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INHIBITION OF PROTEIN KINASE C PHOSPHORYLATION BY MONO- AND DIVALENT CATIONS

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Summary. The effect of a matrix of concentrations of Ca^{2+} (0.01, 0.1, 0.5, 5 mM), Mg^{2+} (0.2, 0.5, 1, 2, 5, 10 mM), and Na^{+} (50, 100, 150 mM) on the phosphorylation of histone H-1 by protein kinase C was measured in the presence of 5 mol % diacylglycerol and Mg-ATP in both phosphatidylserine micelles and liposomes formed from a 1:4 mixture of phosphatidylserine and phosphatidylcholine. Monovalent cations (150 mM) reduced activity by 60 and 84% in the micelle and liposome assay systems, respectively. Inhibition was also observed with 5 mM Ca^{2+} and 10 mM Mg^{2+}. The phosphorylating activity was compared with computer calculations of the negative electrostatic potentials (Ψ_o) of the phospholipid membranes in the presence of the cations.

Activation of phospholipid and Ca^{2+}-dependent protein kinase (PKC) requires the presence of Ca^{2+}, a negative phospholipid such as phosphatidylserine (PS), Mg-ATP, and a specific activator such as diacylglycerol (DAG) (1-4). PKC activity is usually measured *in vitro* under conditions optimized to give the highest phosphorylation rate; these conditions are far removed from physiological conditions. The components of the assay; 50 ug/ml histone H-1, 0.5 ug/ml PKC, 5 ug/ml PS, 20 mM Tris/HCl, pH 7.5, 5 mM Mg^{2+}, and, typically, 10 μM Ca^{2+} induce aggregation of the phospholipid vesicles or phospholipid multi-lamellar liposomes with the histone H-1 (5,6). It has recently been shown that liposomes composed of mixtures of PS and phosphatidylcholine (PC) could be substituted for the pure PS micelles in the original assay (5,7,8). A particular advantage of the mixed phospholipid liposomes is that it is possible to vary the surface potential (Ψ_o) of the liposomes according to the percentage of negatively charged phospholipids in the bilayer and the mono- and divalent ions in the buffer (9-12).

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Abbreviations: PKC, Protein kinase C; PS, Phosphatidylserine; DAG, Diacylglycerol; PC, Phosphatidylcholine.

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In an initial series of experiments on the effect of surface potential, the activity of PKC with PS:PC liposomes (1:4, 50 μg PS and 200 μg PC/ml) was compared to that of PKC with pure PS (50 ug/ml) micelles. Surprisingly, the addition of 100 mM KCl inhibited phosphorylation of histone H-1 by PKC by 72 and 63%, respectively (13). Recently, similar inhibition by cations of phosphorylation of substrates has been reported by several authors. The inhibition of phosphorylation of troponin I by PKC with high salt concentrations has been described (5); in this study loss of phosphorylating activity appeared to coincide with loss of the aggregation of troponin I with PS, whereas dissociation of PKC from membranes required much higher salt concentrations (0.7 M). In another study it was found that the KCl used to elute PKC from a DEAE-cellulose column displayed competitive inhibition of histone H-1 phosphorylation (14). Both pH and ionic strength have been shown to inhibit phosphorylation of B-50 protein in synaptosomal plasma membranes (15).

In vitro assays for PKC activity are important in understanding a variety of biological control mechanisms. However, our observations and those cited above raised a question about whether these *in vitro* results could be extrapolated to intracellular conditions of 150 mM KCl and 100 nM Ca^{2+}. In order to understand the electrostatic aspects of this inhibition, we studied the effects of a matrix of concentrations of K^+, Ca^{2+}, and Mg^{2+} on the activity of PKC in both PS micelles and in mixed PS:PC liposomes. Then Ψ_o, as well as the electrostatic potentials at 5 Å (Ψ_5) and 10 Å (Ψ_{10}) from the phospholipid surface (16), were calculated as a function of mono- and divalent ion concentrations.

Materials and Methods

Histone H-1 (IIIS), PS (bovine brain), PC (egg), and diolein (DAG) were obtained from Sigma (St. Louis, MO), [γ-32P]ATP was from New England Nuclear, Boston, MA. PKC was purified from rat brain cytosol by a three-step procedure. Activity was monitored at all steps with a histone H-1 kinase assay. Protein kinase activity eluted from a DEAE Cellulose (DE-52, Whatman, Maidstone, England) at 0.05 - 0.15 M NaCl. The pooled fractions were made 1.0 M with solid NaCl and were passed over a Phenyl Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column equilibrated with 1.0 M NaCl and eluted with a decreasing NaCl gradient. The PKC activity that eluted at 0.3 - 0.1 M was pooled and passed over a PS affinity column using the method of Uchida and Filburn (17). PKC activity was eluted with 1.5 mM EGTA and concentrated with an Amicon Centricon system (30 kDa cutoff). The concentrated PKC was stored at -70°C in the following buffer system: 20 mM Tris, pH 7.5, 0.5 mM EDTA, 1.5 mM EGTA, 1 mM dithiothreotol, 0.02% sodium azide, 1 ug/ml leupeptin, and 10% glycerol. The purity of the PKC was checked by SDS-PAGE and was found to contain a single band at 80 kDa.

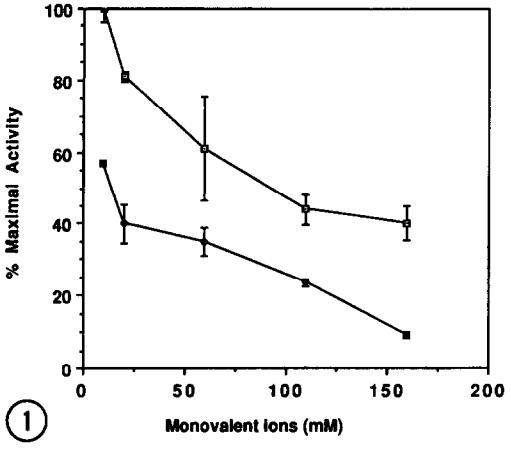
PKC activity was assayed by measuring the incorporation of ^{32}P into histone H-1 and was essentially as reported by Kikkawa *et al.* (18). Incubation mixtures comprised: PS (50 μg/ml) or PS:PC (50 μg PS and 200 μg PC/ml), 20 mM Tris-HCl, pH 7.5, 20 μM ATP, PKC (20 pmoles/ml), 3 mM Mg^{2+}, 1 mM EDTA (2 mM free Mg^{2+}), and varying amounts of free Ca^{2+} as calculated by iterative

application of the association constants (19,20), in a total volume of 0.2 ml. Both PS and PS:PC liposomes contained 5 mol % DAG. The lipids were combined and dissolved in $CHCl_3$, evaporated to dryness under nitrogen, and dried under vacuum overnight. To form PS micelles the PS:DAG mixture was allowed to swell in 20 mM Tris pH 7.5 and then sonicated for 1 min in a bath. To form reverse evaporation vesicles (REV's) the PC:PS:DAG mixture was dissolved in ether, 20 mM Tris pH 7.5 buffer was added, and the ether was removed under vacuum on a rotary evaporator (21). Large unilamellar liposomes (LUVET's) were prepared by swelling the PS:PC:DAG mixture in 20 mM Tris pH 7.5 buffer, performing five freeze/thaw cycles, and then extruding the suspension through 100 nm pore size Nuclepore filters (Nuclepore Corp., Pleasanton, CA) (22). All incubations were pre-incubated for 2 min at 30°C, the reaction was initiated by the addition of ^{32}P-ATP and allowed to proceed for 3 min at 30°C, after which 0.2 ml of ice-cold 50% trichloroacetic acid was added to halt the phosphorylation. Each sample was vacuum filtered on a 96-well Millipore HA filter (Millipore, Bedford, MA) and then each filter was washed 4x with ice-cold 10% trichloroacetic acid. The filters were solubilized with NCS tissue solubilizer, the solution was neutralized with acetic acid, Packard Opti-Fluor (Packard, Downers Grove, Il) was added, and the ^{32}P incorporation was assessed using a Packard Liquid Scintillation Counter. The background activity of the proteolytic fragment of PKC was assessed in the absence of Ca^{2+} and phospholipid; this was reproducibly less than 10% of the total activity. This background activity was subtracted before graphical presentation of the data.

The Ψ_0, Ψ_5 and Ψ_{10} of the PS:PC liposomes and the PS micelles in the presence of various combinations of monovalent and divalent ions were calculated numerically with a computer program (Zeta 8B) generously provided by Dr. Stuart McLaughlin. The intrinsic association constants: K_1, K_2, $K_3(Ca^{2+})$, and $K_3(Mg^{2+})$, representing monovalent ion binding to PS, divalent ion binding to PC, Ca^{2+} ion binding to PS, and Mg^{2+} ion binding to PS, were taken as 0.6, 1.3, 12, and 8 M^{-1}, respectively (11). The bilayer surface area per molecule was taken as 70 A^2 (23). The 20 mM Tris buffer was assumed to contribute 10 mM monovalent cations, and the 3 mM Mg^{2+} with 1 mM EDTA used in the calcium buffer was assumed to contribute 2 mM divalent cations. Anions were not considered because it has been shown that they make a negligible contribution to Ψ in systems containing negative phospholipids (11). The Ψ_0 as well as Ψ_5 and Ψ_{10} were calculated as a function of mono- and divalent ions and PS concentration.

Results

As seen in Fig. 1, the addition of 10-150 mM KCl to the assay mixture severely attenuated the activity of PKC in both PS micelles and PS:PC liposomes (REV's). In one set of experiments, NaCl was substituted for KCl and an identical decrease in PKC activity was measured (data not shown). In the experiments shown in Fig. 2, PS:PC liposomes (LUVET's) were used rather than REV's in order to be certain that no residual ether affected the results. Because Ca^{2+} may compete with Mg^{2+} for binding to ATP (20) and Mg-ATP is essential for kinase activity, the effect of Ca^{2+} was measured at 0.2, 0.5, 1, 2, 5, and 10 mM Mg^{2+}. For reference, the very low activities of PKC in the presence of 150 mM KCl, 10 μM Ca^{2+}, and 0.2 as well as 2 mM Mg^{2+} are plotted in Fig. 2 as single points. In Fig. 3 the same data as in Fig. 2 is plotted as a function of Mg^{2+} concentration. It is apparent that Ca^{2+} is a more potent inhibitor of PKC than Mg^{2+}, even though they have similar association constants with PS (13 and 8 M^{-1}, respectively) (11). This effect may be due to competition with Mg^{2+} for binding to ATP (20).



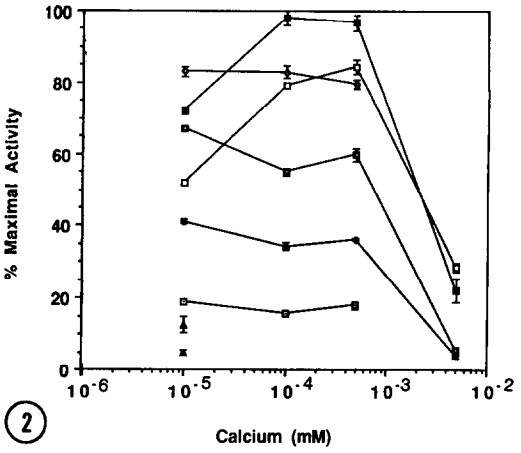
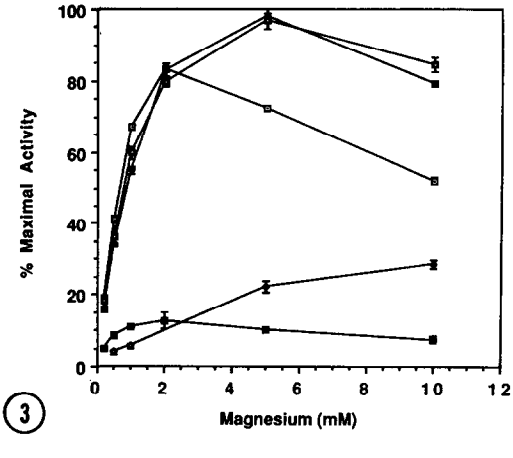


Figure 1. The per cent of maximal activity of PKC phosphorylation of histone H-1 as a function of monovalent ion concentration (as described in Methods) in suspensions of either PS micelles (-□-) or 1:4 PS:PC REV's (-◆-).

Figure 2. The per cent of maximal activity of PKC phosphorylation of histone H-1 as a function of Ca^{2+} in assay systems (described in Methods) that contain 1:4 PS:PC LUVET's and the following Mg^{2+} concentrations; 0.2 mM (-□-), 0.5 mM (-◆-), 1 mM (-■-), 2 mM (-◆-), 5 mM (-■-), 10 mM (-□-). In addition, at only 10^{-5} M Ca, PKC activity is shown as single points in the presence of 0.2 mM Mg^{2+} + 150 mM KCl (-▲-), and 2 mM Mg^{2+} + 150 mM KCl (-▲-).



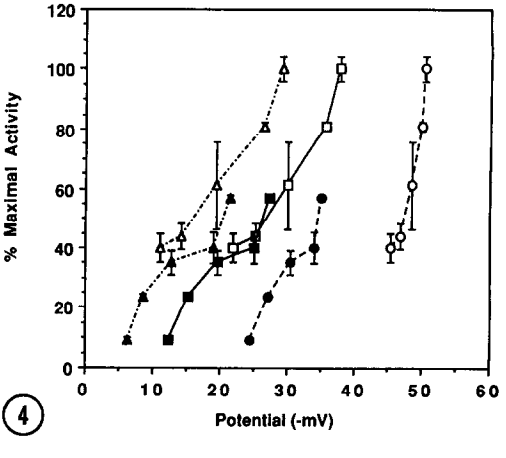


Figure 3. The per cent of maximal activity of PKC phosphorylation of histone H-1 as a function of Mg^{2+} in assay systems (described in Methods) that contain 1:4 PS:PC LUVET's and the following Ca^{2+} concentrations; 10 μM (-□-), 100 μM (-◆-), 500 μM (-■-), 5 mM (-◆-), and 10 μM + 150 mM KCl (-■-). This graph is drawn from the same data set as Fig. 2.

Figure 4. The per cent of maximal activity of PKC phosphorylation of histone H-1 in assay systems (described in Methods, data from Fig. 1) in suspensions of either PS micelles or 1:4 PS:PC REV's, respectively, as a function of: surface potential (Ψ_o), (-○- and -●-); electrostatic potential 5 Å from the phospholipid surface (Ψ_5), (-□- and -■-); and electrostatic potential 10 Å from the phospholipid surface (Ψ_{10}), (-△- and -▲-). It can be seen that the greatest overlap between micelles and PS:PC REV's with equal potential and activity occurs in the case of those with potential calculated at Ψ_5.

The steep dependence of activity on monovalent ion concentration, as well as the inhibition by both divalent ions, suggested that the inhibitory effect could be related to the electrostatic potential of the PS surface (9,11). In Fig. 4 the PKC activity of PS micelles and PS:PC liposomes, in which the total PS, PKC, and ATP were identical, are plotted as a function of surface potential as well as Ψ_5 and Ψ_{10} (16). In the case of Ψ_o, it is seen that the curves are very steep and there is a large discontinuity between the activity of the PS micelles and the PS:PC liposomes at equal surface potentials. However, in the case of Ψ_5, there is considerable overlap between the activity of the two assay systems at equal potentials.

The interaction of the divalent ions in this assay is too complex to carry out a similar comparison of activity with potential. As mentioned above, Ca^{2+} may compete with Mg^{2+} for formation of Mg-ATP (20), thereby additionally inhibiting the activity. However, it is interesting to note that in Fig. 2 the values that correspond most closely with those in Fig. 1 are: 10 μM Ca^{2+} at 2 mM Mg^{2+} (57% of maximal activity of a PS micelle, Ψ_5 = -27.3 mV); 10 μM Ca^{2+} at 5 mM Mg^{2+} (52 % of maximal activity, Ψ_5 = -18.0 mV); and 10 μM Ca^{2+} at 10 mM Mg^{2+} (36% of maximal activity, Ψ_5 = -11.4 mV).

Discussion

The inhibition of PKC by mono- and divalent ions reported here confirm our preliminary report of PKC inhibition by monovalent ions (13) as well as the recent reports of the 35% inhibition of phosphorylation of the neural protein B-50 by 100 mM NaCl (15), the 45% inhibition of histone H-1 phosphorylation by 150 mM NaCl (5), the 60% inhibition of troponin I by 150 mM NaCl (24), and the 50% inhibition of histone H-1 phosphorylation by 150 mM KCl in a DEAE-cellulose elution buffer (14).

Bazzi and Nelsestuen have recently shown that up to 20 min are required for fully irreversible binding of PKC to a membrane (3). They have used fluorescence energy transfer to show that the inhibition of PKC by cations is not due to disruption of the binding of PKC to PS (5). They suggest that high salt concentrations interfere with the binding of histone H-1 to PS and the resulting aggregation of histone H-1 with the lipids and PKC. Our studies suggest that this binding and aggregation are a function of the negative electrostatic potential provided by acidic phospholipids.

It should be emphasized that the electrostatic potentials shown in Fig. 4 were calculated in the absence of bound protein. We suggest that the amount of histone H-1 bound near the PKC on the surface is a function of the initial negative electrostatic potential. Binding of the positively charged histone H-1 would lower the electrostatic potential of the surface, possibly to neutralization. The steepness of the curves in Fig. 4 could be a result of the simultaneous interaction of more than one charge pair per complex.

In conclusion, the complex results of the inhibition experiments suggest that care should be taken in optimizing systems with regard to concentrations of cations and their mutual interactions. The strong inhibition of PKC activity by 160 mM monovalent cations questions the relevance of some previous studies to *in vivo* phosphorylation. Of particular importance is the suggestion that physiological concentrations of both mono- and divalent ions should be used if the results are to extrapolated to the *in vivo* functions of PKC.

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