CGP 41251 and Tamoxifen Selectively Inhibit Mitogen-activated Protein Kinase Activation and c-Fos Phosphoprotein Induction by Substance P in Human Astrocytoma Cells

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Abstract
The substance P (SP) receptor (NK-1 subtype) is widely expressed in primary human astrocytomas and glioblastomas and many brain tumor-derived cell lines. SP receptor activation stimulates the mitogen-activated protein (MAP) kinase pathway and the expression of immediate-early genes (e.g., c-Fos and c-Myc), resulting in an increase in DNA synthesis in human astrocytoma U-373 MG cells. In this study, we investigated the role of protein kinase C (PKC) in SP receptor activation of the MAP kinase pathway. SP peptide, epidermal growth factor, and the PKC activator 12-O-tetradecanoylphorbol-13-acetate (TPA) induced the tyrosine phosphorylation of the Erk1 and Erk2 MAP kinases in a concentration-dependent manner in U-373 MG cells. Pretreatment of the cells with PKC inhibitors, CGP 41251 or tamoxifen, inhibited tyrosine phosphorylation of Erk1 and Erk2 MAP kinases induced by low concentrations of SP or TPA and significantly attenuated phosphorylation at high concentrations of SP or TPA. The inhibitory effect exhibited by tamoxifen on SP-induced MAP kinase activation is similar to that exhibited by the selective PKC inhibitor CGP 41251, suggesting that the PKC enzyme is the in situ target for both inhibitors. Furthermore, SP-induced c-Fos phosphoprotein expression is inhibited by CGP 41251 or tamoxifen with similar efficacy. Importantly, neither CGP 41251 nor tamoxifen has any detectable effect on the MAP kinase activation by epidermal growth factor, consistent with the ability of this growth factor to activate the MAP kinase pathway by a PKC-independent mechanism.

Prolonged treatment with TPA resulted in down-regulation of PKC and selective inhibition of TPA- and SP-induced Erk1 and Erk2 tyrosine phosphorylation in U-373 MG cells. Consistent with the in situ results, CGP 41251 and tamoxifen significantly inhibited endogenous PKC enzymatic activity from U-373 MG cells in vitro. In contrast to CGP 41251 and tamoxifen, G6 6976, a highly selective inhibitor for PKCo and PKCβ isoforms, did not inhibit SP- or TPA-induced tyrosine phosphorylation of Erk1 and Erk2 MAP kinases; rather, it inhibited a signaling pathway leading to the phosphorylation of cAMP-responsive element binding protein in U-373 MG cells. To investigate whether selective PKC isoform(s) are involved in the activation of the MAP kinase pathway by SP, we determined the expression of PKC isoforms in U-373 MG cells. We found that U-373 MG cells express nine different PKC isoforms (α, β, βII, ε, δ, η, ζ, ι, and μ) and that stimulation with SP results in significant and selective translocation of PKCe isoform from cytosolic to membrane fraction. This establishes a correlation between the ability of SP to activate the MAP kinase pathway and its ability to translocate PKCe. In conclusion, the results presented in this study demonstrate that SP receptor activation of PKC, possibly PKCe, leads to the activation of the MAP kinase pathway, and that this pathway can be inhibited by known PKC inhibitors.

Introduction
The SP3 neuropeptide preferentially binds to and acts through the SP receptor (NK-1 subtype; Refs. 1 and 2). In mammals, SP peptide regulates various important functions such as neurotransmitter release, smooth muscle contraction, and cell growth (1, 2). Activation of the SP receptor, which belongs to the superfamily of G protein-coupled receptors (3, 4), induces the hydrolysis of phosphoinositides, leading to the mobilization of intracellular Ca2+ and activation of PKC (5). PKC is a phospholipid-dependent serine/threonine kinase family that can be activated by Ca2+, phospholipids, and DAG (6–8).

The involvement of SP peptide in stimulating mitogenesis in various cell types has been documented by our group.

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3 The abbreviations used are: SP, substance P; PKC, protein kinase C; DAG, diacylglycerol; MAP, mitogen-activated protein; SK, substance K; EGF, epidermal growth factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; MBP, myelin basic protein; CaM, calmodulin; CRE, cAMP-responsive element; CREB, cAMP-responsive element binding.
(9–12) and others (13–17). Furthermore, the ectopic expression of the rat SP receptor in NIH3T3 fibroblasts leads to a SP-induced mitogenesis and ligand-dependent transformation (18, 19). The functional expression of the NK-1 receptor is common in human astrocytic/glial-derived cell lines (approximately 43% of lines examined) and has been identified in adult (e.g., U-373 MG and UC-11 MG; Refs. 20–22) and pediatric astrocytic-derived cell lines (e.g., SJ-S6 and SJ-G4).4 Recently, our group characterized the role of SP peptide and its receptor (NK-1) in the induction of mitogenesis and activation of the MAP kinase pathway in the human astrocytoma cell line U-373 MG (9–11). We demonstrated that activation of the SP receptor stimulates the MAP kinase signaling pathway and induces DNA synthesis and that blocking the MAP kinase pathway by a selective MEK1 inhibitor (PD 098059) blocks SP-induced DNA synthesis (9, 11). These results demonstrated that SP peptide is a strong mitogen for the U-373 MG human astrocytoma cells and established a correlation between SP-induced mitogenesis and activation of the MAP kinase signaling pathway (9, 11). However, the pathway by which SP receptor stimulation activates MAP kinase signaling in human astrocytoma cells is not yet known and is the subject of this study.

Induction of mitogenesis by SP peptide may be of importance because many primary human tumors express the SP receptor (NK-1; Ref. 23). Autoradiography with radiolabeled SP peptide has identified SP receptor in the tumor cells and blood vessels of 75% of astrocytomas and 100% of glioblastomas (23). More importantly, of all tumors investigated, primary samples of human glioblastoma were shown to have the highest expression of SP receptor (23). In addition, the UC-11 MG cell line (24), which is derived from a human glioblastoma multiforme brain tumor sample, has been reported to express high levels of SP receptor (>150,000 binding sites/cell; Ref. 21). The implication of SP receptor overexpression in the pathophysiology of brain tumors is of significance because SP peptide and its receptor may play a role in vasodilation (5, 25, 26) and angiogenesis (27). Furthermore, we demonstrated recently that SK peptide stimulates the SP receptor (NK-1), suggesting that SK peptide may play a role in SP receptor-associated effects in astrocytic cells (9, 11). Therefore, the widespread expression of the SP receptor among many primary tumors (23) and its ability to activate mitogenic signaling upon stimulation by either SP or SK peptide may have diagnostic and therapeutic implications (11). In addition, the ability of SP peptide to stimulate protein synthesis in U-373 MG astrocytoma cell line in a rapamycin-sensitive signaling pathway implicates this peptide in the regulation of cellular hypertrophy (12). This strongly suggests that mitogenic signaling and induction of cellular hypertrophy by SP peptide in astrocytic-derived cells may be an important mechanism for triggering the growth of astrocytic brain tumor cells in vivo (9, 11, 12).

The signaling mechanism by which growth factor receptors (e.g., EGF receptor) activate the MAP kinase pathway has been studied extensively and is adequately understood (28, 29). In contrast, the exact molecular mechanism by which SP receptor activates the MAP kinase signaling pathway requires further examination and remains to be elucidated. Of primary interest to the present study is our previous results, demonstrating that the SP receptor in U-373 MG astrocytoma cells activates the MAP kinase pathway and that this stimulation correlates with the induction of mitogenesis (9–11). Activation of the SP receptor stimulates hydrolysis of phosphoinositides, which leads to PKC activation (5). Thus far, 11 PKC isozymes have been described (30); however, the specific PKC isozyme(s) that play a role in the activation of the MAP kinase pathway by neuromodulin receptor stimulation [e.g., SP (9) and bombesin receptors (31)] in brain tumor-derived cell lines is yet to be elucidated. Although not completely resolved, accumulating reports suggest that some PKC isozymes (Ca2+ dependent or Ca2+ independent; Refs. 32–34) in certain types of cells may phosphorylate c-Raf-1, leading to its activation and subsequently to the activation of the MAP kinase pathway (PKC → Raf-1 → MEK1 → MAP kinases). Therefore, we used three selective PKC inhibitors [CGP 41251 (35), tamoxifen (36), and G6 6976 (37)] to investigate whether SP-induced activation of the MAP kinase pathway is dependent on PKC activation. The SP receptor activation of PKC is particularly important in light of the finding that malignant gliomas express higher PKC activity (100–1000-fold) in comparison to normal astroglial cells (38, 39). The underlying mechanism(s) that cause an increase in PKC activity in brain tumors is an important question requiring further investigations and is likely to involve autocrine and/or paracrine stimulation by brain tumor-associated mitogens.

In the present study, we examined the molecular mechanism by which SP receptor stimulation activates the MAP kinase signaling pathway in a human astrocytoma cell line using selective PKC inhibitors CGP 41251 and tamoxifen. Both drugs are reported to block the growth of brain tumor-derived cell lines (40, 41) and are potentially important for the treatment of brain tumors. In this study, we focused on the involvement of PKC in SP peptide-, TPA-, or EGF-induced activation of the MAP kinase pathway and the induction of c-fos phosphoprotein.

Results

SP-induced Tyrosine Phosphorylation of Erk1 and Erk2 Kinases Is Inhibited by the Selective PKC Inhibitor CGP 41251 in U-373 MG Cells. The human astrocytoma cell line U-373 MG expresses functional SP receptor of NK-1 subtype (9–11, 20, 22). Activation of this receptor by nanomolar concentrations of SP or SK peptides rapidly induces tyrosine phosphorylation and enzymatic activity of Erk1 and Erk2 MAP kinases (9, 11). To address the signaling pathway by which SP peptide activates MAP kinases in human astrocytoma cells, we used two known PKC inhibitors [CGP 41251 (35) and tamoxifen (36)] to examine whether PKC is involved. CGP 41251 and tamoxifen were selected because both compounds exhibit growth-inhibitory effects against cell lines derived from brain tumors (40, 41), thus making them more relevant to studies focused on mitogenic stimulation in astrocytic-derived cells. Serum-starved U-373 MG cells have

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4 M. Sharif, unpublished data.
Fig. 1. Induction of tyrosine phosphorylation of Erk1 and Erk2 MAP kinases by SP and inhibition by the selective PKC inhibitor CGP 41251 in U-373 MG cells. Serum-starved U-373 MG cells were stimulated with indicated concentrations of SP for 10 min at 37°C in the absence (A) or presence (B) of 1 μM CGP 41251 added 15 min prior to SP stimulation. Cell lysates were subjected to SDS-PAGE and Western analysis as described in “Materials and Methods.” Phosphorylated Erk1 (p44) and Erk2 (p42) were detected with phospho-specific MAP kinase antibody using chemiluminescence methods. Bacterially expressed and purified nonphosphorylated (P-) and tyrosine phosphorylated (P+) human Erk2 (p42) were used as negative and positive controls, respectively. Right, relative positions of molecular weight markers.

low levels of tyrosine-phosphorylated Erk1 (p44) and Erk2 (p42) MAP kinases (Fig. 1A, Lane 1). However, tyrosine phosphorylation of the MAP kinases was rapidly induced by SP peptide stimulation in a concentration-dependent manner (Fig. 1A). Although stimulation of starved U-373 MG cells with 1 nM SP peptide resulted in Erk1 and Erk2 phosphorylation levels reaching approximately 90% of maximal, the maximum phosphorylation of these kinases was attained using ≤10 nM of SP (Fig. 1A). We have demonstrated previously that the increase in SP-induced band intensity for the Erk1 and Erk2 kinases in Western blots is due to the induction of tyrosine phosphorylation rather than to new synthesis of these proteins (9). Preincubation of serum-starved U-373 MG cells with 1 μM of the selective PKC inhibitor CGP 41251 (35, 42, 43) almost completely inhibited phosphorylation of MAP kinases induced by low physiological SP concentrations (1 nM) but not by high nonphysiological (100 nM) concentration of the peptide (compare corresponding lanes in Fig. 1, A and B). The CGP 41251 inhibitor itself did not have any detectable effect on the basal levels of tyrosine phosphorylation of Erk1 and Erk2 proteins (Fig. 1, A and B, Lanes 1). This result strongly suggests that SP-induced MAP kinases phosphorylation is activated by a PKC-dependent mechanism in the U-373 MG astrocytoma cells.

Increasing Concentrations of CGP 41251 Significantly Inhibited SP- or TPA-induced Tyrosine Phosphorylation of Erk1 and Erk2 Kinases in U-373 MG Cells. The ability of CGP 41251 to inhibit PKC activity and the subsequent effect it may have on the stimulation of tyrosine phosphorylation of Erk1 and Erk2 kinases by SP or TPA was further examined in titration experiments. As shown in Fig. 2A, CGP 41251 inhibited SP-induced tyrosine phosphorylation of Erk1 and Erk2 kinases in a concentration-dependent manner with the maximum inhibition achieved at 10 μM CGP 41251 (Fig. 2A). To demonstrate that the inhibition of tyrosine phosphorylation of Erk1 and Erk2 was due to the inhibition of PKC activity by CGP 41251, we treated the U-373 MG cells with TPA. TPA, a potent functional analogue of DAG, activates PKC enzyme (44) by direct interaction with the C1 region in the NH2-terminal regulatory half of the protein (45–47). Subsequently, PKC activates the MAP kinase pathway, as detected by increased tyrosine phosphorylation of Erk1 and Erk2 MAP kinases in U-373 MG cells. To avoid using an unnecessary high concentration of this activator, we experimentally determined the minimum concentration of TPA required to
maximally activate phosphorylation of MAP kinases (~90–100% activation, Fig. 3A) to be approximately 10 nM. The addition of 10 nM of TPA resulted in MAP kinase phosphorylation levels similar to that attained by stimulation with 1 nM of SP peptide (compare Fig. 2, A and B, Lanes 2). We then used 10 nM of TPA to examine the effect of increasing concentrations of CGP 41251 on MAP kinase phosphorylation. The MAP kinases phosphorylation was inhibited by increasing concentrations of CGP 41251 with maximum inhibition achieved at 10 μM CGP 41251 (Fig. 2B). These results clearly implicate PKC in the stimulation of MAP kinase phosphorylation and demonstrate that CGP 41251 has significant inhibitory effect on TPA- or SP-induced responses. Therefore, we concluded that CGP 41251 inhibits both SP- and TPA-induced phosphorylation of MAP kinases in a concentration-dependent manner. The concentration of CGP 41251 (10 μM) that resulted in maximum inhibition of the MAP kinase phosphorylation was used in subsequent experiments where PKC or non-PKC-mediated Erk1 and Erk2 MAP kinase phosphorylation and enzymatic activation was examined.

Inhibition of SP- or TPA-induced Erk1 and Erk2 Phosphorylation by Tamoxifen in U-373 MG Cells. The antiestrogen drug tamoxifen has been reported to exhibit an inhibitory effect against rat brain PKC enzymatic activity in vitro (36). We, therefore, examined whether tamoxifen could attenuate or inhibit SP- or TPA-induced Erk1 and Erk2 tyrosine phosphorylation in U-373 MG cells. Using titration experiments, we initially determined that 10 μM of tamoxifen has the maximum inhibitory effect on SP-induced Erk1 and Erk2 tyrosine phosphorylation without any detectable cytotoxic effect on U-373 MG cells during the assay time. Tamoxifen concentrations lower than 10 μM resulted in marginal inhibition of SP-induced Erk1 and Erk2 kinase phosphorylation (data not shown). In the presence of 10 μM tamoxifen, phosphorylation of Erk1 and Erk2 was inhibited most significantly at low SP concentrations (e.g., 0.3 and 1 nM; Fig. 4), similar to the results obtained using CGP 41251 (Figs. 1 and 2). The inhibitory effect of tamoxifen was also observed with TPA-induced Erk1 and Erk2 phosphorylation (Fig. 3B). The concentrations of tamoxifen (10 μM) and CGP 41251 (10 μM) examined in these experiments have similar potency with respect to their ability to inhibit either SP- or TPA-induced phosphorylation of Erk1 and Erk2 kinases (Figs. 2–4) with almost complete inhibition at 1 nM SP (Fig. 2A and Fig. 4B) or 10 nM TPA by 10 μM of either inhibitor (Fig. 2B and Fig. 3B). This is the first report to demonstrate that tamoxifen functions in situ to inhibit the tyrosine phosphorylation of MAP.
Inhibition of SP-induced Erk1 and Erk2 tyrosine phosphorylation by CGP 41251 or tamoxifen in UC-11 MG SC-A3 cells. Serum-starved UC-11 MG SC-A3 cells were stimulated with indicated concentrations of SP for 10 min at 37°C in the absence of CGP 41251 or tamoxifen (A) or presence of serially diluted CGP 41251 (B) or tamoxifen (C). CGP 41251 and tamoxifen were added 15 min prior to SP stimulation. Serum-starved UC-11 MG SC-A3 cells were treated with 10 nM TPA for 15 min in the presence of serially diluted CGP 41251 added 15 min prior to stimulation with TPA (D). Cell lysates were subjected to SDS-PAGE and Western analysis as described in "Materials and Methods." Tyrosine-phosphorylated Erk1 (p44) and Erk2 (p42) MAP kinases were detected as described in Fig. 1. Left, relative positions of molecular weight markers.

Inhibition of SP-induced Erk1 and Erk2 Tyrosine Phosphorylation by CGP 41251 or Tamoxifen in UC-11 MG SC-A3 Cells. To demonstrate that the CGP 41251 and tamoxifen ability to inhibit SP-induced Erk1 and Erk2 tyrosine phosphorylation is not restricted to U-373 MG cells but can also be extended to other cell lines expressing SP receptor, we used UC-11 MG SC-A3, a clonal cell line derived in our laboratory from the UC-11 MG cell line (11). The parental UC-11 MG cell line was established from a glioblastoma multiforme brain tumor sample (24). SP stimulation of UC-11 MG SC-A3 induced the tyrosine phosphorylation of the Erk1 and Erk2 in a concentration-dependent manner (Fig. 5A), and this phosphorylation was inhibited by CGP 41251 (Fig. 5B) or tamoxifen (Fig. 5C) as demonstrated in U-373 MG cells. In addition, CGP 41251 inhibited Erk1 and Erk2 tyrosine phosphorylation induced by TPA (Fig. 5D). These results demonstrate that the ability of CGP 41251 and tamoxifen to block SP-induced Erk1 and Erk2 tyrosine phosphorylation is not limited to U-373 MG cells and can be extended to other SP receptor-expressing, brain tumor-derived cell lines such as UC-11 MG SC-A3.

Correlation between SP-induced Tyrosine Phosphorylation and Enzymatic Activity of Erk1 and Erk2 MAP Kinases in U-373 MG Cells. To demonstrate that Erk1 and Erk2 MAP kinase tyrosine phosphorylation assay is a suitable measure of the activation of the MAP kinase pathway, we compared it to the in-gel MAP kinase assay that measures enzymatic activity. Cell lysates were prepared from SP-stimulated or unstimulated U-373 MG cells in the presence or absence of the selective PKC inhibitor CGP 41251. Equal samples from the same cell lysates were subjected to Western analysis using the phospho-specific MAP kinase antibody (to detect tyrosine phosphorylation of Erk1 and Erk2; Fig. 6, A and C) and to in-gel MAP kinase enzymatic assay using MBP as a substrate (Fig. 6, B and D). Consistent with the previous data, the unstimulated U-373 MG cells have very low basal levels of Erk1 and Erk2 phosphorylation and kinase activity (compare Fig. 6, A and B, Lanes 1). However, when cells were stimulated with increasing concentrations of SP for 10 min, a large increase in both tyrosine phosphorylation (Fig. 6A) and enzymatic activity (Fig. 6B) of Erk1 and
Erk2 kinases was observed in a concentration-dependent manner. The increase in tyrosine phosphorylation or enzymatic activity was significantly inhibited when cells were pretreated with 10 μM of CGP 41251 (Fig. 6, C and D). As observed in Fig. 1, the phosphorylation induced by SP at concentrations higher than 1 nM was not completely inhibited, even at 10 μM CGP 41251. Therefore, the residual tyrosine-phosphorylated Erk1 and Erk2 appeared to be sufficient to give reduced but detectable enzymatic activity (Fig. 6, C and D). The slightly lower kinase activity versus phosphorylation of Erk1 and Erk2 kinases at 1 nM SP (Fig. 6, A and B) may reflect the incomplete renaturation of the Erk1 and Erk2 enzymes in the in-gel kinase activity assay. Nonetheless, it is clear that determination of phosphorylation of Erk1 and Erk2 with phospho-specific MAP kinase antibody is a suitable quantitative assay to monitor the activation of the MAP kinases in the U-373 MG cell system. The results presented in Fig. 6 demonstrate an excellent correlation between levels of tyrosine phosphorylation and the enzymatic activity of Erk1 and Erk2 MAP kinases. In conclusion, induction of tyrosine phosphorylation of the Erk1 and Erk2 kinases by stimulants such as SP is a suitable quantitative assay to monitor the activation of the MAP kinase signaling pathway.

SP Peptide Stimulation Induces the c-Fos Phosphoprotein Expression in a Concentration and Time-dependent Manner in U-373 MG Cells. To demonstrate that tamoxifen and CGP 41251 not only attenuate SP-induced phosphorylation and enzymatic activity of Erk1 and Erk2 but also inhibit downstream responses (e.g., expression of immediate-early genes), we examined the induction of the c-Fos phosphoprotein by stimulation with SP peptide. The c-Fos expression is partially regulated by the Erk1 and Erk2 MAP kinases, which are known to phosphorylate and activate the ternary complex factor, Elk-1, leading to the induction of c-Fos expression via the serum response factor binding to the serum response element in the c-Fos promoter (48). The induction of c-Fos expression by SP was clear, both at transcriptional and translational levels in U-373 MG cells. SP peptide-induced expression of c-Fos mRNA was detected as early as 15 min, peaked at 30 min, and completely disappeared by 60 min (data not shown). In contrast to c-Fos mRNA, expression of c-Fos phosphoprotein increased with increasing concentrations of SP, reaching a maximum 1 h after stimulation with 10 nM SP (Fig. 7A). Furthermore, 1 nM SP peptide induced a c-Fos protein expression that was sustained for at least 2 h after stimulation (Fig. 7B). A significant amount of the highly phosphorylated c-Fos protein was also detected during the 1-h stimulation time (Fig. 7A; see band slightly higher than that labeled as c-Fos). Because the induction of the c-Fos protein was maximum at 1 h after SP stimulation, we selected this time point to further examine the effect of CGP 41251 and tamoxifen on c-Fos protein induction by SP peptide (Fig. 8).

**Inhibition of SP-induced c-Fos Protein Expression by Tamoxifen or CGP 41251 in U-373 MG Cells.** To examine whether tamoxifen or CGP 41251 has any effect on SP-induced expression of the c-Fos protein, U-373 MG cells were treated for 1 h with a serial dilution of SP in the absence or presence of 10 μM of either tamoxifen or CGP 41251. Stimulation of cells with increasing concentration of SP for 1 h induced expression of c-Fos phosphoprotein (Fig. 8A). Preincubation with 10 μM tamoxifen completely inhibited c-Fos protein expression at all SP concentrations (Fig. 8C). Of interest is the observation that this concentration of ta-
**Substance P (60 min)**

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**Substance P (1 nM)**

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**Fig. 7.** Induction of c-Fos phosphoprotein expression by SP peptide in a concentration- and time-dependent manner in U-373 MG cells. Serum-starved U-373 MG cells were stimulated with serial dilutions of SP for 1 h (A) or with 1 nM of SP for the time indicated (B). Cell lysates were subjected to SDS-PAGE and Western analysis as described in “Materials and Methods.” c-Fos protein was immunoblotted with antibody specific to the c-Fos protein and detected by chemiluminescence methods. Left, relative positions of molecular weight markers.

**Results and Discussion**

Tamoxifen (10 μM) exhibited incomplete suppression of MAP kinase phosphorylation yet completely inhibited c-Fos protein induction. This may be explained by the fact that tamoxifen has targets in addition to PKC that are involved in regulating c-Fos expression at the transcriptional level; for example, tamoxifen is an antagonist of CaM (49). CaM regulates cellular responses controlled by Ca2+-dependent signaling via activating kinases such as the Ca2+/CaM-dependent protein kinases (e.g., CaMKI, CaMKII, and CaMKIV). The Ca2+/CaM-dependent protein kinases are among the multiple calcium-regulated kinases that can phosphorylate the transcription factor CREB protein at serine 133 (50). Phosphorylated CREB binds to Ca2+/CRE in the c-Fos promoter and activates transcription in cooperation with the serum response element transcription complex (50). Similarly, CGP 41251 inhibited c-Fos protein expression, suggesting that it may also inhibit c-Fos promoter through the CRE element (Fig. 8D). The c-Fos inhibition data further substantiate our findings that tamoxifen and CGP 41251 attenuate SP-induced phosphorylation and enzymatic activity of Erk1 and Erk2 in U-373 MG cells.

**SP and EGF Activate the Erk1 and Erk2 MAP Kinases by Different Pathways in U-373 MG Cells.** The results obtained with CGP 41251 and tamoxifen implicate PKC in the activation of the MAP kinase pathway by SP peptide. To investigate whether CGP 41251 and tamoxifen can also attenuate the ability of receptors with intrinsic tyrosine kinases known to activate the MAP kinase pathway, we stimulated U-373 MG cells with EGF and examined the effect of either CGP 41251 (10 μM) or tamoxifen (10 μM) on EGF-induced phosphorylation of Erk1 and Erk2. A wide range of EGF concentrations were used to ensure detection of any subtle inhibition of activation of the MAP kinase pathway by PKC inhibitors. Serum-starved U-373 MG cells were stimulated with increasing concentrations of EGF for 10 min. Under these conditions, the induction of tyrosine phosphorylation of Erk1 and Erk2 MAP kinases was apparent at 0.1 ng/ml of EGF (Fig. 9, A and B). EGF concentration at 0.1 or 0.3 ng/ml induced Erk1 and Erk2 phosphorylation to a level similar to that obtained with 1 nM SP peptide (Fig. 4A) or 10 nM TPA (Fig. 3A). We speculated that CGP 41251 or tamoxifen may attenuate EGF-induced tyrosine phosphorylation of Erk1 and Erk2 if PKC is involved in the activation of this pathway. However, neither CGP 41251 nor tamoxifen exhibited any detectable inhibitory effect on the phosphorylation of Erk1 and Erk2 induced by the EGF concentrations examined (Fig. 9, C and D). In summary, these results clearly demonstrate that SP and EGF activate the MAP kinase pathway by two different mechanisms; SP-induced MAP kinase pathway is PKC independent, whereas the EGF-induced MAP kinase pathway is PKC dependent.

**Selective Inhibition of SP-induced Erk1 and Erk2 Tyrosine Phosphorylation by Prolonged TPA Treatment in U-373 MG Cells.** CGP 41251 and tamoxifen results presented thus far implicated PKC enzyme in the activation of the MAP kinase pathway by SP peptide but not by EGF. To further demonstrate that SP peptide, unlike EGF, is dependent on PKC enzyme for the activation of the MAP kinase pathway, we down-regulated PKC enzyme by prolonged TPA (16 h) treatment (Fig. 10). The prolonged treatment with various concentrations of TPA (1-1250 nM) did not have a significant effect on Erk1 and Erk2 MAP kinase tyrosine phosphorylation (Fig. 10D). To demonstrate that PKC enzyme is down-regulated as a result of the prolonged TPA treatment, TPA-pretreated cells were stimulated with fresh TPA (25 nM for 15 min) to activate PKC and subsequently the phosphorylation of the Erk1 and Erk2 MAP kinases. The ability of the freshly added TPA to induce Erk1 and Erk2 tyrosine phosphorylation was clearly inhibited by increasing concentrations of prolonged TPA pretreatment (Fig. 10C). This clearly demonstrates that prolonged TPA treatment down-regulates PKC enzyme in U-373 MG. Similarly, SP-induced Erk1 and Erk2 MAP kinase tyrosine phosphorylation was inhibited by prolonged TPA treatment (Fig. 10A). The inhibition was clearly detected at 2.5 nM TPA pretreatment (Fig. 10A). The down-regulation of PKC enzyme by pro-
Fig. 8. Induction of c-Fos phosphoprotein expression and phosphorylation by SP and inhibition by tamoxifen or CGP 41251 in U-373 MG cells. Serum-starved U-373 MG cells were stimulated with serial dilutions of SP for 1 h in the absence (A and B) or presence of 10 μM tamoxifen (C) or 10 μM CGP 41251 (D). Tamoxifen or CGP 41251 were added 15 min prior to SP stimulation. Cell lysates were subjected to SDS-PAGE and Western analysis as described in "Materials and Methods." c-Fos protein was detected as described in Fig. 7. Left, relative positions of molecular weight markers.

CGP 41251 and Tamoxifen Inhibit Endogenous PKC Enzyme Activity from U-373 MG Cells in Vitro. Our data demonstrated that CGP 41251 and tamoxifen inhibited activation of MAP kinases by SP, presumably by inhibiting PKC activity in vivo. To further strengthen the link of CGP 41251 and tamoxifen inhibitory effect on MAP kinases to inhibition of PKC, we examined the effect of these two drugs on the activity of endogenous PKC present in the cytosol fraction of unstimulated U-373 MG cells in vitro. The analysis was carried out using an ELISA, where the enzymatic activity was monitored by a color reaction.

The endogenous cytosolic PKC activity was increased by 3-fold in comparison to control upon the addition of phosphatidylserine (Fig. 11), an activator of many PKC isozymes (51) expressed in brain tumor cells. The increase in PKC activity was completely inhibited by preincubation with 10 μM CGP 41251. This finding is consistent with our earlier observation that activation of MAP kinases could be inhibited by CGP 41251, supporting our hypothesis that this drug inhibited MAP kinases via interference with PKC. In contrast, only about 35% of the elevated PKC activity was suppressed in the presence of 10 μM tamoxifen, a concentration at which MAP kinase activation and c-Fos protein induction was almost entirely inhibited in previous experiments. The weaker inhibitory effect of tamoxifen in comparison to CGP 41251 on endogenous PKC activity was confirmed in four independent experiments using freshly prepared cytosolic fractions (n = 16). Nevertheless, the degree of inhibition by tamoxifen is statistically significant (P < 0.001). The different potencies exhibited by tamoxifen and CGP 41251 may suggest that tamoxifen is a PKC isozyme-selective inhibitor (e.g., PKCa), whereas the effect of CGP 41251 is less isozyme selective. The tamoxifen result also suggests that 35% reduction in elevated PKC activity may be the portion responsible for the SP-induced MAP kinase pathway in U-373 MG cells in situ.

Inability of G6 6976, a Selective Inhibitor of the PKCα and PKCβI Isozymes, to Inhibit SP- or TPA-Induced Tyrosine Phosphorylation of Erk1 and Erk2 MAP Kinases in U-373 MG Cells. The ability of G6 6976, a PKC isozyme-selective inhibitor, to inhibit the stimulation of tyrosine phosphorylation of Erk1 and Erk2 kinases by SP or TPA was
Fig. 9. Inability of tamoxifen or CGP 41251 to inhibit EGF-induced tyrosine phosphorylation of Erk1 and Erk2 MAP kinases in U-373 MG cells. Serum-starved U-373 MG cells were stimulated with increasing concentrations of EGF for 10 min in the absence (A and B) or presence of 10 μM tamoxifen (C) or 10 μM CGP 41251 (D). Tamoxifen or CGP 41251 were added 15 min prior to stimulation with EGF. Cell lysates were subjected to SDS-PAGE and Western analysis as described in "Materials and Methods." Tyrosine phosphorylated Erk1 (p44) and Erk2 (p42) MAP kinases were detected as described in Fig. 1. Bacterially expressed and purified nonphosphorylated (P−) and phosphorylated (P+) human Erk2 (p42) proteins were used as negative and positive controls, respectively (B and D). Left, relative positions of molecular weight markers.

examined in titration experiments. This PKC inhibitor has been reported to be selective for the Ca²⁺-dependent PKCa and PKCβ isoforms (IC₅₀ = 2.3 nM and 6.2 nM, respectively; Ref. 37). Serum-starved U-373 MG cells were treated with 1 nM SP for 10 min (Fig. 12A) or 10 nM TPA for 15 min (Fig. 12B) in the presence of increasing concentrations of Gö 6976 added 15 min prior to stimulation with SP or TPA. None of the concentrations of Gö 6976 tested inhibited either SP- or TPA-induced tyrosine phosphorylation of Erk1 and Erk2 kinases (Fig. 12). The Gö 6976 inhibitor itself had no detectable effect on the basal level of Erk1 and Erk2 phosphorylation in starved U-373 MG cells (Fig. 12C). The fact that even in the presence of high concentrations of Gö 6976 (5 μM) there was no attenuation of SP- or TPA-induced phosphorylation of Erk1 and Erk2 kinases suggests that PKCa and PKCβ isoforms are probably not involved in the activation of the MAP kinase pathway by SP or TPA. These results clearly indicate that in contrast to tamoxifen and CGP 41251 inhibitors, the Gö 6976 compound is incapable of inhibiting the phosphorylation of MAP kinases induced by SP or TPA, and that MAP kinase activation by SP or TPA is independent of PKCa and β isoforms in U-373 MG cells.

**Ability of Gö 6976, a Selective Inhibitor of the PKCa and PKCβ, to Inhibit SP- or TPA-induced CREB Phosphorylation of U-373 MG Cells.** To demonstrate that Gö 6976 was active in vivo, its ability to inhibit the pathway leading to the phosphorylation of CREB protein on serine 133 by SP or TPA was examined in titration experiments. Serum-starved U-373 MG cells were treated with 1 nM SP for 10 min (Fig. 13A) or 10 nM TPA for 15 min (Fig. 13B) in the presence of increasing concentrations of Gö 6976 added 15 min prior to stimulation with SP or TPA. In contrast to data presented in Fig. 12, Gö 6976 inhibited SP- or TPA-activated pathways, leading to CREB phosphorylation in a concentration-dependent manner (Fig. 13, A and B). The Gö 6976 inhibitor itself had no detectable effect on the basal level of CREB phosphorylation in U-373 MG cells (Fig. 13C). The observation that Gö 6976 inhibitor is able to inhibit TPA-induced phosphorylation of CREB protein suggests that PKC activation is involved, directly or indirectly, in stimulating the signaling pathway, leading to CREB protein phosphorylation. The fact that Gö 6976 inhibitor is able to attenuate of SP- or TPA-induced phosphorylation of CREB protein suggests that PKCa and PKCβ isoforms are probably involved, inducing the pathway leading to the phosphorylation of CREB protein. In summary, although the Gö 6976 drug is unable to inhibit the MAP kinase pathway (Fig. 12), it is an active drug capable of blocking another signaling pathway (CREB phosphorylation; Fig. 13).

**SP Treatment Selectively Stimulates the Translocation of PKCa Isozyme from Cytosolic to Detergent-soluble Membrane Fraction in U-373 MG Cells.** To date, it is not known which PKC isoform(s) are distributed or translocated to the membrane fraction by the activation of SP receptor.
Identification of such an isozyme(s) may establish a correlation between a specific PKC isozyme translocation/activation and the activation of the MAP kinase pathway by the SP receptor. To that end, the expression of various PKC isozymes in U-373 MG cells was determined by Western blotting using PKC isozyme-specific antibodies. Antibody specificity was confirmed using baculovirus-expressed and -purified PKC isozymes and/or mouse brain lysate. We discovered that U-373 MG cells express nine different PKC isozymes (α, βI, βII, ε, δ, η, ζ, ι, and μ; Fig. 14). Among the 11 different isozymes examined, only PKC γ and θ were not detected in U-373 MG cell lysates (data not shown).

Distribution of PKC isozymes in fractions prepared from unstimulated U-373 MG cells was examined, indicating that the isozymes mainly appear in the cytosolic fraction, with various amounts in the detergent-soluble membrane fraction, except for PKCβII and perhaps PKCθ isozymes, which are found mainly in the membrane fraction (Fig. 14, control lanes). To examine the ability of PKC isozymes to translocate, U-373 MG cells were stimulated with TPA, SP, or EGF for 10 min. Stimulation with TPA (10 nM) leads to clear translocation of PKCe from the cytosolic fraction to the detergent-soluble membrane fraction (Fig. 14, TPA lanes). A similar but less intense effect is seen with PKCα and βI isozymes after treatment with TPA. Stimulation with SP (10 nM) for 10 min results in significant and selective translocation of the PKCe isozyme from the cytosolic fraction to the detergent-soluble membrane fraction (Fig. 14, SP lanes). The distribution of

**Fig. 10. Inhibition of Erk1 and Erk2 tyrosine phosphorylation as a result of PKC down-regulation by prolonged TPA treatment.** Cells were incubated with the indicated concentrations of TPA for 16 h (A–D) prior to stimulation with either 10 nM of SP for 10 min (A), 5 ng/ml of EGF for 10 min (B), or 25 nM of TPA for 15 min (C) at 37°C. Cell lysates were prepared as described in "Materials and Methods." Tyrosine phosphorylated Erk1 (p44) and Erk2 (p42) MAP kinases were detected as described in Fig. 1. Left, relative positions of molecular weight markers.
other PKC isozymes was not affected by the 10-min SP treatment (Fig. 14, SP lanes). In contrast, treatment with EGF (5 ng/ml) for 10 min has no detectable effect on the translocation of any of the PKC isozymes examined (Fig. 14, EGF lanes). The results clearly show that SP treatment, like TPA, stimulates the translocation of PKCe in U-373 MG, suggesting an important role for this isozyme in SP-induced signaling and possibly in the activation of the MAP kinase pathway.

**Discussion**

By activating cell surface receptors, mitogens stimulate networks of signaling pathways such as the MAP kinases, the c-Jun N-terminal kinases/stress-activated protein kinases, p38 kinase, and Fos-regulating kinase pathways (52). These pathways, which involve complex and not fully understood steps, transmit cell surface signals into nuclear activities that regulate gene expression (48, 52). Among these signaling pathways, thus far, only the MAP kinase signal pathway has clearly been associated with the induction of mitogenesis (53) and/or oncogenesis (54–56). The activation of the MAP kinase pathway results in the phosphorylation of threonine and tyrosine residues of the MAP kinases (Erk1 and Erk2) in the sequence T–E–Y by the dual specificity kinase MEK1 (57–59). The phosphorylation activates the MAP kinases (Erk1 and Erk2), which in turn phosphorylate many substrates including important transcription factors (60).

The expression of the SP receptor is common in primary astrocytomas/glioblastomas (23) and astrocytic/glial-derived cell lines (9, 20–22). Recently, we demonstrated that SP and SK peptides stimulate mitogenesis in astrocytoma cells by activating the SP receptor (NK-1; Refs. 9, 11). Furthermore, we have determined that activation of the SP receptor stimulates the MAP kinase signaling pathway in astrocytoma cells, and that blocking the MAP kinase pathway by cAMP (inhibits c-Raf-1; Ref. 11) or PD 098059 (inhibits MEK1; Refs. 9 and 11) blocks SP-induced DNA synthesis. These results have established a correlation between SP-induced mitogenesis and activation of the MAP kinase signaling pathway (9, 11). The signaling pathway by which growth factor receptors activate the MAP kinase pathway is adequately understood (28, 29, 61). In contrast, the signaling pathway by which SP receptor activates the MAP kinase signaling path-
the activation of c-Raf-1 kinase and in turn the MAP kinase pathway by neuropeptide receptor stimulation is yet to be elucidated. Accumulating reports, however, suggest that PKCa (Ca$^{2+}$-dependent isozyme; Refs. 32 and 63) and PKCε (Ca$^{2+}$-independent isozyme; Refs. 33 and 34) in certain cell types may phosphorylate and activate c-Raf-1, which in turn activates the MAP kinase pathway (PKC → Raf-1 → MEK1 → MAP kinases).

To investigate whether SP receptor-induced activation of the MAP kinase pathway is dependent on PKC activation, we used three PKC inhibitors (CGP 41251, tamoxifen, and G6 6976) in this study (35–37). Tamoxifen and CGP 41251 were selected because both have been reported to inhibit PKC (35, 36) and to block the growth of brain tumor-derived cell lines in vitro (40, 41). Furthermore, tamoxifen is presently in clinical trials for the treatment of recurrent malignant gliomas using dosages calculated to be sufficient to inhibit PKC activity in tumor cells (38, 64). SP receptor activation of PKC may be important in view of the finding that malignant gliomas express higher PKC activity (2–3 orders of magnitude) in comparison to normal astrocytes (38, 39). The underlying mechanism that results in increased PKC activity in brain tumors is an important question requiring further investigations and is likely to involve autocrine and/or paracrine stimulation by growth factors and possibly by neuropeptides (9, 31).

To elucidate the signaling by which SP peptide activates the MAP kinase pathway in astrocytoma cells, we examined whether PKC inhibitors can block this pathway. The phosphorylation of the MAP kinases was rapidly induced in the presence of TPA, SP peptide, or EGF in U-373 MG cells. TPA, like DAG, activates PKC by direct interaction (44) with the cysteine-rich sequence of the C1 region in the aminoterminal regulatory half of the protein (45–47). In fact, PKC is the major intracellular receptor for tumor-promoting phorbol esters (65, 66). The PKC enzyme in turn phosphorylates and activates c-Raf-1 kinase (32). Subsequently, c-Raf-1 kinase activates MEK1, leading to the activation of the MAP kinase pathway (53). Consistent with a previous report (67), TPA treatment induces the hyperphosphorylation of c-Raf-1 in U-373 MG cells.4 The TPA-induced phosphorylation of MAP kinases was inhibited by CGP 41251 or tamoxifen. These results clearly implicate PKC in the activation of MAP kinases by TPA and demonstrate that PKC inhibitors block TPA activation of the MAP kinase pathway. Similarly, CGP 41251 and tamoxifen inhibited the SP-induced MAP kinase pathway. In contrast, G6 6976, a selective inhibitor of the PKCa and PKCβII isozymes (37), did not inhibit TPA- or SP-induced tyrosine phosphorylation of MAP kinases in U-373 MG cells. These results suggest that TPA- or SP-induced MAP kinase phosphorylation is activated by a PKC-dependent mechanism, and that PKCa and PKCβII isozymes are not involved in this activation in U-373 MG cells. Both PKCa (32, 63) and PKCe (33, 34) have been reported to phosphorylate and activate c-Raf-1. The G6 6976 inhibitor data suggest that PKCe is unlikely to play a role in TPA or SP induction of the MAP kinase pathway in U-373 MG cells. In contrast, we have determined that U-373 MG cells express high level of PKCe isozyme in comparison to normal astrocytes.4 Furthermore,
Fig. 14. Expression, distribution, and translocation of PKC isozymes in U-373 MG cells. Confluent U-373 MG cells were either left unstimulated (Control) or stimulated with TPA (10 nM), SP (10 nM), or EGF (5 ng/ml) for 10 min at 37°C prior to cell fractionation as described in “Materials and Methods.” Equal amounts of proteins (9 μg) from cytosolic (C) and detergent-soluble membrane (M) fractions of each treatment were subjected to SDS-PAGE and Western analysis as described in “Materials and Methods.” PKC isozymes were detected with isozyme-specific PKC antibodies using chemiluminescence methods. Left, relative position of the band corresponding to each specific PKC isozyme.

down-regulation of PKC expression by prolonged TPA treatment (e.g., using 10 nM for 16 h) inhibited SP- or TPA- but not EGF-induced Erk1 and Erk2 MAP kinase phosphorylation (Fig. 10). In this study, we also determined the pattern of expression of 9 PKC isozymes (α, β, βII, ε, δ, η, ζ, ι, and μ) present in U-373 MG cells and demonstrated that SP treatment selectively stimulates the translocation of PKCc isozyme, suggesting an important role for this isozyme in SP-induced signaling and possibly in the activation of the MAP kinase pathway. The selective translocation of PKCc as a result of SP stimulation and its high expression in U-373 MG cells are significant in view of the recent report by Cacace et al. (34), which demonstrated that PKCc functions as an oncogene in rodent fibroblasts by enhancing the c-Raf-1 kinase activity, leading to an increase in MAP kinase activity.

Tamoxifen has been reported to exhibit an inhibitory effect against PKC in vitro (36). In this study, we showed that tamoxifen at concentrations reported previously to block PKC activity and the growth of glioma cell lines in culture (IC50 = 10 μM; Ref. 41) inhibits SP- or TPA-induced phosphorylation of MAP kinases in U-373 MG cells. Therefore, the phosphorylation of MAP kinases would be a suitable assay for evaluating the efficacy of tamoxifen analogues or selective PKC inhibitors in blocking signaling in response to agonists that activate PKC.

The results obtained with CGP 41251 and tamoxifen implicate PKC in the activation of the MAP kinase pathway by SP. However, neither drug exhibited any inhibitory effect on EGF-induced phosphorylation of MAP kinases. This is consistent with the notion that EGF activates the MAP kinase pathway via Ras (Ras → Raf-1 → MEK1 → MAP kinases), and that PKC activation by EGF is either a redundant pathway and/or does not significantly contribute to the activation of MAP kinase pathway. In summary, our results demonstrate that SP and EGF activate the MAP kinase pathway by two different mechanisms; the SP-induced MAP kinase pathway is PKC dependent, whereas the EGF-induced MAP kinase pathway is PKC independent.

Stimulation of U-373 MG cells with SP induced the expression of high levels of c-Fos phosphoprotein that was inhibited by tamoxifen or CGP 41251. Of interest is the observation that although the inhibitory potency of tamoxifen exhibited incomplete suppression of MAP kinase phosphorylation at a high concentration of SP (e.g., 100 nM), it completely inhibited c-Fos protein induction. This may be interpreted by the fact that tamoxifen has additional targets that participate in regulating c-Fos expression. For example, the calmodulin antagonism by tamoxifen (40) is likely to attenuate the c-fos promoter activity by inhibiting transcription activation via the CRE element. The clear efficacy of tamoxifen and CGP 41251 in inhibiting c-Fos protein expression, a protein required for cell cycle progression in certain cells (68, 69), may explain why both drugs exhibit strong growth-inhibitory activity against many brain tumor-derived cell lines in culture (40, 41).

Materials and Methods

Chemicals and Reagents. CGP 41251, a generous gift from D. Fabbro (Ciba-Geigy Limited, Basel, Switzerland), was dissolved in DMSO. Tamoxifen and MBP were purchased from Sigma Chemical Co. (St. Louis, MO). Tamoxifen was dissolved in ethanol/DMSO (9:1), and MBP was dissolved in water. TPA, which is also known as phorbol 12-myristate 13-acetate (30), was purchased from Calbiochem (La Jolla, CA). SP was purchased from Peninsula Laboratories (Belmont, CA). Recombinant human EGF was purchased from R&D Systems (Minneapolis, MN). All reagents were dissolved in appropriate solvents and stored at −80°C according to the manufacturer’s instruction. [γ-32P]ATP (specific activity, 6000 Ci/mmol) was purchased from Amerham Corp. (Arlington Heights, IL). MESCUP protein kinase assay kit was purchased from Medical and Biological Laboratories Co. (Nagoya, Japan). The phospho-specific MAP kinase antibody, phospho-specific CREB (serine 133) antibody, and Pho-
totope chemiluminescent Western detection system were purchased from New England Biolabs (Beverly, MA). The rabbit polyclonal c-Fos antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bacterially expressed and purified nonphosphorylated or phosphorylated human EGF receptor were purchased from New England Biolabs. Fetal bovine serum and MEM with Earle’s salts and 25 mM HepES were purchased from Life Technologies, Inc. (Grand Island, NY). All other chemicals and reagents were of highest grade and purchased from Sigma.

PKC Isozyme-specific Antibodies. Isozyme-specific PKC polyclonal antibodies for PKCα (1:500 dilution), PKCβ (1:250), PKCγ (1:2500), PKCδ (1:750), PKCη (1:250), and PKCζ (1:500) were purchased from Santa Cruz Biotechnology and were used with the secondary anti-rabbit IgG AP-linked (1:1000) from New England Biolabs. Isozyme-specific PKC monoclonal antibodies for PKCα (1:250), PKCβ (1:500), and PKCζ (1:500) were purchased from Transduction Laboratories (Lexington, KY) and were used with the secondary anti-mouse IgG AP-linked (1:30000) purchased from Sigma.

Cell Culture. Human astrocytoma cell line U-373 MG was purchased from American Type Culture Collection (Rockville, MD). The UC-11 MG SC-A3 clonal cell line was isolated in our laboratory from the parental line UC-11 MG, derived from a glioblastoma multiforme brain tumor sample (24). U-373 MG and UC-11 MG SC-A3 were maintained in growth medium (MEM supplemented with 10% fetal bovine serum, 100 μM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin) in a humidified 37°C atmosphere.

In Gel Kinase Activity Assay. The in-gel kinase activity assay was conducted primarily as described previously (70–72) with slight modifications. Lysates prepared from cells preincubated with or without CGP 41251 and stimulated with SP were electrophoretically separated by 10% polyacrylamide gel polymerized in the presence of 0.3 mg/ml MBP and 0.1% SDS as described above. The gel was then washed with 50 mM Tris-HCl (pH 8.0), 0.1% (v/v) 2-isopropanol for 1 h at room temperature. The resolved proteins were denatured with 50 mM Tris-HCl (pH 8.0), 6.0 μg guanidine-HCl, and 2 mM DTT for 1 h at room temperature; washed three times with 50 mM Tris-HCl (pH 8.0), 0.04% (v/v) Tween-40, and 2 mM DTT (renaturation buffer); and renatured in renaturation buffer for 16 h at 4°C. The gel was washed once with 40 mM HEPES [pH 8.0, 5 mM MgCl₂, 0.1 mM EGTA, and 2 mM DTT (phosphorylation buffer) for 1 h at room temperature. Phosphorylation of MBP by MAP kinases took place in 10 ml of phosphorylation buffer supplemented with 10 μM ATP and 5 μg/ml (γ-32P)ATP (6000 Ci/mmol; Amersham) for 1 h at room temperature and was terminated by washing the gel five times with 5% (v/v) trichloroacetic acid, 1% (w/v) sodium PP, (Sigma). Autoradiograms were obtained by exposure of the dried gel to Kodak XAR-2 film at room temperature with an intensifying screen.

Cell Fractionation. To separate PKC isoforms between the cytosol and the membrane, U-373 MG cells were plated at a density of 1.25 × 10⁶ cells/ml in 100-mm tissue culture plates (10 ml/plate) and incubated at 37°C for 3 days prior to stimulation with the appropriate agent for 10 min. Stimulation was terminated by removing the medium and washing cells once with ice-cold PBS. Cells were then scraped into buffer A [20 mM Tris-HCl (pH 7.5), containing 0.25 mM sucrose, 2 mM EDTA, 2 mM EGTA, and Complete Mini EDTA-free protease inhibitor cocktail tablets from Boehringer Mannheim Corp. (Indianapolis, IN)], briefly sonicated and centrifuged at 100,000 × g for 1 h. The supernatant containing the soluble cytoplasmic proteins was designated as cytosolic fraction. The pellet containing all membranes and the insoluble cytoskeletal components was subjected to extraction by buffer B [20 mM Tris-HCl (pH 7.5), containing 1% NP40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and the protease inhibitor cocktail tablets] on ice for 30 min, followed by centrifugation as described above. The supernatant containing the membrane proteins was designated as a detergent-soluble membrane fraction. The pellet containing the denaturing detergent-resistant cell cytoskeleton was discarded.

References


