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VERBASCOSIDE ISOLATED FROM *LANTANA CAMARA*, AN INHIBITOR OF PROTEIN KINASE C

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ABSTRACT.—Verbascoside [**1**] isolated from *Lantana camara* is an inhibitor of protein kinase C (PKC) from the rat brain. Half-maximal inhibition of the kinase occurs at 25 μM. Verbascoside interacted with the catalytic domain of PKC and was a competitive inhibitor with respect to ATP (Ki = 22 μM) and a non-competitive inhibitor with respect to the phosphate acceptor (histone IIIS). This effect was further evidenced by the fact that verbascoside inhibited native PKC and its catalytic fragment identically and did not affect [^{3}H]-phorbol-12,13-dibutyrate binding to PKC. The antitumor activity of verbascoside measured in vitro might be due at least in part to inhibition of PKC.

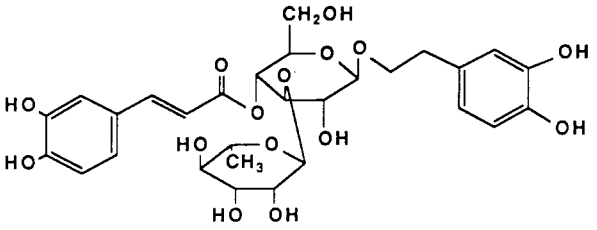
The Ca^{++}/phospholipid-dependent protein kinase, protein kinase C (PKC), plays a crucial role in signal transduction, cellular proliferation, and differentiation (1), but the pleiotropic effects of this enzyme in cellular regulation and its involvement in tumor promotion underscore the importance of understanding its mechanism of regulation. Therefore, newly developed inhibitors of PKC should contribute to an understanding of its mechanism of regulation and constitute useful pharmacological tools.

We now report that verbascoside [**1**] isolated from *Lantana camara* L. (Verbenaceae) is a potent inhibitor of PKC. Verbascoside, also named acteoside, has been isolated from various plants (2–4). Antimicrobial, immunosuppressive, and antitumor activities of verbascoside are well described (5–7), supporting the uses of *L. camara* in traditional medicine (8).

EXPERIMENTAL

PLANT MATERIAL AND ISOLATION OF VERBASCOSIDE.—*L. camara* was collected in July 1987 in the Ivory Coast, Africa. A voucher specimen was deposited (no. 87/1025) at the Faculté de Pharmacie, Toulouse. Verbascoside [**1**] was isolated from the MeOH extract of dried leaves. The extraction, purification, and identification of verbascoside were carried out as previously described (3).

CHEMICALS.—Histones (type IIIS), phosphatidyl serine, phorbol-12,13-dibutyrate (PDBu), ATP, 1,2-dioleine, poly (Glu, Tyr) 4:1, glycogen synthase, cAMP-dependent protein kinase, and Triton X100 were from Sigma Chemical Co., France. [γ-^{32}P]-ATP (3000 Ci/mmol) and [^{3}H]-phorbol-12,13-dibutyrate ([^{3}H]-PDBu) (17.5 Ci/mmol) were purchased from Amersham, France. Dulbecco's modified Eagle



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medium, fetal calf serum, glutamine, penicillin, and streptomycin sulfate were from Boehringer Mannheim, France. H-7 was obtained from Seikagaku Kogyo, Tokyo, Japan. All other chemicals were from Prolabo, France.

MIXED MICELLAR ASSAY FOR PROTEIN KINASE C.—The purified protein kinase C was prepared from rat brain and assayed as described by Kikkawa *et al.* (9). Briefly, the incubation mixture (200 μl) contained 20 mM Tris/HCl buffer (pH 7.5), 10 mM $MgCl_2$, 200 μg/ml histones (Type IIIS), micelles made with 700 μM phosphatidyl serine and 180 μM 1,2-dioleine in 0.3% triton X100, 0.2 mM $CaCl_2$, 100 μM ATP, [γ-^{32}P]-ATP (10^5 dpm), the compound to be tested (solubilized in DMSO), and the enzyme (0.5 μg protein). After incubation at 30° for 3 min, the reaction was terminated by the addition of 3 ml of 20% trichloroacetic acid (TCA). Acid-precipitable materials were collected on Whatman GF/C filters and extensively washed with ice-cold 20% TCA. The radioactivity on the filters was measured using a liquid scintillation counter (LKB, model 1215 Rackbeta). PKC activity was corrected for nonspecific activity by assaying in the absence of micelles and $CaCl_2$.

ASSAYS FOR OTHER PROTEIN KINASES.—Tyrosine protein kinase was extracted, purified from a lymphoma cell line, and assayed according to Casnellie *et al.* (10) with poly (Glu, Tyr) 4:1 as substrate. cAMP-dependent protein kinase was assayed as described by Hidaka *et al.* (11) with histone IIIS as substrate.

[^{3}H]-PDBu BINDING ASSAY.—[^{3}H]-PDBu binding to PKC was determined according to Tanaka *et al.* (12) with slight modifications. Assays were carried out in a final volume of 250 μl in 50 mM Tris/HCl (pH 7.4), $CaCl_2$ (1 mM), [^{3}H]-PDBu (39.2 nM), 50 μg/ml PKC, and the compound to be tested. Nonspecific binding was defined as the portion of total binding not displaceable by a 100-fold excess of unlabelled PDBu. After 1 h at 30°, 4 ml of ice-cold 50 mM Tris/HCl (pH 7.4) was added. The mixture was poured onto a glass fiber filter (Whatman GF/C) and washed 3 times with buffer, and the bound radioactivity was determined by scintillation counting.

L1210 CELL PROLIFERATION.—The lymphocytic mouse leukemia L1210 cells (ATCC, CCL 219) were plated sparsely at 10^4 cells per well in 24-well cluster plates (Nunc, Denmark) in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and the compound to be tested (solubilized in DMSO). After a 2-day incubation period at 37° in a humidified atmosphere (5% CO_2 in air), growth was monitored by counting cell numbers in a Coulter-counter (Coultronics, France). IC_{50} values were calculated on the basis of the linear regression lines established for each compound tested.

RESULTS AND DISCUSSION

Verbascoside [**1**] inhibited PKC in a concentration-dependent manner (Figure 1)

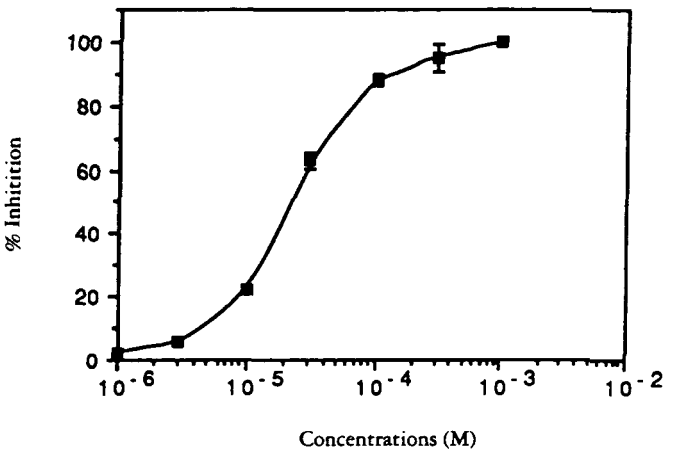

FIGURE 1. Inhibition of rat brain PKC by verbascoside [**1**]. Effects of increasing concentrations of verbascoside on the activity of PKC were evaluated using mixed micelles of triton X100 as described in Experimental. Points shown are mean values ±SD of 6 replicates.

with an IC_{50} value (concentration causing a 50% inhibition) of 25 μM. Basal activity of the enzyme (the activity in the absence of Ca^{++}, phosphatidylserine, and dioleine) was not affected. The inhibitory effect of verbascoside could be overcome by increasing the amounts of ATP added, indicating that the interaction between the compound and the enzyme was reversible. Kinetics analysis by double-reciprocal plots revealed a competitive type of inhibition with respect to ATP and non-competitive with respect to phosphate acceptor (Figure 2). Ki values of verbascoside determined from the secondary replots were 22 and 28 μM with respect to ATP and histone, respectively.

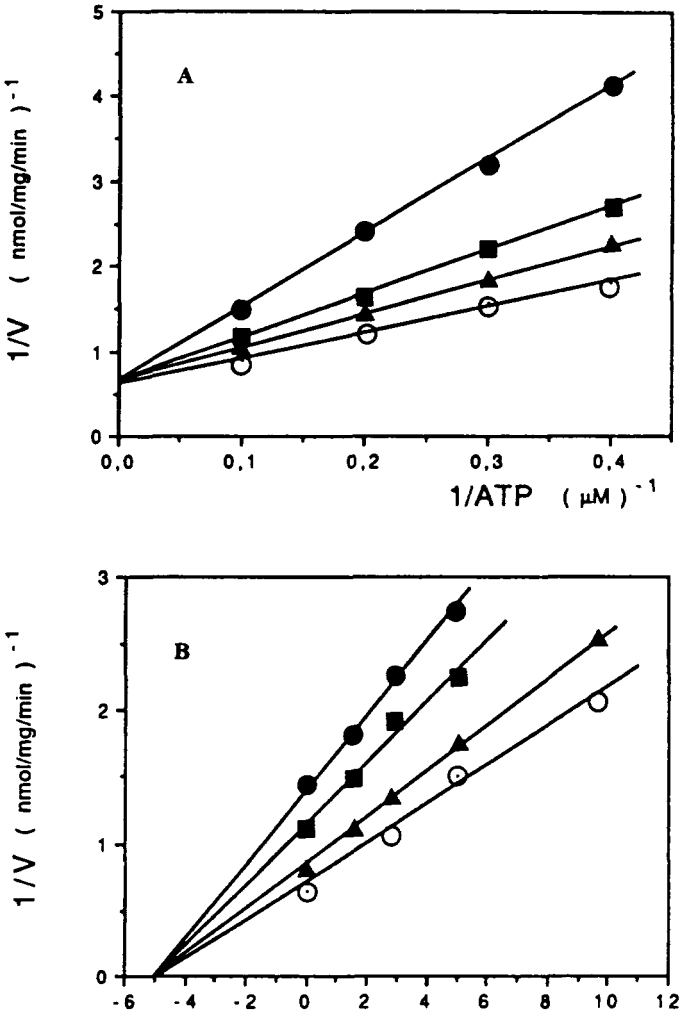

FIGURE 2. Lineweaver-Burk plots for the inhibition of PKC by verbascoside [1]. A) Verbascoside inhibition of PKC with respect to ATP. ATP varied from 0.5 to 2 μM. Histone concentration was held constant at 200 μg/ml, and 0.5 μg of PKC was used. Verbascoside concentrations were: 0 (○), 5 (▲), 10 (■), and 15 (●) μM. Apparent Ki was obtained from the intersections of the lines by an unweighted method of a least squares fit of data. B) Verbascoside inhibition with respect to histones. Double reciprocal plots obtained with histones varied from 100 to 400 μg/ml. [γ-^{32}P]-ATP final concentration was 10 μM. Verbascoside concentrations were: 0 (○), 10 (▲), 20 (■), and 30 (●) μM.

To gain further insight into the mechanism of verbascoside-mediated inhibition, we sought to identify the site or sites on the enzyme with which verbascoside directly interacts to produce inhibition. The inhibitory potency of verbascoside was assessed in terms of another function residing in the regulatory subunit, the region of PKC recognized by diacylglycerol, and by phorbol esters such as phorbol 12,13-dibutyrate (PDBu). By using [^{3}H]-PDBu as a probe, we examined the ability of verbascoside to displace this radioactive marker from the intact enzyme. In this experiment, unlabelled PDBu competed with [^{3}H]-PDBu for binding to PKC with an IC_{50} value of 68 nM, but verbascoside failed to alter [^{3}H]-PDBu binding (9% inhibition of specific binding at 1 mM). This result suggests that verbascoside binds to a site different from that of PDBu or diacylglycerol and that this site might be at the catalytic center of PKC. PKC was reported to be alternatively activated, in an irreversible manner, by limited proteolysis with trypsin. In this process, the catalytically active fragment produced was entirely independent of calcium and enzyme cofactors. To define unequivocally the mode of action of verbascoside on PKC, the catalytic fragment of PKC was prepared by trypsin digestion according to Lee and Bell (13). After incubation for 10 min at 30° with 1 μg/ml trypsin, PKC became alternatively activated in an irreversible manner and exhibited an activity entirely independent of calcium and lipid cofactors (not shown). When tested on the catalytic fragment of PKC, verbascoside displayed the same inhibitory capacity and a dependence on ATP or histone similar to that observed for the native enzyme, indicating the the activity of this compound was not due to effects on the enzyme activating process. With respect to the catalytic fragment of PKC, verbascoside exhibited an IC_{50} value of 21 μM.

Because it is now clear that staurosporine blocks not only PKC but also a variety of other protein kinases (14), the question of whether verbascoside might inhibit other protein kinases arose. Therefore, the selectivity of verbascoside was investigated. When tested on tyrosine protein kinase (TPK), or cAMP-dependent protein kinase (PKA), verbascoside did not alter any activity of these various protein kinases (less than 10% inhibition at 100 μM), showing that this compound was a PKC-specific inhibitor.

Previous studies have clearly established the PKC plays a key role in cellular growth and differentiation (1). Therefore, we examined the effects of verbascoside on tumor cell growth in vitro. Under our experimental conditions, verbascoside showed a potent antiproliferative effect against L-1210 cells with an IC_{50} value of 13 μM (Figure 3). In the same experiment, H-7, another PKC inhibitor (15), affected L-1210 cell growth with an IC_{50} value of 23.5 μM, a result in agreement with already published data (16). In order to document adequately the effect of verbascoside on cell proliferation, we used a colorimetric assay developed by Mosmann (17). The assay is dependent on cellular reduction of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by the mitochondrial succinate-dehydrogenase of viable cells to a blue formazan product which can be measured photometrically. Around the concentration giving a 50% inhibition of cell proliferation, verbascoside or H-7 (10 and 25 μM respectively) did not show any detectable difference in MTT reduction compared with the same number of untreated cells (not shown). These results indicated that no cytotoxicity of the tested compounds was observed. Therefore, it is conceivable that the antiproliferative and antitumor activity of verbascoside in vitro is due to inhibition of PKC. Further studies are under way to confirm that verbascoside specifically inhibits PKC in vivo.

The discovery and development of inhibitors of PKC should permit critical analysis of specific functions of PKC in cell systems and in animals. A number of PKC inhibitors have been reported, but except for H-7 and staurosporine (15,18), which appear to be competitive inhibitors with respect to ATP, few data are available regarding the

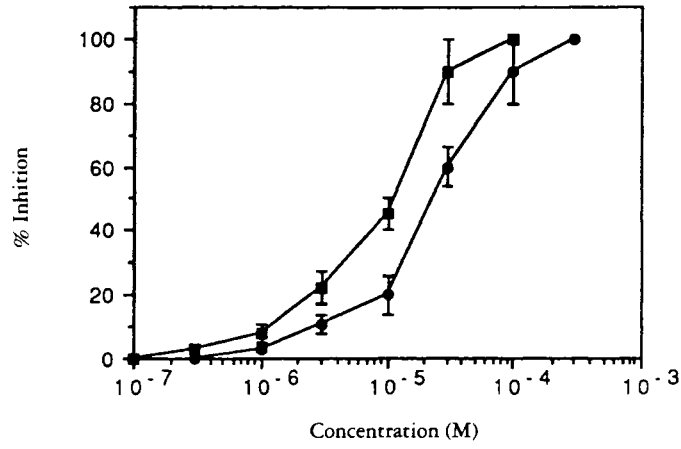

FIGURE 3. Effect of verbascoside [**1**] and H-7 on the growth of L1210 cells. Logarithmically growing L1210 cells were plated in 24-well cluster plates (10^4 cells/well) and incubated with verbascoside (■) or H-7 (●) for 48 h. Number of cells was counted 48 h after addition of compounds. Results are expressed as mean ± SD ($n = 6$).

mechanisms by which other inhibitors affect PKC activity. This has led to the current assumption that most of these molecules "perturb" the lipid bilayer and consequently inhibit enzyme activity nonspecifically.

The results presented in this paper demonstrate the effects of a new PKC inhibitor which interacts directly with the catalytic domain of PKC. Verbascoside may therefore prove to be a biological tool for examining the physiological role of phosphorylation by PKC and a putative pharmacological modulator of PKC in vivo.

The possibility of using verbascoside for therapeutic intervention in a wide range of diseases including cancer, inflammation, or immune disorders will be further evaluated in cellular systems and in animal models of diseases.

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