, but a quaternary complex of this enzyme, diacylglycerol or phorbol esters, Ca^{2+}, and membrane phospholipids is required for enzyme activation (Nishizuka, 1986; Bell, 1986).

In order to study the effect of aliphatic polyamines on the protein kinase C activation process, we have utilized a model system consisting of protein kinase C purified from rat brain and human inside-out erythrocyte vesicles. This experimental system has been shown to be useful for studying physiologically relevant protein–lipid interactions (Wolf *et al.*, 1985*b*), and it represents a good model to investigate the mechanism of protein kinase C association with the membrane, the step required for enzyme activation (Wolf *et al.*, 1985*a*).

Protein kinase C associated with membrane can be quantitatively assayed as phorbol ester receptor, i.e. for its [^{3}H]PDBu-high-affinity-binding activity in the presence of physiological concentrations of Ca^{2+} (Leach *et al.*, 1983; Wolf *et al.*, 1985*b*; Cochet *et al.*, 1986). Furthermore, the fact that human erythrocyte membrane vesicles lack endogenous phorbol ester-binding activity (Palfrey & Waseem, 1985) allows an accurate estimate of protein kinase C associated with membranes.

As shown in Fig. 1, under our experimental conditions

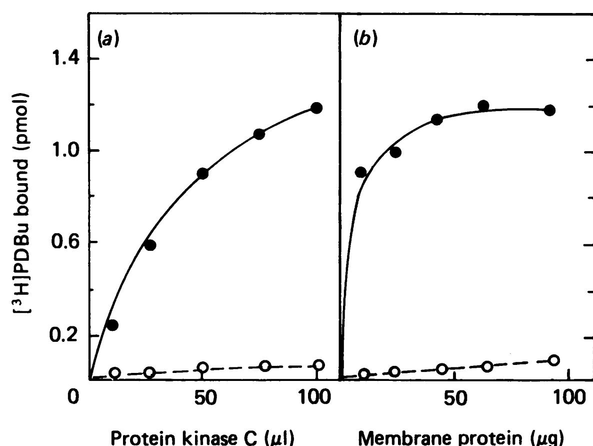

Fig. 1. Association of protein kinase C with inside-out erythrocyte membrane vesicles

(*a*) Membrane suspensions (50 µg of protein) were incubated with various amounts of protein kinase C preparation (0.25 mg/ml). (*b*) Enzyme (100 µl; 0.25 mg of protein/ml) was incubated with various amounts of membranes. After the association reaction, 50 µl of the resuspended membranes was incubated with 50 µl of reaction mixture containing 40 nM-[^{3}H]PDBu in the absence (●) or presence (○) of 500-fold excess of radioinert PDBu. Incubation conditions and measurement of bound [^{3}H]PDBu were as described in the Experimental section. Results are means for three separate experiments.

total [^{3}H]PDBu-binding activity increased as a function of enzyme concentration (Fig. 1*a*) and of the amount of membranes in the assay (Fig. 1*b*). In both cases the binding activity appeared highly specific, since [^{3}H]PDBu bound to protein kinase C was almost completely displaced in the presence of excess radioinert PDBu.

For this and subsequent experiments the pure enzyme was not required, since we have obtained similar results with either the homogeneous preparation or an enzyme partially purified (up to 50%) and devoid of other contaminating protein kinases.

Fig. 2(*a*) shows that specific [^{3}H]PDBu binding is saturable by increasing concentrations of phorbol ester. Scatchard analysis of specific-binding data yielded a straight line, indicating a single class of non-interacting binding sites (Fig. 2*b*). Under our experimental conditions an apparent K_d of 10 nM was calculated from the slope of the line. Extrapolation of the line to the abscissa yielded a $N_{max.}$ of 39 pmol/mg of protein.

The ability of certain substances to affect association of the enzyme to membrane, measurable by variations in radioactive phorbol-ester-binding activity, provides a unique tool to examine potential modulators of protein kinase C. In this light, the effect of polyamines on [^{3}H]-PDBu-binding activity of protein kinase C was studied.

In the experiment of Fig. 3, a time-course study of association of protein kinase C with membranes in the absence or presence of spermine is depicted. In both cases membrane association of the enzyme was very rapid, being almost complete in 1 min, and it was constant for at least 8 min more. From the results shown in Fig. 3 and from other experiments performed at lower temperature (10 °C) (results not shown), it was not possible to define rigorously the slopes of the curves for this system. However, results of the kinetics obtained in the absence or in the presence of spermine (25–200 µM) suggest that the principal action of polyamine was an almost immediate inhibition of the maximal extent of the active membrane-associated enzyme complex.

It is known that polybasic amines can interact with a variety of cellular polyanions, including acidic membrane phospholipids (Mezzetti *et al.*, 1980; Schuber *et al.*, 1983; Tabor & Tabor, 1984; Tadolini *et al.*, 1985; Tadolini & Varani, 1986; Meers *et al.*, 1986). With the

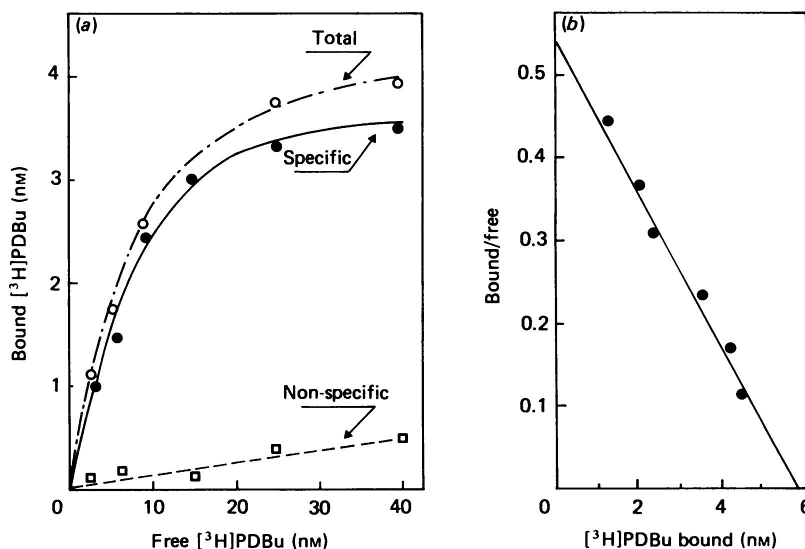

Fig. 2. Saturation analysis of [^{3}H]PDBu binding by membrane-associated protein kinase C

Enzyme preparation (90 µl; 30 µg of protein) was associated with 110 µg of protein membrane pelleted and resuspended as described in the Experimental section. (*a*) Portions (50 µl) of resuspended membranes were incubated with increasing concentrations of [^{3}H]PDBu alone (○) or plus 500-fold excess of unlabelled PDBu (□). Incubation conditions and measurement of bound [^{3}H]PDBu were as described in the Experimental section. Non-specific binding, that unaffected by a 500-fold excess of unlabelled PDBu, was subtracted from the total binding to derive specific binding (●). (*b*) Scatchard analysis of specific binding.

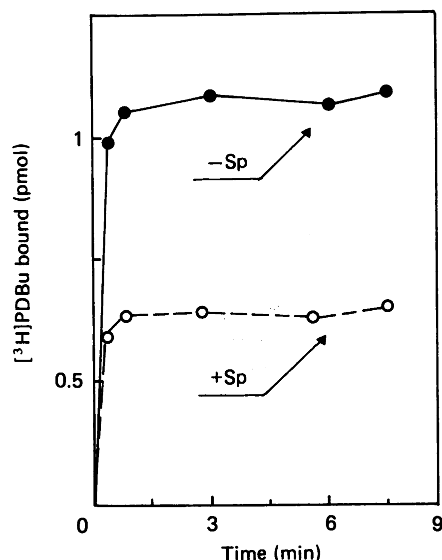

Fig. 3. Time course of association of protein kinase C with inside-out erythrocyte membrane vesicles

Membrane vesicles (100 μg of protein) were associated with 100 μl of enzyme preparation (0.3 mg/ml) for various times at 25 °C in the absence (●) or presence (○) of 100 μM-spermine (Sp). At the indicated times the tubes were centrifuged at 40000 *g* for 30 s, and portions (50 μl) of resuspended membranes were incubated with 40 nM-[^{3}H]PDBu. Assay conditions and measurement of bound [^{3}H]PDBu were as described in the Experimental section. Results are means for three separate experiments.

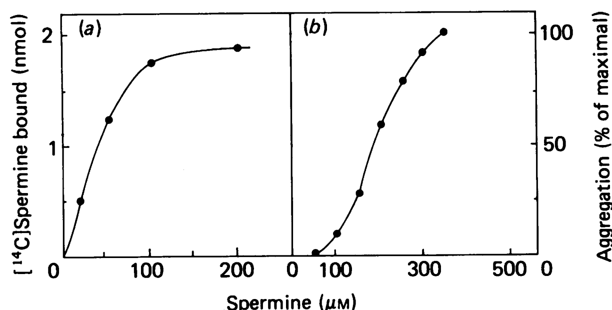

Fig. 4. Interaction of spermine with inside-out erythrocyte membrane vesicles

(*a*) Membrane suspensions (95 μg of protein) were incubated with increasing concentrations of [^{14}C]spermine for 10 min at 25 °C and processed as described in the Experimental section. (*b*) Membrane suspensions (85 μg of protein) were incubated for 10 min at 25 °C with increasing concentrations of unlabelled spermine. The extent of membrane aggregation was evaluated by measuring turbidity changes as described in the Experimental section. Data are expressed as percentages of maximal aggregation (A_{500} = 0.69). Results are means for three separate experiments.

aim of elucidating whether the inhibitory effect of spermine on protein kinase C association with membranes could be physiologically relevant, we have first undertaken studies on spermine binding to inside-out vesicles and their aggregation under our standard experimental conditions. As shown in Fig. 4(*a*), specific [^{14}C]spermine binding to inside-out vesicles was saturable by increasing amounts of spermine, and the concentration of total spermine able to saturate 50% of the available sites on the membrane was about 50 μM. The amount of [^{14}C]spermine bound at saturating conditions was approx. 20 nmol/mg of inside-out-vesicle protein.

Spermine bound to phospholipid vesicles and to subcellular organelles causes a neutralization of the negative charges and a decrease in the net repulsive forces (Tadolini, 1980; Schuber *et al.*, 1983; Tadolini *et al.*, 1986). This fact leads to a lower solubility of the membranes that may precipitate as aggregates. A spermine-induced aggregation of inside-out vesicles would decrease the available binding sites for protein kinase C; thus it was necessary to take into account the effect of increasing concentrations of polyamine on membrane aggregation. As shown in Fig. 4(*b*), aggregation of inside-out vesicles was minimal in the presence of spermine up to 100 μM, increasing sharply thereafter, the half-maximal point being at approx. 200 μM-spermine.

Taken together, these data indicate the reliability of the results obtained by using polyamines at low concentrations (25–150 μM) which are effective in binding to membrane phospholipids without causing, as a side effect, a significant loss of the functional conformation of this cellular structure.

As shown in Fig. 5(*a*), polyamines at various concentrations did not affect [^{3}H]PDBu binding when added after association of the enzyme with the membrane, ruling out the possibility of direct interference of these polycations in the [^{3}H]PDBu-binding assay. On the other hand, addition of 25–200 μM-spermine during the step of association of protein kinase C with membranes (Fig. 5*b*) greatly and selectively decreased [^{3}H]PDBu-binding activity, indicating an effect of this polyamine in the association process of the enzyme to membranes. Interestingly, the concentration of spermine that gave 50% of its maximal effect on protein kinase C association was about 40 μM, which is approximately the same concentration required for half-maximal saturation of the available sites for spermine on the membranes (as shown in Fig. 4*a*).

In addition, the inhibitory effect of spermine appears to be quite specific, since, among the other polyamines tested, only spermidine was slightly effective. The fact that the aliphatic tetramine spermine was a much better effector than spermidine (a triamine) and putrescine (a diamine) suggests the importance of the internal amino groups in the polyamine for an optimal inhibitory effect on the [^{3}H]PDBu-binding activity.

In these experiments, the same amount of enzyme protein was added to the assay and a saturating concentration of radioactive binder (40 nM) was used, so that changes in [^{3}H]PDBu bound were likely to be indicative of change of phorbol ester receptor activity of membrane-associated protein kinase C. Thus it was important to assess the possibility that the polyamine-induced decrease in [^{3}H]PDBu binding observed was the result either of the decrease in the amount of protein kinase C associated with membranes or of a diminished affinity of the phorbol ester for its binding site. Table 1 shows such experimental data obtained in the presence of 100 μM-polyamines in the association reaction. The results were analysed by Scatchard plot, which gave an estimated number of phorbol-ester-binding sites of the membrane ($N_{max.}$) and also allowed assessment of any

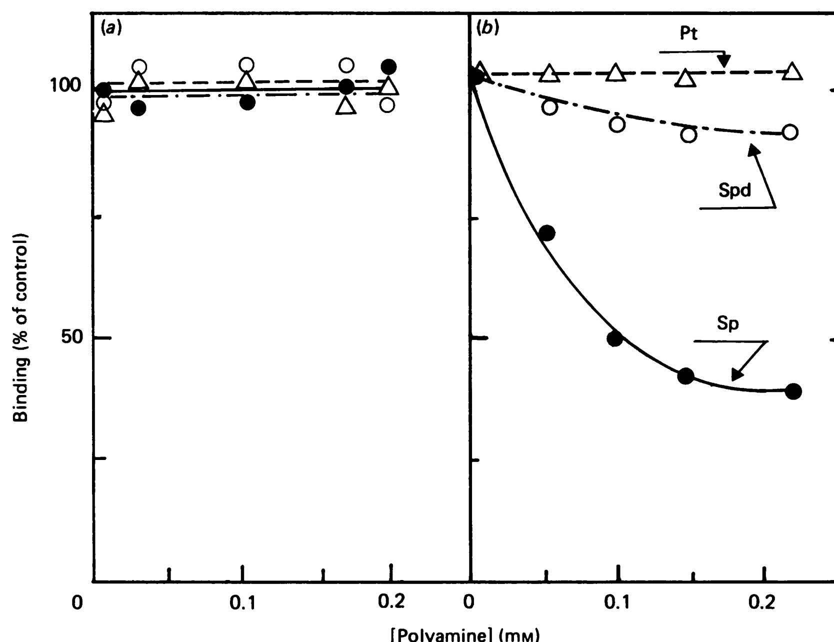

Fig. 5. Effect of polyamines on the association of protein kinase C with inside-out erythrocyte membrane vesicles

Enzyme preparation (100 μl; 0.3 mg/ml) was associated with membranes (100 μg of protein) in the absence or presence of various concentrations of spermine (Sp; ●), spermidine (Spd; ○) and putrescine (Pt; △). In (*a*) polyamines were added after association of protein kinase C with membranes (i.e. in the [^{3}H]PDBu-binding assay). In (*b*) polyamines were added before the association reaction. Results are expressed as percentages of the amount of [^{3}H]PDBu bound in the absence of polyamines (1.4 pmol). Results are means for four separate experiments.

Table 1. Effect of polyamines on the number and dissociation constant of [^{3}H]PDBu specific binding sites on inside-out erythrocyte membrane vesicles

Enzyme preparation (100 μl; 0.27 mg/ml) was associated to membranes (100 μg of protein) in the absence or presence of 100 μM-polyamines as described in the Experimental section. Portions (50 μl) of resuspended membranes were incubated with 40 nM-[^{3}H]PDBu in the presence or absence of 500-fold excess of radioinert PDBu. Incubation conditions and procedures used to determine specific binding were as described in the Experimental section. Specific binding data were subjected to Scatchard analysis; the apparent dissociation constant (K_d) and the maximal number of specific binding sites ($N_{max.}$) were calculated. Results are means ± S.E.M. for three separate experiments.

Addition	K_d (nM)	$N_{max.}$ (pmol/mg of protein)
None	10.1 ± 0.2	41 ± 3
Spermine	10.0 ± 0.3	17 ± 2
Spermidine	9.9 ± 0.3	35 ± 3
Putrescine	10.1 ± 0.2	39 ± 2

changes in the ligand affinity. The results showed that the K_d was not significantly different in the absence or presence of the polycations, whereas the $N_{max.}$ was remarkably decreased in the presence of polyamines, the order of potency being spermine > spermidine > putrescine.

DISCUSSION

Activation of protein kinase C is coupled to association of soluble enzyme with the cell membrane, a step which enables it to phosphorylate specific proteins, yielding ultimately to the desired physiological response. So far, the intimate molecular mechanism involved in the association of protein kinase C with the plasma membrane has not yet been clarified. In the present paper we show that polyamines, particularly spermine, greatly interfere with the activation process of the enzyme. In fact this polycation is able to inhibit the association reaction, by decreasing the amount of activated enzyme available for binding to radioactive PDBu.

The specificity and stoichiometry of protein kinase C activation by phosphatidylserine, diacylglycerol and Ca^{2+} has been studied in a model system (Bell, 1986). The mechanism proposed evokes first the binding of protein kinase C to a cluster formed by four phosphatidylserine molecules and Ca^{2+}. The succeeding binding of diacylglycerol to the preformed complex causes a dramatic decrease in Ca^{2+} requirement, possibly owing to a reorganization of the intra-complex bonds. These observations allow us to envisage a possible molecular mechanism for understanding spermine action on the protein kinase C activation process. The correlation between spermine binding to inside-out erythrocyte vesicles and the inhibitory action on the protein kinase C activation process suggests that the polyamine may exert its effect by an interaction with the membrane. Spermine is known to bind liposomes, forming complexes with acidic phospholipids, in particular with phosphatidic acid and phosphatidylserine (Tadolini *et al.*, 1985; Chung *et al.*, 1985); thus electrostatic interactions of spermine with phospholipids may decrease the pool of phosphatidylserine involved in the formation of binding sites. Spermine aligning parallel to the membrane may bind with its four charges three to four phosphatidylserine molecules (Tadolini *et al.*, 1985; Chung *et al.*, 1985),

forming a cluster with no binding properties for the enzyme. In this cluster spermine may substitute for the Ca^{2+} molecule. A direct competition between spermine and Ca^{2+} for binding to the phosphatidylserine head group was shown (Meers *et al.*, 1986); in this case Ca^{2+} would compete with spermine for its binding site. However, under our experimental conditions a 10-fold increase in free Ca^{2+} concentration did not significantly affect spermine action on the activation process (results not shown). This could be due to the fact that during the association reaction diacylglycerol, which modulates the Ca^{2+} requirement (Hannun *et al.*, 1986), was always omitted. Thus it is possible that in our assay much higher concentrations of Ca^{2+} than those employed may be required to displace spermine.

However, the results presented here cannot rule out a direct interaction between the polyamine and the enzyme itself, as has been described for other protein kinases, which might lead to conformational changes in the binding sites (Hathaway & Traugh, 1984; Mezzetti *et al.*, 1985*b*).

The inhibitory effect of polyamines which we have described appears to be quite specific, since it was dependent on the chemical structure of the polycation and it was manifest at concentrations compatible with physiological conditions.

The identification of inhibitors of protein kinase C activation process can be helpful in the elucidation of the molecular mechanism by which this enzyme is regulated *in vivo*. Our data point out that other factors, besides the phospholipid composition of the membrane and the availability of Ca^{2+} or diacylglycerol at the proper site, can be deeply involved in the control of the association and activation of protein kinase C. In this light we have clearly demonstrated that micromolar concentrations of polyamines are active in preventing the association of this enzyme to membranes, strongly suggesting that these polycations, particularly spermine, may act as regulators of protein kinase C functioning *in vivo*. The perspective that spermine may be part of a cellular mechanism by which the cell can regulate its ability to respond to extracellular messengers is very appealing.

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