Vol. 147, No. 1, 1987
August 31, 1987

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 248-253

SUBSTRATE-SPECIFIC STIMULATION OF PROTEIN KINASE C BY POLYVALENT ANIONS

Mohammad D. Bazzi and Gary L. Nelsestuen

Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108

Received June 19, 1987

Summary. The activity of protein kinase C (PKC) toward arginine-rich substrates was greatly stimulated by sulfate and phosphate, but not by monovalent anions. This stimulation did not require phospholipid, calcium, or diacylglycerol, and appeared to mimic the stimulation by phospholipid. Anionic proteins such as bovine serum albumin also promoted PKC activity toward certain substrates that were characterized by either high arginine or high lysine content. The mechanism of both of these stimulations appeared to be related to formation of a substrate-PKC complex which is essential to phosphorylation by PKC. Polyvalent anions bind the cationic substrate and, together with PKC, form an aggregate which allows phosphorylation. Potential physiological relevance of this stimulation is discussed.

Protein kinase C is an important intracellular regulatory enzyme, believed to be the primary site of action of the second messenger, diacylglycerol (DAG), as well as one site of action of phorbol esters (1,2). In vitro properties of the enzyme show that maximum activity of PKC can require DAG, Ca^{2+}, and acidic phospholipids (1,2). Recently, we identified three categories of in vitro substrates of PKC based cofactor requirements (3): category A substrates were phosphorylated without cofactors and were characterized by formation of PKC-substrate aggregates. Category B substrates required only the phospholipid cofactor and were characterized by substrate-PKC binding without aggregation. Category C substrates required all three cofactors, DAG, Ca^{2+}, and phospholipid, and were characterized by lack of direct substrate-PKC binding. This finding may account for the observation that PKC shows a wide specificity for in vitro substrates (1) while only a small number of in vivo substrates have been identified (1, 4).

These findings suggested that PKC may be a very nonspecific enzyme which requires the delivery of substrate to its active site. In vitro, aggregation of substrate-enzyme or substrate-phospholipid-enzyme complex is an effective means of substrate delivery to PKC (3, 5). Activation and inhibition of PKC is therefore possible with many materials that influence aggregation. For example, sphingosine (6), lysosphingolipids (7), and polyamines (8) are antagonists of the enzyme while fatty acids are agonists (9, 10). Gangliosides have been reported to be agonists (11, 12) or antagonists of PKC (13). Extension of these findings to the in vivo circumstance has been proposed (6, 7, 9, 14). However, the agonists or antagonists might exert their influence by altering the in vitro substrate-enzyme-phospholipid aggregation event. This could be a relatively non-specific process that may have little relationship to in vivo events. This mode of action has been proposed for sphingosine (15) and polyamines (3). To date, in vitro

0006-291X/87 $1.50

studies provide a large number of potential modulators of PKC activity and better understanding of their mechanism of action is needed to assess their effect under physiological conditions.

This study was initiated to help our understanding of the mechanism of agonist influence on the activity of PKC. A new finding was that polyvalent anions including sulfate, phosphate, and bovine serum albumin (BSA) were strong, substrate-specific agonists of PKC. The mechanism of their action appeared to correlate with the formation of a PKC-substrate complex which allowed delivery of substrate to PKC.

Materials and Methods

Protamine sulfate, protamine base, random copolymer of poly(Arg, Ser) (PAS), and random copolymer of poly(Lys, Ser) (PLS) were purchased from the Sigma Chemical Co, and were used without further purification. [γ-^{32}P]ATP (3 mCi/mM) was from Amersham corporation. Nitrocellulose filters were from Millipore corporation. All other chemicals were obtained from Sigma Chemical Company. PKC was purified to apparent homogeneity from bovine brain (5). Phospholipid vesicles were formed by brief, direct probe sonication of the appropriate mixtures of phospholipids (5). PKC activity was assayed as described by Kikkawa et al.(16)

Light scattering intensity at 90^{o} was used to provide qualitative evidence of aggregation events. Upon aggregation, large changes in this parameter are observed. Detailed explanation of this process and examples of the types of data obtained have been presented previously (3). Although the changes in this parameter were large, the results are interpreted in a qualitative manner (i.e. did aggregation occur) and the absolute intensities are not presented here.

Results

It is well known that protamine sulfate is phosphorylated by PKC in a cofactor-independent manner (16, 17). This phosphorylation was thought to be highly anomalous but was recently shown to correlate with the ability of protamine sulfate to interact with PKC and to form an aggregate in which phosphorylation could occur (in this previous study (3), the term protamine was used to refer to protamine sulfate). The results in figure 1A show that cofactor-independence was

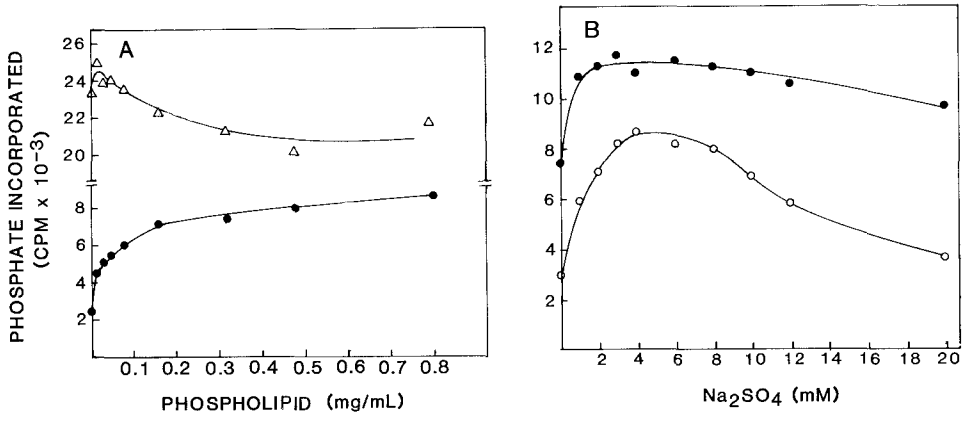

Figure 1. Phospholipid and sulfate stimulation of protamine phosphorylation by PKC. Panel A shows the effect of phospholipids (30% PS: 70% PC) on the phosphorylation of Protamine base (●) or protamine sulfate (Δ). Panel B shows the effect of sulfate on the phosphorylation of protamine base in the absence (O) or the presence (●) of 320 μg/mL phospholipids. Assays in both panels contained 20 mM HEPES (pH 7.5), 32 ng of PKC, 5 mM Mg acetate, 2.0 mM EGTA, 20 μM ATP, and 0.2 mg/mL protamine base or protamine sulfate.

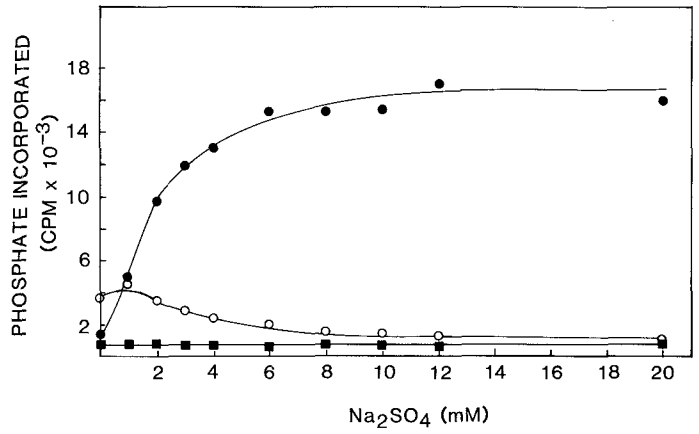

Figure 2. Influence of sulfate on phosphorylation of PKC substrates. Assay conditions were as in figure 1 with no phospholipid added to the reaction. Substrate concentrations in each case were 0.2 mg/mL of either PAS (●), PLS (O), or histone III-S (■).

unique to protamine sulfate and that protamine base was quite different. Phospholipid was a major cofactor for phosphorylation of protamine base. The pH of the reaction was not altered by the addition of protamine base or sulfate. Thus, protamine base was a category B substrate (requiring phospholipid) while protamine sulfate was a category A substrate (3). Addition of 0.2 mM Ca^{+2} caused slight inhibition of phosphorylation of protamine base (data not shown).

These results suggested that sulfate was a stimulator of PKC activity. The results in figure 1B show that sodium sulfate stimulated the phosphorylation of protamine base to essentially the same extent as phospholipid (compare Fig. 1A and B). The concentration of sulfate providing optimal phosphorylation was dependent on the concentration of protamine; at 0.2 mg protamine base/mL, optimum sulfate was 5 mM while at 0.4 mg protamine base/mL the optimum sulfate concentration was 7 mM (data not shown). High concentrations of sulfate inhibited activity in agreement with previous studies which showed that salt inhibited PKC activity in a substrate-dependent manner by interfering with association events (3).

Figure 2 shows sulfate stimulation of the phosphorylation of other substrates. A high degree of stimulation was observed with PAS, but not with PLS or histone. This suggested that sulfate stimulation was specific for substrates containing large amounts of arginine (protamine and PAS). Sulfate inhibition of phosphorylation of PLS (Fig. 2) appeared to be due to the general effect of high ionic strength which inhibits phosphorylation (3).

Various anions were tested and the results (Fig. 3) showed that monovalent anions were ineffective in stimulation of PKC with any of the four substrates used. However, phosphate served as a major promoter for phosphorylation of protamine base and PAS. Again, stimulation by phosphate was dependent on a high arginine content in the substrate. PAS formed aggregates with these anions, and this was especially apparent for sulfate which formed visibly turbid solutions when added to PAS. No aggregation by sulfate or phosphate, detected by light scattering intensity changes or visible turbidity, was observed with histone or PLS (data not shown).

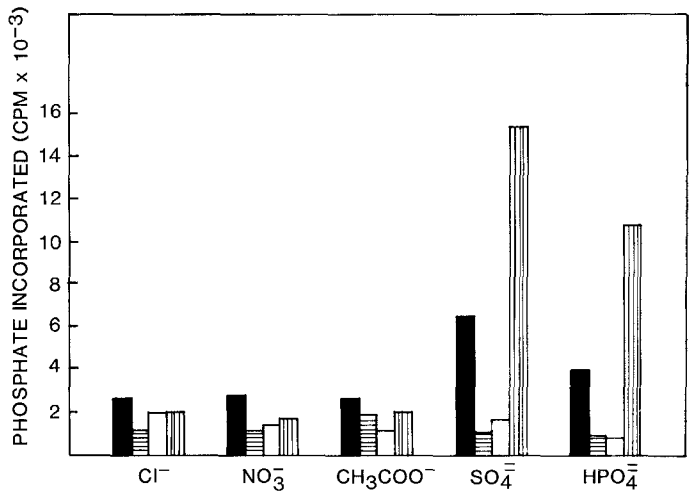

Figure 3. Effect of different anions on PKC activity toward various substrates. Assay conditions were as in figure 1 except without phospholipid. Four substrates were tested with each anion listed at the bottom of the graph. The substrates (left to right) were: protamine base (solid bars), histone (horizontal scored bars), PLS (open bars), and PAS (vertical scored bars). In each case, the substrate concentration was 0.2 mg/mL and the anion concentration was 5 mM.

Since the basis for PKC stimulation by multivalent inorganic anions was thought to involve simple substrate aggregation thereby delivering substrate to the active site of the enzyme, other multivalent anions should also promote PKC activity. The results in figure 4 showed bovine serum albumin (BSA) promoted phosphorylation of three substrates: protamine, PAS, and PLS. Light scattering intensity measurements or simple visual inspection for turbidity showed that aggregation occurred when BSA was added to the effective substrates but not when BSA was added to histone.

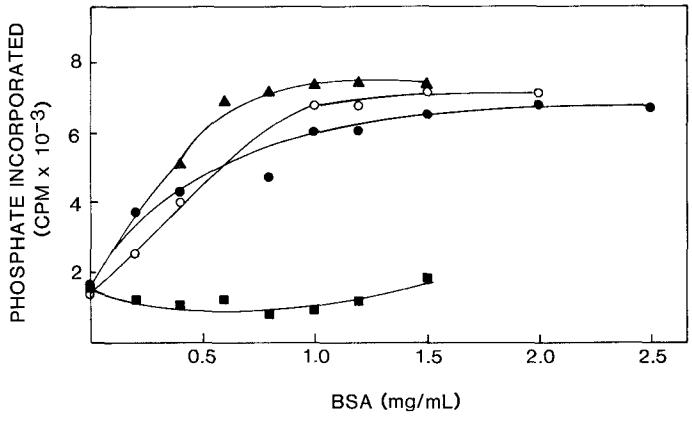

Figure 4. Stimulation of PKC by bovine serum albumin. Assay conditions are as in figure 1 except that bovine serum albumin was added. The four different substrates used were: protamine base (▲), PLS (O), PAS (●), and histone (■).

Discussion

These studies showed a high degree of substrate-specific stimulation of PKC by polyvalent inorganic or organic anions. At least ten fold stimulation was observed without attempts to optimize this effect. Stimulation of this magnitude could greatly alter the activity of an enzyme and provide a major regulatory function. In addition, specificity is often construed as evidence for a biological recognition process and it is possible that the anion composition of a cell might influence the population of protein substrates which are phosphorylated by PKC. For example, sulfate and phosphate might promote phosphorylation of arginine-rich substrates. This might provide a regulatory role for anions which is exerted through the PKC pathway. However, the specificity which sulfate and phosphate displayed for arginine-containing substrates appeared to be due to a general interaction with arginine rather than to an enzyme process involving binding or allosteric behavior.

Previous studies (3, 5, 15, 18) have indicated a direct relationship between aggregation events and the appearance of in vitro PKC activity. Consequently, the large stimulation by polyvalent anions was compared to aggregation properties. The arginine-rich substrate, PAS, is a good example of this general behavior. PAS showed phospholipid-dependent phosphorylation by PKC in the presence of monovalent anions (3, 19), but was phosphorylated in a cofactor-independent manner in the presence of polyvalent anions (Fig. 2, 3, & 4). In the presence of monovalent anions, PAS was freely soluble in aqueous buffers, but addition of sulfate or BSA to the medium resulted in turbid aggregates that were visible. Thus, stimulation by these polyvalent anions may constitute another property of an in vitro activation process that requires substrate-PKC aggregation.

The cofactor-independent phosphorylation of protamine sulfate by PKC, a well known process (16,17), appeared to rely on aggregation with PKC (3). Protamine base, buffered with monovalent anions, did not produce the same extensive aggregation with PKC that was observed when PKC was added to protamine sulfate. However, the rate and the extent of protamine base-PKC aggregation was greatly enhanced by soluble sulfate salts. While addition of sulfate to protamine base did not alter the light scattering intensity of the solution, sulfate apparently reduced overall solubility so that extensive aggregation occurred when PKC was added. Thus, some promoters of PKC activity may function by simply reducing substrate solubility.

Recent studies with peptide substrates have shown cofactor-independent phosphorylation of a peptide derived from a sequence of protamine (20). The results indicated that residues surrounded by two extended blocks of arginine are phosphorylated by PKC in a cofactor-independent manner (20). The results presented here indicated that sequence alone may be insufficient to produce cofactor-independent phosphorylation (e.g. PAS); factors that reduce substrate-enzyme solubility also appeared to be required. In fact, it may be possible to create peptide substrates of each category. Predictions based on the findings to date suggest that category A substrates should require intense positive charge provided by arginine residues as well as conditions that reduce peptide solubility. Category B substrates should have intense positive charge provided by either lysine or arginine. Category C peptides will probably display the lowest charge density.

It should be emphasized that the aggregation event discussed here is viewed as an in vitro means for delivery of the substrate to the active site of PKC. This delivery process could be a

general mechanism that is applicable to in vivo circumstances as well, but via means other than aggregation. Quantitative differences existed in the stimulation of PKC by various compounds. For example, PAS phosphorylation was stimulated most by sulfate, while the phosphorylation of PLS was stimulated most by phospholipids. The basis for these differences could be related to any of a number of factors such as quantitative properties of the components in the aggregate. Quantitation of these properties in an aggregate would appear to be difficult. In any event, substrate-delivery via aggregation appeared to be the primary mechanism for all in vitro substrates we have studied to date. Identification of an in vivo counterpart to the aggregation process will require further studies.

Acknowledgement. This work was supported in part by grant HL15728 from the National Institutes of Health.

References cited

1) Nishizuka, Y. (1986) *Science (Washingto D.C.) 233*, 305-312.
2) Kikkawa, U. & Nishizuka, Y. (1986) *Ann. Rev. Cell Biol., 2*, 149-178.
3) Bazzi, M. D. & Nelsestuen, G. L. (1987) *Biochemistry 26*, 1974-1982.
4) Woodgett, J. R., Gould, K. L., & Hunter, T. (1986) *Eur. J. Biochem. 161*, 177-184.
5) Bazzi, M. D. & Nelsestuen, G. L. (1987) *Biochemistry 26*, 115-126.
6) Hannun, Y. A., Loomis, C. R., Merrill, A. H., & Bell, R. M. (1986) *J. Biol. Chem. 261*, 12604-12609.
7) Hannun, Y. A. & Bell, R. M. (1987) *Science (Washington, D. C.) 235*, 670-674.
8) Qi, D.-F., Schatzman, R. C., Mazzie, G. J., Turner, S., Raynor, R. L., Liao, S., & Kuo, J. F. (1983) *Biochem. J. 213*, 281-288.
9) Murakami, K. & Routtenberg, A. (1985) *FEBS Lett. 192*, 189-193.
10) Murakami, K., Chan, S. Y., & Routtenberg, A. (1986) *J. Biol. Chem. 261*, 15424-15429.
11) Momoi, T. (1986) *Biochem. Biophys. Res. Comm. 138*, 865-871.
12) Chan, K-F. J. (1987), *J. Biol. Chem. 262*, 2415-2422.
13) Kreutter, D., Kim, J. Y. H., Goldenring, J. R., Rasmussen, H., Ukomadu, C., DeLorenzo, R. J., & Yu, R. K. (1987) *J. Biol. Chem. 262*, 1633-1637.
14) Thames, P., Capito, K., Hedeskov, C. J. (1986) *Biochem. J. 237*, 131-138.
15) Bazzi, M. D., and Nelsestuen, G. L. (1987) *Biochem. Biophys. Res. Comm.* , in press.
16) Kikkawa, U., Minakuchi, R., Takia, Y., & Nishizuka, Y. (1983) *Methods Enzymol. 99*, 288-298.
17) Takia, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., & Nishizuka, Y. (1979) *J. Biol. Chem. 254*, 3692-3695.
18) Bazzi, M. D. & Nelsestuen, G. L. (1987) *Biochemistry 26*, in press.
19) Komuro, T., Nakamura, S.-I., Mizuta, K., Sakanoue, Y., Hashimoto, E., & Yamamura, H. (1987) *Biochem. Int. 14*, 63-70.
20) Ferrari, S., Marchiori, F., Marin, O., and Pinna, L. A. (1987) *Eur. J. Biochem. 163*, 481-487.