Benzene-Mediated Protein Kinase C Activation

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Extracellular ligands transfer information into the cell through several pathways that operate in an integrated fashion. Protein kinase C, and enzyme that plays a pivotal role in signal transduction, is the molecular target for tumor promoters from the series of phorbol esters. A number of structurally unrelated tumor promoters also enhance protein kinase C, interacting or not interacting with the phorbol ester binding site. Evidence is provided that benzene potently activate protein kinase C in vitro, as well as in intact platelets. The drug does not compete for the phorbol ester binding site and probably affects the hydrophobic environment requires for full enzyme activation. Toluene is equally active. The relevance of the presented findings in the carcinogenic effects of benzene is discussed.

Introduction

There is clinical as well as experimental evidence that cancer is a multistep process that may involve several causal agents. Two stages have been characterized from the experimental model designed by Berenblum (1) and Mottram (2) in mouse skin: initiation and promotion. More recently, models of cultured cells have also provided evidence that cell transformation by physical and chemical carcinogens requires at least two steps. The initial one is a frequent event that gives rise to potentially transformed cells or "initiated" cells, whereas the second one is a rare event that may occur each time an initiated cell divides (3). The requirement for two genetic events apparently corroborates the result of transfection experiments that indicate that at least two cooperating oncogenes are needed to convert primary embryo fibroblasts into tumor cells (4).

The two classes of carcinogenic agents can be distinguished on the basis of their molecular targets. Initiators include chemicals that affect DNA and elicit irreversible effects as well as agents that generate genetic damage (i.e., mutation, deletion, insertion, translocation or amplification) such as UV light, ionizing radiation, various retroviruses and DNA-containing viruses. Promoters evoke epigenetic changes and reversibly modify the cellular phenotype, including chemicals as well as physical agents causing chronic tissue injury.

Tumor promoters are not carcinogenic by themselves but increase the probability of an initiator-treated cell to become malignant. Despite their lack of carcinogenicity, tumor promoters play a key role in the etiology of cancer. In fact, genotoxic carcinogens are generally present at harmless doses in the environment and only become nocive when associated with tumor promoters, which dramatically lower a carcinogen's threshold of action (5).

Two findings have led to the indentification of the major molecular target of phorbol esters and have shed light on the mechanism by which these potent tumor promoters modify the cellular phenotype. First, Driedger and Blumberg, using (3H)phorbol esters, were able to detect high-affinity binding sites for phorbol esters in various cell lines (6). Second, as a result of a collaborative work with Y. Nishizuka, we have contributed to the indentification of protein kinase C, a phospholipid and Ca\(^{2+}\) -activated enzyme involved in signal transduction, as the high-affinity binding site for phorbol esters (7-9).

In this presentation we wish to provide evidence that protein kinase C is not an exclusive molecular target for phorbol ester tumor promoter and that its enzyme activity may be affected by benzene and various other tumor promoters.

Protein Kinase C Activation by Tumor-Promoting Phorbol Esters

Signal transduction from extracellular ligands into the cell follows different strategies. The two best known signaling pathways are shown schematically in Figure 1.
A number of hormones and neurotransmitters generate cyclic AMP, which serves as a second messenger and triggers protein phosphorylation through activation of a cyclic AMP-dependent protein kinase, while two GTP-binding proteins negatively or positively modulate the response. Alternatively, a large series of ligands that control growth and cellular functions, including neurotransmitters, regulatory peptides, hormones, releasing factors, platelet activators and growth factors, proceeds through mediation by two second messengers. Upon ligand-receptor interaction, phosphatidylinositol 4,5-bisphosphate is hydrolyzed and two second messengers are formed: diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP3). The signaling pathway divides into two branches, the second messengers initiating two sets of cellular events. The former mediates protein kinase C activation, and the latter triggers Ca\(^{2+}\) mobilization from internal stores and consequently raises cytosolic Ca\(^{2+}\). A GTP-binding protein functionally similar to those that have been involved in the adenylate cyclase system modulates signal transduction at this step. The two branches act separately but both seem to be required for full cellular response (8–11).

The two pathways may not be independent and frequently affect each other. Cyclic AMP-mediated signals negatively control the protein kinase C pathway. Conversely, IP3/DG-mediated signals block adenylate activity. Also, both series of signals potentiate each other in various cell types or cell lines.

Tumor-promoting phorbol esters substitute for the second messenger DG in activating protein kinase C and subsequently bypass the upper part of the pathway. These agents compete with DG for its binding site and display a higher affinity than the natural effector. However, phorbol esters do not fully mimic physiological stimuli. We have recently suggested that these ligands trigger quantitatively and qualitatively different protein kinase C responses due to their specific properties which differ in several respects (9): a) the half-life of DG is less than 1 min in platelets, whereas phorbol esters like TPA are slowly metabolized in cells; b) phorbol esters do not mobilize Ca\(^{2+}\) from internal stores; c) phorbol esters can bypass some regulatory mechanisms that control the IP3/DG pathway, as illustrated in platelets where cyclic nucleotides inhibit thrombin-mediated and not phorbol ester-mediated protein kinase C activation (12); and d) phorbol esters can diffuse in all the cellular compartments and as a consequence may evoke a different localization of protein kinase C compared to physiological ligands, thus providing additional substrates to the enzyme. It is likely that these distinctive features might account, at least partly, for their promoting effects.

**Benzen and Toluene-Mediated Protein Kinase C Activation**

Some reports have supported the possibility that benzene may act as a tumor promoter in its neoplastic effects (19). This prompted us to investigate whether this compound affected protein kinase C activity.

The first experiments were designed to test the potency of benzene and its alkylated derivative toluene on protein kinase C activity in intact platelets. In response to physiological ligands, protein kinase C phosphorylates in platelets a set of specific protein substrates. The preferential substrate of the enzyme is a 43 KD protein, which serves as an index of protein kinase C activity in these cells. Figure 2 shows the autoradiogram of SDS-polyacrylamide gel electrophoresis of platelet proteins after the cells were exposed for 2 min to either 12-O-tetradecanoylphorbol 13-acetate (TPA), benzene, or...
Table 1. Toluene- and benzene-protein kinase C activity in vitro.

| Addition, v/v | Enzyme activity, cpm/assay | Increase, % |
|---------------|-----------------------------|-------------|
| None          | 3280 ± 246                  | 100         |
| Toluene       |                             |             |
| 0.8%          | 6432 ± 407                  | 196         |
| 2.4%          | 8859 ± 520                  | 270         |
| 4.8%          | 7993 ± 559                  | 243         |
| Benzene       |                             |             |
| 0.8%          | 5050 ± 376                  | 154         |
| 2.4%          | 7077 ± 529                  | 215         |
| 4.8%          | 7219 ± 415                  | 220         |

*Assays were carried out in the presence of 0.1 mM CaCl₂. Data are means + SE of three experiments in duplicate.

toluene. It can be seen that the two latter compounds activate 43 KD protein phosphorylation and give a phosphorylation pattern similar to that evoked by TPA, suggesting that these phosphorylation events were mediated by the same enzyme.

We have investigated the possibility that benzene interacts with protein kinase C on the phorbol ester/DG binding site. As a result of binding studies conducted on protein kinase C according to a previously described technique using 3H-TPA (15), we were unable to show any significant alteration of phorbol ester binding in the presence of either benzene or toluene (data not shown).

To further substantiate these findings, the action of benzene and toluene was studied in the in vitro assay described in Roghani et al. (14), using a preparation of protein kinase C from mouse brain. Results show that both chemicals directly activated protein kinase C in the presence of phosphatidylserine and Ca²⁺ at concentrations below 1% (Table 1). The dose-response curve of toluene-triggered protein kinase C activation in the presence of 0.5 mM EGTA is shown in Figure 3. Upon addition of TPA at 50 ng/mL, a slight stimulation could also be detected. Benzene and toluene-mediated enzyme activation was not dependent on Ca²⁺. It should be pointed out that the omission of phospholipid in the incubation mixture prevented enzyme activation, suggesting that the two drugs might interfere with the enzyme-phospholipid interaction.

We have recently shown that mixtures of sodium deoxycholate and phosphatidylserine were much more potent than phosphatidylserine alone in supporting protein kinase C activity and that sodium deoxycholate alone was an enzyme activator (manuscript in preparation). Surprisingly, using such mixtures containing a ratio of phosphatidylserine/deoxycholate of 1/9 (w/w), we could not get any benzene-mediated enzyme activation. Furthermore, benzene inhibited sodium deoxycholate-mediated protein kinase C activation. Results displayed in Figure 4 show the differential effects of benzene on protein kinase C activation depending on the hydrophobic environment. The complexity of the reaction is even more emphasized in experiments where benzene inhibition was studied as a function of deoxycholate concentrations, where at low concentrations of deoxycholate and benzene, an activation of the enzyme can be detected (Fig. 5). The results suggest that benzene and toluene...
reaction requires phospholipid although it was not dependent on Ca^{2+}. Thus, chloroform and its derivatives most likely act in a benzene-like fashion on phospholipid-enzyme interaction.

In summary, several tumor promoters can activate protein kinase C, presumably through interactions at different sites of the molecule. In contrast, well-characterized tumor promoters such as anthralin, tetrachlorodibenzoepin, phenobarbital, palmitoxin, benzoyl peroxide, and 1,25-dihydroxyvitamin D$_3$ do not directly activate the enzyme. However, the possibility that these tumor promoters act downstream from protein kinase C or through the Ca^{2+} branch of the IP$_3$/DG pathway is currently under investigation.

**Discussion and Conclusion**

The present results provide evidence that benzene and toluene activate in platelets, as well as in the *in vitro* assay, protein kinase C alone, or in the presence of phorbol ester TPA. The site of action of these organic solvents on the enzyme are presumably different. What is the physiological relevance of these findings? Is benzene-mediated protein kinase C activation relevant in its effect on malignancy?

The effects of benzene on cell proliferation were tested on the fibroblastic cell line Balb/c-3T3. Preliminary results have shown an initial marked increase of $^3$H-thymidine incorporation into DNA, up to 0.25%, followed by inhibition of growth at higher concentrations. Phorbol ester TPA at low doses has similar effects on this cell line, suggesting that benzene may similarly affect the IP$_3$/DG pathway.

The concentrations used in our experiments are generally higher than the low daily doses used in experimental carcinogenesis. However, it should be stressed that the real amount of benzene intervening under our conditions is much less, since most of the added benzene is bound to the tube or the dish walls.

Taking phorbol ester-mediated protein kinase C activation as a model, by analogy it is hypothesized that benzene may trigger an enzyme activation qualitatively distinct from that mediated by physiological stimuli. As reviewed by Hunter (19), the site of action of oncogene proteins is probably associated with signaling pathways and most likely with the IP$_3$/DG pathway. It is highly probable that the elucidation of how oncogene proteins and tumor promoters cooperate in signal transduction will considerably help to understand how a cell progresses to transformation.

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