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SYNERGISTIC FUNCTIONS OF PHORBOL ESTER AND CALCIUM IN SEROTONIN RELEASE FROM HUMAN PLATELETS*

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SUMMARY: In human platelets, thrombin activates Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) and mobilizes Ca^{2+} concomitantly, whereas 12-*O*-tetradecanoylphorbol-13-acetate (TPA) may be intercalated into membranes and directly activates protein kinase C without mobilization of Ca^{2+} in sufficient quantities. A series of experiments with TPA and Ca^{2+}-ionophore (A23187) indicates that activation of protein kinase C is a prerequisite requirement for release of serotonin, and that this enzyme activation and Ca^{2+} mobilization act synergistically to elicit a full cellular response. Both cyclic AMP and cyclic GMP inhibit activation of protein kinase C by prohibiting the signal-dependent breakdown of inositol phospholipid to produce diacylglycerol, but none of these cyclic nucleotides prevents the TPA-induced activation of this enzyme.

TPA[1/] is well known as a potent tumor promoter, and a number of kinetic studies with various cell types appear to suggest that the biochemical target of this phorbol ester is located on membranes (for a review, see Ref. 1). A recent analysis in this labo-

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1/ The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; protein kinase C, Ca^{2+}-activated, phospholipid-dependent protein kinase; PI, phosphatidylinositol; SNP, sodium nitroprusside; DBcAMP, dibutyryl cyclic AMP; 8BrcGMP, 8-bromo-cyclic GMP; PGE1, prostaglandin E1; SDS, sodium dodecyl sulfate.

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ratory (2) has suggested that protein kinase C-phospholipid complex is a TPA receptor itself[2/], and evidence has been presented that the tumor promoter may be intercalated into the phospholipid bilayer, where it activates this enzyme directly. Under normal conditions protein kinase C is activated by diacylglycerol, which is transiently produced from PI in a signal-dependent manner (4-6). In general, such PI breakdown is associated with immediate mobilization of Ca^{2+}. The present studies with human platelets will show that, under appropriate conditions, TPA and Ca^{2+}-ionophore selectively and independently induce protein kinase C activation and Ca^{2+} mobilization, respectively. Thus, it is possible to demonstrate that activation of protein kinase C is a prerequisite requirement for release of serotonin, and that protein kinase C activation and Ca^{2+} mobilization act synergistically to elicit a full cellular response. This communication will also show that the TPA-induced activation of protein kinase C is not susceptible to feedback control by cyclic nucleotides, although the thrombin-induced activation of this enzyme is inhibited by both cyclic AMP and cyclic GMP. These cyclic nucleotides prevent the signal-dependent phospholipid breakdown, and thereby counteract activation of cellular functions and proliferation (7-9).

EXPERIMENTAL PROCEDURES

Washed human platelets were prepared by the method of Baenziger and Majerus (10). 40K protein was partially purified from human platelets as described earlier (6). A homogeneous preparation of protein kinase C was prepared from rat brain soluble fraction as described (11). Bovine thrombin and SNP were products of Mochida Pharmaceutical Co. and Nakarai Chemicals, respectively. TPA, DBcAMP and 8BrcGMP were products of Sigma. PGE1 was donated by Ono Pharmaceutical Co., and A23187 was a product of Calbiochem. Other chemicals and materials were obtained from commercial sources. The platelets were prelabeled with either [^{3}H]arachidonic acid, ^{32}Pi or [2-^{14}C]serotonin under the conditions described by Rittenhouse-

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2/ This proposal has been supported recently by Niedel et al. (3), who show that a phorbol ester binding protein may be copurified with protein kinase C.

Simmons (12), Haslam et al. (13) and Haslam and Lynham (14), respectively. The radioactive platelets were stimulated as indicated in each experiment. The radioactive phospholipids and diacylglycerol were directly extracted from the platelets by the method of Bligh and Dyer (15), subjected to silica gel G plate thin layer chromatography, and quantitated as described by Rittenhouse-Simmons (12). Radioactive platelet proteins were subjected to SDS-polyacrylamide slab gel electrophoresis, stained, dried on a filter paper, and then exposed to an X-ray film to prepare an autoradiograph. The electrophoresis was carried out under the conditions specified by Laemmli (16). The relative intensity of each band was quantitated by densitometric tracing at 430 nm using a Shimadzu chromatogram scanner, Model CS-910. The release of radioactive serotonin from the platelets was determined by the method of Costa and Murphy (17). The radioactive protein was isolated by SDS-polyacrylamide gel electrophoresis, and two-dimensional mapping of the tryptic peptides was performed by the method of Beemon and Hunter (18).

RESULTS AND DISCUSSION

Although both thrombin and TPA equally induced rapid phosphorylation of an endogenous platelet protein having a molecular weight of about 40,000 dalton (40K protein), TPA did not provoke PI breakdown nor produce diacylglycerol as described earlier (2). In addition, when platelets were stimulated with TPA alone, the rate and extent of release of serotonin were insufficient, and the full physiological response was not observed. To ascertain whether protein kinase C lies on a common pathway leading to the phosphorylation of 40K protein, human platelets were labeled with ^{32}Pi and stimulated by either thrombin or TPA. The radioactive 40K protein was isolated by SDS-polyacrylamide gel electrophoresis, and then subjected to tryptic digestion. The fingerprint mapping patterns of tryptic phosphopeptides shown in Fig. 1 suggest that the sites in 40K protein phosphorylated during TPA and thrombin actions were identical. A similar mapping pattern was also obtained for the 40K protein which was phosphorylated in a purified cell-free system with a homogeneous preparation of protein kinase C. The results provide an additional evidence that TPA may be intercalated into platelet membranes and directly activate protein kinase C.

Next, a series of experiments was conducted to examine if the protein phosphorylation catalyzed by protein kinase C is a pre-

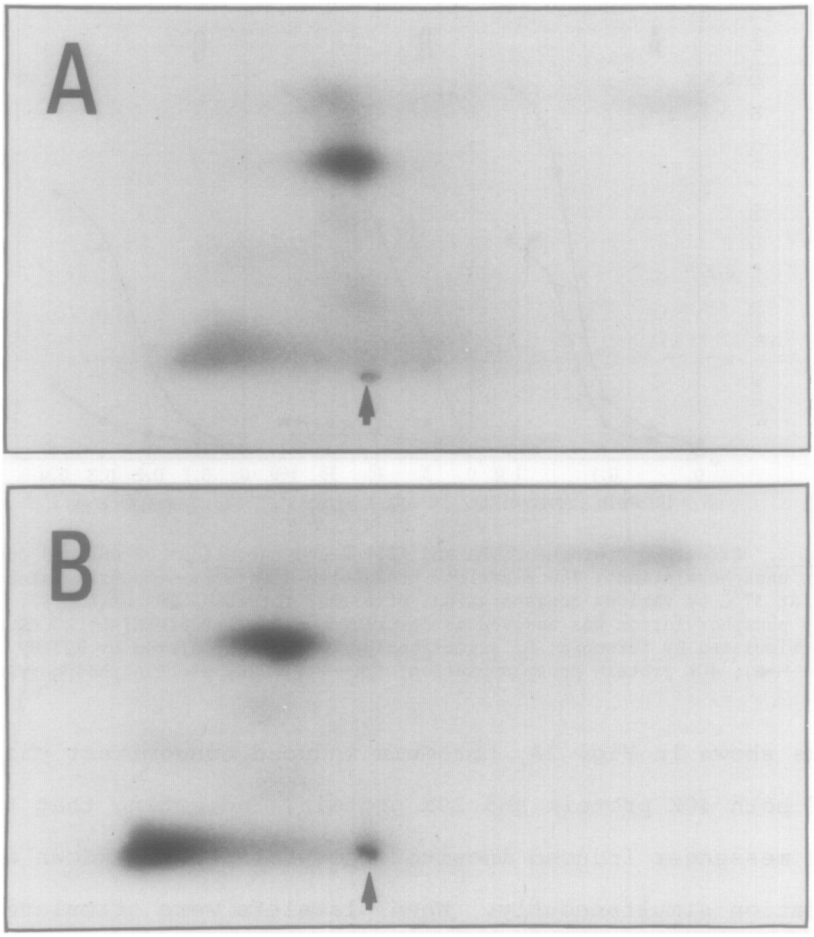

Fig. 1. Tryptic phosphopeptide mapping of 40K protein phosphorylated in intact platelets which were stimulated by either thrombin or TPA. The platelets prelabeled with ^{32}Pi were stimulated for 30 s at 37°C by either thrombin (0.4 unit/ml) or TPA (200 ng/ml). The radioactive 40K protein was extracted from the gel, digested with trypsin, and subjected to cellulose-coated thin layer plate electrophoresis in the horizontal dimension (negative pole left; positive pole right) followed by ascending chromatography in the vertical dimension as described under "EXPERIMENTAL PROCEDURES". A, stimulated by thrombin; B, stimulated by TPA. Arrowhead indicates the origin.

requisite requirement for eliciting a physiological response such as release reaction. It has been repeatedly shown that, when platelets are activated by natural messengers such as thrombin, myosin light chain that has a molecular weight of 20,000 dalton (20K protein) is also heavily phosphorylated (14,19). A specific calmodulin-dependent protein kinase is responsible for this protein phosphorylation, and the reaction absolutely depends upon Ca^{2+} mobilization (20). In

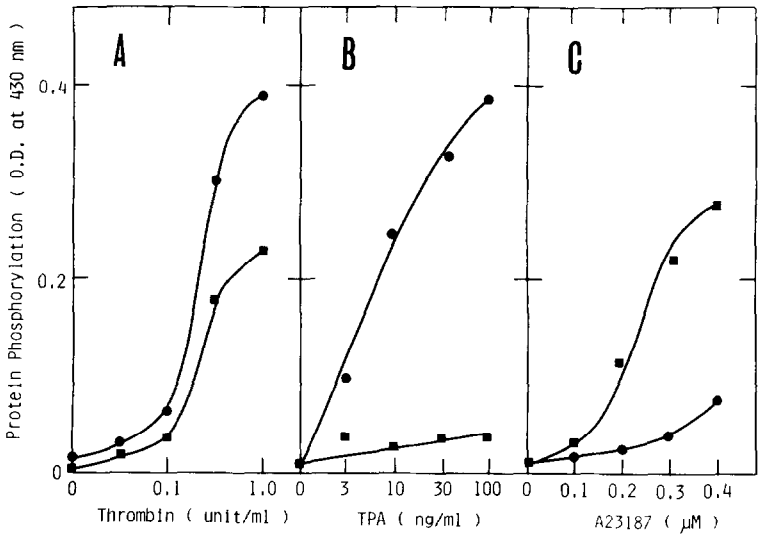

Fig. 2. Effects of thrombin, TPA and A23187 concentrations on 40K and 20K protein phosphorylation. The platelets prelabeled with ^{32}Pi were stimulated for 1 min at 37°C by various concentrations of either thrombin, TPA or A23187. Protein phosphorylation was assayed as described under "EXPERIMENTAL PROCEDURES". A, stimulated by thrombin; B, stimulated by TPA; C, stimulated by A23187. (●——●), 40K protein phosphorylation; (■——■), 20K protein phosphorylation.

fact, as shown in Fig. 2A, thrombin induced concomitant phosphorylation of both 40K protein and 20K protein, indicating that the natural messenger induced inositol phospholipid breakdown and Ca^{2+} mobilization simultaneously. When platelets were stimulated by TPA, 40K protein was phosphorylated to an extent that was very similar to that induced by thrombin, whereas 20K protein was phosphorylated only slightly as shown in Fig. 2B. Conversely, 20K protein was phosphorylated preferentially by the addition of A23187, and the phosphorylation of 40K protein was less significant particularly at lower concentrations of the ionophore as shown in Fig. 2C. The results suggest that under appropriate conditions activation of protein kinase C and mobilization of Ca^{2+} may be induced selectively and independently by TPA and A23187, respectively, without interaction with natural cell surface receptors.

In the experiment given in Fig. 3, human platelets were incubated with various concentrations of TPA in the presence and absence of a low concentration of A23187. Under these conditions

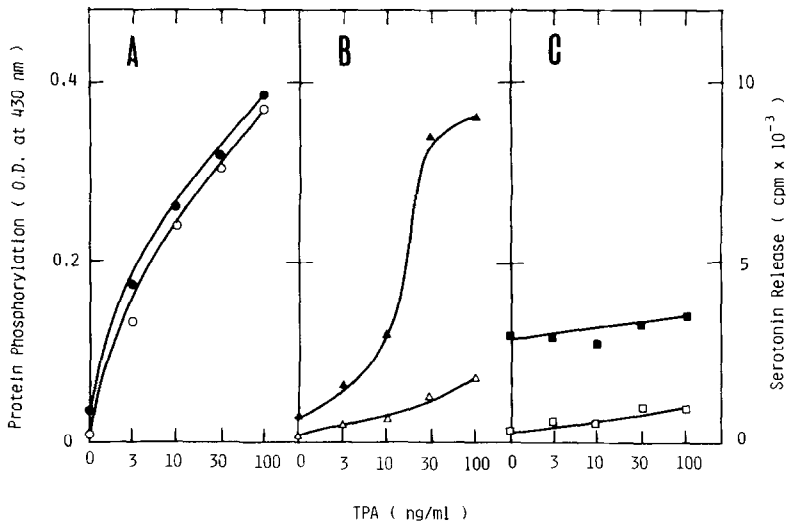

Fig. 3. Effects of TPA and A23187 on 40K and 20K protein phosphorylation and serotonin release. The platelets prelabeled with ^{32}Pi or [^{14}C]serotonin were preincubated with various concentrations of TPA for 30 s at 37°C, and then stimulated by 0.2 μM A23187 for 30 s at 37°C in the presence of 0.5 mM $CaCl_2$. Protein phosphorylation and serotonin release were assayed as described under "EXPERIMENTAL PROCEDURES". A, 40K protein phosphorylation; B, serotonin release; C, 20K protein phosphorylation. (●——●, ▲——▲, ■——■), stimulated by TPA plus A23187; (o——o, △——△, □——□), stimulated by TPA.

40K protein was phosphorylated by the addition of TPA irrespective of the presence and absence of A23187 (Fig. 3A). In contrast, serotonin was not released sufficiently by the addition of TPA alone, and the full cellular response was observed in the simultaneous presence of TPA and Ca^{2+}-ionophore (Fig. 3B). This ionophore alone at the concentration employed (0.2 μM) did not cause PI breakdown, diacylglycerol formation and 40K protein phosphorylation, nor did it induce release of serotonin. However, A23187 at higher concentrations *per se* induced the phosphorylation of 40K protein in addition to 20K protein as well as the release reaction. This is presumably owing to an enhancement of non-specific degradation of phospholipid and also to activation of protein kinase C by a large increase in Ca^{2+} concentrations (21,22). Likewise, TPA alone at higher concentrations (more than 100 ng/ml) caused release of serotonin in significant quantities, but the exact reason of this enhanced release reaction is not known. Perhaps, TPA itself per-

turbed the membrane structure, resulting in the degradation of various phospholipids. The phosphorylation of 20K protein that was induced by A23187 was not significantly affected by TPA under the given conditions (Fig. 3C). The results presented above seem to indicate that activation of protein kinase C is a prerequisite requirement for release of serotonin, and that under certain conditions protein phosphorylation and Ca^{2+} mobilization are equally indispensable and act synergistically for eliciting a full cellular response.

Bidirectional control systems occur in many tissues such as platelets. In these tissues receptors that produce cyclic AMP generally antagonize activation of cellular functions and proliferation (for a review, see Ref. 23). It has been shown earlier (7) that PGE1 as well as DBcAMP inhibits the thrombin-induced PI breakdown to produce diacylglycerol, and thereby counteracts protein kinase C activation. Evidence has been also presented suggesting that cyclic GMP similarly inhibits the thrombin-induced PI breakdown, and thus provides an immediate feedback control that prevents over-

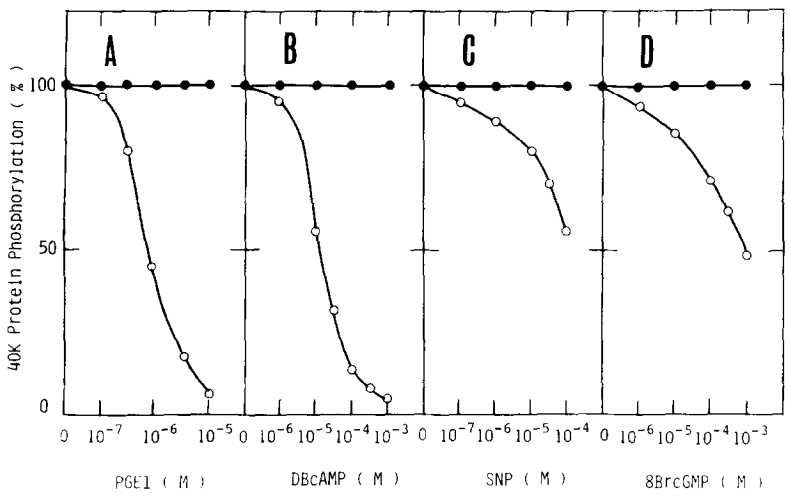

Fig. 4. Effects of PGE1, DBcAMP, SNP and 8BrcGMP on thrombin-induced and TPA-induced 40K protein phosphorylation. The radioactive platelets were pre-incubated with either PGE1, DBcAMP, SNP or 8BrcGMP as indicated for 5 min at 37°C, and then stimulated by thrombin (0.1 unit/ml) or TPA (200 ng/ml) for 1 min at 37°C. Protein phosphorylation was assayed as described under "EXPERIMENTAL PROCEDURES". A, with PGE1; B, with DBcAMP; C, with SNP; D, with 8BrcGMP. (o——o), stimulated by thrombin; (●——●), stimulated by TPA.

response (8). The results summarized in Fig. 4 show that PGE1, DBcAMP, SNP and 8BrcGMP were all inhibitory for the thrombin-induced phosphorylation of 40K protein, whereas none of these agents blocked the TPA-induced reaction. Thus, it may be conceivable that, under physiological conditions, diacylglycerol is produced only transiently and disappears rapidly when cell surface receptors are stimulated. In contrast, TPA is intercalated into the membrane for a prolonged period, and when Ca^{2+} is available, the cell tends to function and proliferate since protein kinase C is always active despite the feedback control by cyclic nucleotides.

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REFERENCES

1. Blumberg, P.M. (1980) CRC Crit. Rev. Toxicol. 8, 153-234
2. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851
3. Niedel, J.E., Kuhn, L.J., and Vandenbark, G.R. (1983) Proc. Natl. Acad. Sci. USA 80, 36-40
4. Kawahara, Y., Takai, Y., Minakuchi, R., Sano, K., and Nishizuka, Y. (1980) Biochem. Biophys. Res. Commun. 97, 309-317
5. Ieyasu, H., Takai, Y., Kaibuchi, K., Sawamura, M., and Nishizuka, Y. (1982) Biochem. Biophys. Res. Commun. 108, 1701-1708
6. Sano, K., Takai, Y., Yamanishi, J., and Nishizuka, Y. (1983) J. Biol. Chem. 258, 2010-2013
7. Takai, Y., Kaibuchi, K., Sano, K., and Nishizuka, Y. (1982) J. Biochem. 91, 403-406
8. Takai, Y., Kaibuchi, K., Matsubara, T., and Nishizuka, Y. (1981) Biochem. Biophys. Res. Commun. 101, 61-67
9. Kaibuchi, K., Takai, Y., Ogawa, Y., Kimura, S., and Nishizuka, Y. (1982) Biochem. Biophys. Res. Commun. 104, 105-112
10. Baenziger, N.L., and Majerus, P.W. (1974) Methods Enzymol. 31, 149-155
11. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 13341-13348
12. Rittenhouse-Simmons, S. (1979) J. Clin. Invest. 63, 580-587
13. Haslam, R.J., Salam, S.E., Fox, J.E.B., Lynham, J.A., and Davidson, M.M.L. (1980) Cellular Response Mechanism and their Biological Significance (Eds. Rotman, A., Meyer, F.A., Gilter, C., and Silberberg, A.) pp. 213-231, John Wiley and Sons Ltd., New York
14. Haslam, R.J., and Lynham, J.A. (1977) Biochem. Biophys. Res. Commun. 77, 714-722
15. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917

16. Laemmli, U.K. (1970) Nature 227, 680-685
17. Costa, J.L., and Murphy, D.L. (1975) Nature 255, 407-408
18. Beemon, K., and Hunter, T. (1978) J. Virol. 28, 551-566
19. Lyons, R.M., Stanford, N., and Majerus, P.W. (1975) J. Clin. Invest. 56, 924-936
20. Hathaway, D.R., and Adelstein, R.S. (1979) Proc. Natl. Acad. Sci. USA 76, 1653-1657
21. Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T., and Nishizuka, Y. (1979) Biochem. Biophys. Res. Commun. 91, 1218-1224
22. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692-3695
23. Berridge, M.J. (1975) Adv. Cyclic Nucleotide Res. 6, 1-98