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Serum-Dependent Effects of Tamoxifen and Cannabinoids upon C6 Glioma Cell Viability

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ABSTRACT. In the present study, the effects of the combination of tamoxifen ((Z)-2[*p*-(1,2-diphenyl-1-butenyl)phenoxy]-*N*,*N*-dimethylamine citrate) and three cannabinoids (Δ^9-tetrahydrocannabinol [Δ^9-THC], cannabidiol, and anandamide [AEA]) upon the viability of C6 rat glioma cells was assessed at different incubation times and using different culturing concentrations of foetal bovine serum (FBS). Consistent with previous data for human glioblastoma cells, the tamoxifen sensitivity of the cells was increased as the FBS content of the culture medium was reduced from 10 to 0.4 and 0%. The cells expressed protein kinase C α and calmodulin (the concentration of which did not change significantly as the FBS concentration was reduced), but did not express estrogen receptors. Δ^9-THC and cannabidiol, but not AEA, produced a modest reduction in cell viability after 6 days of incubation in serum-free medium, whereas no effects were seen in 10% FBS-containing medium. There was no observed synergy between the effects of tamoxifen and the cannabinoids upon cell viability. BIOCHEM PHARMACOL **60**;12:1807–1813, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. tamoxifen; Δ^9-tetrahydrocannabinol; anandamide; C6 glioma; cell viability

Despite advances over the last few decades in the diagnosis and treatment of many forms of cancer, individuals with malignant gliomas still have a poor prognosis [1]. Tamoxifen‡, a synthetic, non-steroidal antiestrogen which is used extensively in the treatment of breast cancer [2], has received interest as a possible therapeutic agent in the treatment of glioma [3, 4]. *In vitro*, tamoxifen has been shown to block the cell proliferation of human U138 glioblastoma cells in a manner dependent upon the serum content of the culture medium [5]. In addition, tamoxifen affects the sensitivity of glioma cells to other antiproliferative treatments [6–8].

In a recent study, it was found that Δ^9-THC affected C6.9 glioma (a subclone of C6 rat glioma cells) cell viability by a mechanism not involving cannabinoid receptor activation [9]. These authors demonstrated that in serum-free medium, cell viability, assessed as the rate of reduction of MTT, decreased after incubation with 1 μM Δ^9-THC for a period of ≥ 5 days. This was accompanied by DNA fragmentation, suggestive of apoptosis, and a stimulation of sphingomyelin hydrolysis. Human U373MG astrocytomas, but not primary astrocytes, were affected by Δ^9-THC [9].

In the present study, three questions were investigated using the C6 rat glioma cell line:

1. Is the serum-dependent cytotoxicity of tamoxifen restricted to human glioblastoma cells, or is it a general phenomenon that is even seen in glioma cells from other species?
2. Is the effect of Δ^9-THC upon cell viability seen with other cannabinoids, and is it dependent upon the serum content of the culture medium?
3. Is the effect of Δ^9-THC upon cell viability affected by concomitant tamoxifen treatment? Synergistic effects of tamoxifen and Δ^9-THC might provide a new therapeutic approach for the treatment of glioma.

MATERIALS AND METHODS

Materials

Anti-human estrogen receptor (287–300) from mouse (cat no. SRA-1000) was obtained from StressGen. Anti-bovine calmodulin (128–148) mouse monoclonal IgG_1 (cat no. 05-173) was obtained from Upstate Biotechnology. Anti-PKC α (662–672) from rabbit (cat no. 539601) was obtained from Calbiochem. The secondary antibodies (sheep anti-mouse IgG, peroxidase-linked; donkey anti-rabbit Ig, peroxidase-linked) were obtained from Amersham Pharmacia Biotech. Tamoxifen, calmodulin (from human erythrocytes), and MTT were obtained from the

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‡ *Abbreviations:* tamoxifen, ((Z)-2[*p*-(1,2-diphenyl-1-butenyl)phenoxy]-*N*,*N*-dimethylamine citrate); Δ^9-THC, Δ^9-tetrahydrocannabinol; AEA, anandamide, arachidonyl ethanolamide; FBS, foetal bovine serum; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); PKC, protein kinase C; IgG, immunoglobulin G; PVDF, Polyvinylidene fluoride; and TBS-T, 20 mM Tris, 150 mM NaCl, 0.05% Tween.

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Sigma Chemical Co. AEA was obtained from Tocris Cookson. R-1 methanandamide was obtained from the Cayman Chemical Company. The compounds (with the exception of initial experiments with AEA where it was dissolved in Ham's F-10 medium) were dissolved in ethanol. The assay ethanol concentration did not exceed 1% and was kept constant in all cases (except for the untreated cells). C6 rat glioma cells were obtained from the American Type Culture Collection. Ham's F-10 medium + 25 mM HEPES buffer, L-glutamine, FBS, and PEST (penicillin/streptomycin) were obtained from GIBCO BRL, Life Technologies.

Cell Culturing Conditions

C6 rat glioma cells were used between passages 41 and 80. Stocks of cells were cultured in 75-cm^2 flasks in F-10 Ham's medium supplemented with 25 mM HEPES buffer, L-glutamine, 10% FBS, and 1% PEST. Cells were maintained at 37° at 96% relative humidity in an atmosphere of 5% CO_2 and 95% air. For the Western blot experiments using different FBS concentrations, the stocks were cultured in the appropriate FBS concentrations for 3 days. Cells were harvested using a rubber policeman and pelleted by centrifugation for 10 min at 125 *g*. The pellets were resuspended in ice-cold lysis buffer (20 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton, 0.1 mM phenylmethylsulphonyl fluoride, 5 μg/mL of leupeptin, 5 μg/mL of pepstatin A, 5 U/mL of aprotinin) and incubated for 20 min at 4°, after which samples were sonicated and then centrifuged for 10 min at 10,000 *g* (4°) to remove cellular debris. The supernatant was collected and used for Western blot analyses after the protein content was determined [10], using BSA as a standard.

MTT Assay

To determine the effects of cannabinoids and tamoxifen upon cell viability, we measured the redox-mediated reduction of MTT [11]. Cells (usually ~1×10^5/mL) were plated out in 24-well culture plates in medium containing the appropriate FBS concentration. After 24-hr incubation at 37°, the medium was removed and new culture medium containing the test compounds was added (assay volume 500 μL). The cells were then further incubated at 37° for the times indicated in the figures and text, after which 50 μL of a 5 mg/mL solution of MTT dissolved in Ham's F-10 medium was added. After 3 hr of incubation, 500 μL of 0.1 M HCl in isopropanol containing 10% Triton X-100 was added to each well, and the plates were mixed thoroughly for 40–60 min using a multitube vortex mixer. Aliquots (300 μL) from all wells were transferred to optically clear 96-well flat-bottomed microtiter plates and read spectrophotometrically at 570 nm using a ThermoMax microplate reader (Molecular Devices). The optical densities were then expressed as % of the corresponding optical densities for samples assayed concomitantly (and with the same incubation time) where neither test compound nor ethanol carrier had been added.

Estrogen Receptor Western Blot

Rat brain homogenate and C6 cell lysate samples were mixed with sample buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, 5% mercaptoethanol, and 0.05% bromophenol blue) and boiled for 5 min. The samples were run on 10% polyacrylamide gel electrophoresis [12] in running buffer (24 mM Tris, 196 mM glycine, 17 mM SDS) at 140 V for ~100 min. After the electrophoresis, the gels were incubated in Laemmli buffer (48 mM Tris, 39 mM glycine, 20% methanol, 1.3 mM SDS) for 15 min, and PVDF membranes (Micron Separations Inc.) were incubated in methanol for 5 min and Laemmli buffer for ~10 min. The gels were blotted (Trans-Blot SD, Semi-Dry Electrophoretic Transfer cell, Bio-Rad) to the PVDF membranes at 0.2 A for 35 min, and the membranes were then blocked in TBS-T containing 5% non-fat dry milk (Semper) overnight at 4°. The blots were washed with TBS-T for 2 × 10 min and incubated with the primary antibody (anti-human estrogen receptor [287–300] from mouse) diluted to 1:1000 in TBS-T containing 3% non-fat dry milk for 1 hr at room temperature. After that, the blots were washed for 3 × 15 min with TBS-T and incubated with secondary antibody (sheep anti-mouse IgG, peroxidase-linked) diluted to 1:2000 in TBS-T containing 3% non-fat dry milk for 1 hr at room temperature. They were then washed for 4 × 15 min with TBS-T before being detected with enhanced chemiluminescence (ECL) Western blotting reagents (Amersham Pharmacia Biotech) and exposed to x-ray film.

Calmodulin Western Blot

Rat brain homogenate and C6 cell lysate samples were mixed with sample buffer and boiled for 5 min. The samples were run on 15% polyacrylamide gel electrophoresis in running buffer at 140 V for ~100 min. After the electrophoresis, the gels were incubated in carbonate buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, 20% methanol) for 15 min, and PVDF membranes were incubated in methanol for 5 min followed by carbonate buffer for ~10 min. The gels were blotted to the PVDF membranes at 0.2 A for 25 min, and the blots were then fixed in PBS (1.5 mM KH_2PO_4, 150 mM NaCl, 2.7 mM Na_2HPO_4) containing 0.2% glutaraldehyde for 45 min [13]. They were blocked in PBS containing 3% non-fat dry milk for 20 min at room temperature and were then incubated with the mouse monoclonal anti-bovine calmodulin antibody diluted to 1:2000 in PBS containing 3% non-fat dry milk overnight at 4°. The blots were washed for 2 × 10 min with PBS and incubated with secondary antibody (sheep anti-mouse IgG, peroxidase-linked) diluted to 1:2000 in PBS containing 3% non-fat dry milk for 90 min at room temperature. Then, they were washed for 2 × 10 min with PBS and 2 × 5 min with PBS-T (PBS containing 0.05% Tween) before being detected with ECL Western blotting reagents and exposed

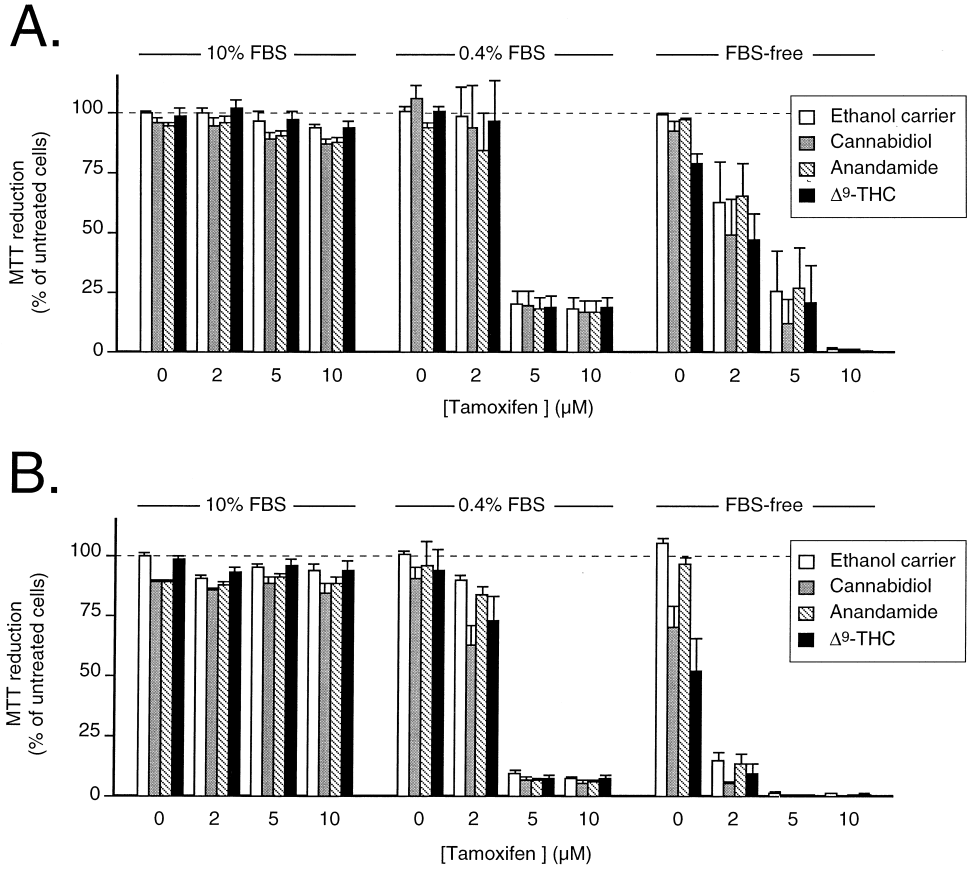

FIG. 1. Effect of cannabinoids upon C6 glioma cell viability. Shown are means ± SEM, N = 4 of the MTT reduction (as % of untreated cells incubated for the same length of time) following treatment for either 3 days (Panel A) or 6 days (Panel B) with tamoxifen and the test cannabinoids (at concentrations of 1 μM). The legends shown in the figure indicate the cannabinoids used at each tamoxifen concentration; thus, for example, columns described as "ethanol carrier" contain the appropriate concentration of tamoxifen. Four-way ANOVA for the entire data set gave highly significant ($P < 0.0001$) contributions of FBS concentration, tamoxifen, days of treatment, FBS × tamoxifen, FBS × days of treatment, and tamoxifen × days of treatment. Significant ($P < 0.01$) contributions of cannabinoid, FBS × cannabinoid, and FBS × tamoxifen × days of treatment were seen. All other interactions were non-significant ($P > 0.3$). *Post hoc* Bonferroni/Dunn indicated significant differences between the three FBS concentrations, between Δ^9-THC and ethanol carrier, between cannabidiol and ethanol carrier, and between all tamoxifen concentrations except for 5 μM vs 10 μM.

to x-ray film. Immunoreactive bands were digitised and pixel intensities quantitated as described previously [14].

PKC α Western Blot

Rat brain homogenate and C6 cell lysate samples were mixed with sample buffer and boiled for 5 min. The procedure was as for the estrogen receptor Western blots, except for a blotting time to PVDF membranes at 0.2 A for 40 min. The primary antibody used was anti-PKC α (662–672) from rabbit diluted to 1:1000 in TBS-T containing 3% non-fat dry milk, and the secondary antibody was donkey anti-rabbit Ig, horseradish peroxidase-linked diluted to 1:1000 in TBS-T containing 3% non-fat dry milk. In the study of the specificity of the PKC α antibody, a PKC α synthetic peptide (no. 539633, Calbiochem) was preincubated with the primary antibody (75 μL PKC α synthetic peptide, 1.5 mL TBS-T, and 10 μL anti-PKC α, mixed end-over-end for 2 hr at room temperature). In the primary antibody incubation, the mixture was diluted to 10 mL with TBS-T containing 3% non-fat dry milk.

Statistical Analyses

ANOVAs and *post hoc* Bonferroni–Dunn analyses were undertaken using the Statview™ computer program (SAS Institute Inc.).

RESULTS

FBS-Dependent Cell Toxicity of Tamoxifen

The time- and concentration-dependent effects of tamoxifen upon cell viability were determined at different culture FBS concentrations using MTT reduction as end point. At 10% FBS, concentrations of tamoxifen ≤ 10 μM did not affect cell viability at incubation times of up to 6 days (Fig. 1 unfilled columns), whilst 20 and 30 μM tamoxifen produced a rapid loss in cell viability that was matched by a loss of cells (data not shown). At lower FBS concentrations, the cells were more sensitive to tamoxifen, and significant decreases in cell viability were seen with 5 and 10 μM tamoxifen (unfilled columns in Fig. 1). At an FBS concentration of 0.4%, 5 μM tamoxifen reduced cell

viability first after 3 days, whereas 10 μM was effective after 2 days, as was both 5 and 10 μM tamoxifen in the serum-free medium (data not shown).

Effect of Cannabinoids upon C6 Glioma Cell Viability

Initial experiments were undertaken using AEA and an FBS concentration of 10%. In these experiments, a single exposure to AEA was used and the cell viability determined 1, 3, and 5 days later. Over a concentration range of 0.03–10 μM, AEA produced no significant effects upon cell viability (data not shown). The stable AEA analogue R-1 methanandamide was also without significant effect; thus, the MTT reduction (as % of control) following treatment with carrier, 1 μM, and 10 μM R-1 methanandamide, respectively, was: 1 day, 104 ± 4, 92 ± 14, and 99 ± 19; 3 days, 116 ± 6, 90 ± 13, and 97 ± 8 (means ± SEM, N = 3; no significant effects seen with two-way ANOVA).

In the next series of experiments, the combination of tamoxifen with either Δ^9-THC or cannabidiol was investigated at an FBS concentration of 10%. Three concentrations of the cannabinoids (0.1, 0.3, and 1 μM) were tested together with 5, 10, 20, and 30 μM tamoxifen following incubations for 1, 3, and 5 days. No significant effects of the cannabinoids were seen (data not shown). However, at a lower FBS concentration (0.4%), a reduction in cell viability was seen after long-term treatment with Δ^9-THC; thus, MTT reduction rates (as % of untreated control) of 121 ± 8, 120 ± 9, and 66 ± 9 were found after incubation for 2, 5, and 7 days, respectively, with 1 μM Δ^9-THC (means ± SEM, N = 4). The corresponding values for 1 μM cannabidiol were: 115 ± 3, 126 ± 5, and 101 ± 5, respectively and for 1 μM AEA 105 ± 6, 105 ± 2, and 99 ± 4, respectively (means ± SEM, N = 4). When the FBS-free culturing medium was used, both Δ^9-THC and cannabidiol, but not anandamide, reduced C6 glioma cell viability after a 6-day incubation (Fig. 1B).

Interaction between Cannabinoids and Tamoxifen

The combination of cannabinoids and tamoxifen upon C6 glioma cell viability following exposure times of 3 and 6 days is shown in Fig. 1. When the entire data set was analysed together, significant effects of tamoxifen, incubation time, and cannabinoid treatment were seen, all three showing significant interactions with the FBS concentration. When compared with the effects of tamoxifen, however, the effects of Δ^9-THC and cannabidiol are rather modest, and no significant interaction between tamoxifen and the cannabinoids was noted.

Expression of Estrogen Receptor, Calmodulin, and PKC α in C6 Cells

Western blot experiments were undertaken using antibodies towards estrogen receptors, calmodulin, and PKC α. Under conditions where estrogen receptor immunoreactivity at ~80 kDa was seen for rat brain homogenates (mean

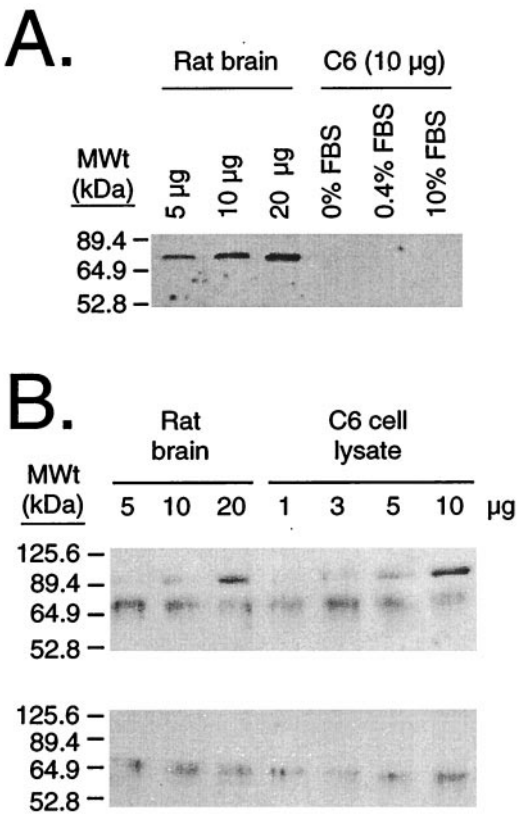

FIG. 2. (A) Western blot of estrogen receptor immunoreactivity for rat brain homogenate and C6 glioma cell lysates. (B) PKC α Western blot for rat brain homogenate and C6 glioma cell lysates. In the lower gel in the panel, the antibody was preincubated with PKC α synthetic peptide.

of two experiments), no immunoreactivity was found for C6 cell lysates, regardless of the FBS concentration used in their culturing (Fig. 2A). PKC α immunoreactivity was seen for both rat homogenate and C6 cell lysates with immunoreactive bands at ~70 and ~90 kDa being found (Fig. 2B). When the antibody was pretreated with a PKC α synthetic peptide, only the band at ~70 kDa was seen on the blot (Fig. 2B). A series of experiments was undertaken to determine whether PKC α expression was dependent upon the FBS concentration used in the culturing conditions. However, no consistent changes could be seen (data not shown).

C6 cells expressed calmodulin immunoreactivity at ~17 kDa (Fig. 3A). Similar molecular weight immunoreactive bands were seen using human calmodulin, and the immunoreactive signal increased as the amount of added calmodulin increased (Fig. 3A). A detailed analysis of the pixel intensities of a series of 17 gels to which 10, 30, and 50 ng calmodulin had been applied showed a reasonable linearity up to 30 ng calmodulin, after which the signal intensity (note that the intensity of the band, rather than its size, is measured) appeared to be saturated (Fig. 3, B and C). Since the absolute pixel intensities varied from gel to gel, we standardised the gels by expressing the pixel intensities as a fraction of the strongest band on each gel.

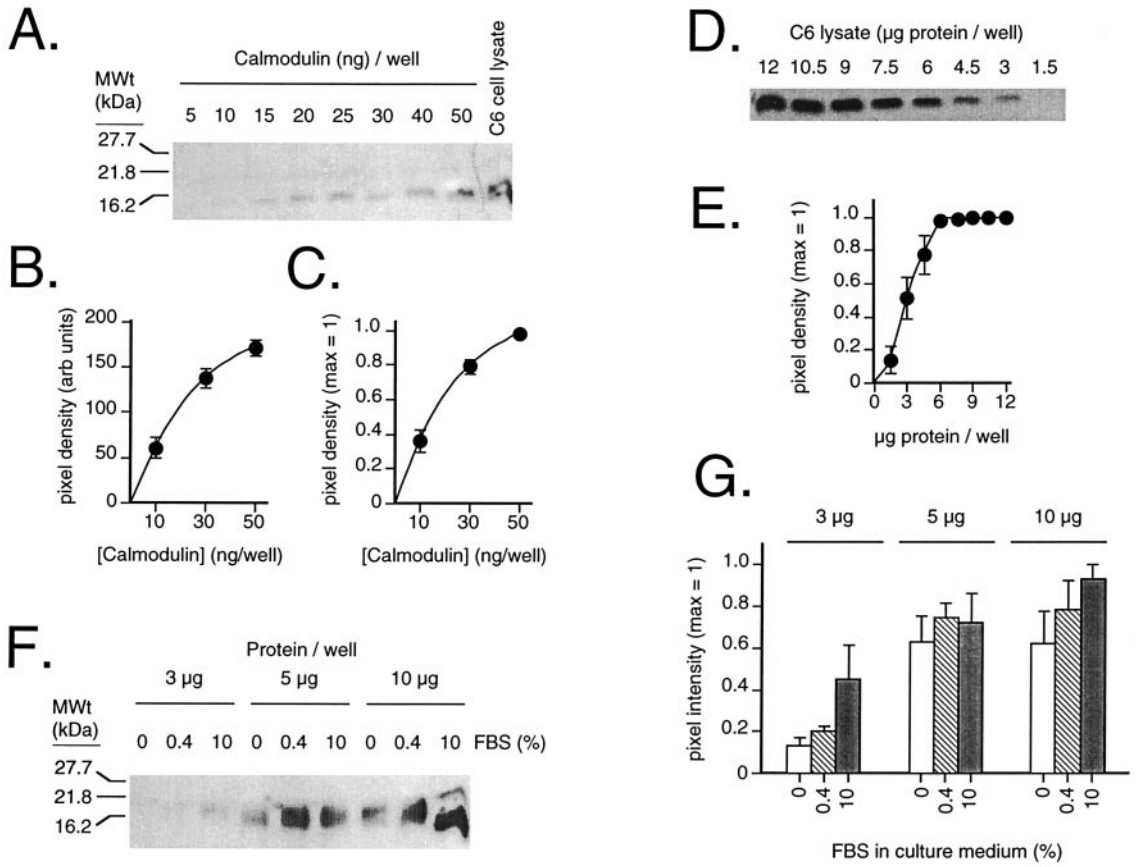

FIG. 3. (A) Western blot of calmodulin immunoreactivity for different concentrations of human calmodulin together with a sample (10 µg) of a C6 glioma lysate. (B and C) Analysis of pixel intensities of a series of gels run with different concentrations of human calmodulin. Data are means ± SEM, N = 17, of the pixel intensities either in arbitrary units (B) or normalised so that the strongest band on each gel was set to unity (C). For both Panels B and C, one-way ANOVA gave highly significant effects of protein loading ($F_{2,48} > 31$, $P < 0.0001$). (D) Western blot of calmodulin immunoreactivity for different applied protein concentrations of a lysate from C6 glioma cells cultured in 10% FBS. (E) Analysis of pixel intensities of a series of gels run with different protein loadings of lysates from C6 glioma cells cultured in 10% FBS. Data are means ± SEM, N = 3. One-way ANOVA indicated a significant contribution of protein concentration ($F_{7,16} = 22$, $P < 0.0001$). (F) Western blot of calmodulin immunoreactivity for samples (3, 5, and 10 µg) of lysates of C6 glioma cells cultured in 0, 0.4, and 10% FBS. (G) Quantification (means ± SEM) of pixel intensities of three separate lysates for each of the three FBS culturing conditions. The pixel intensities were normalised so that the strongest band on each gel was set to unity. Two-way ANOVA of the pixel densities measured at 3 and 5 µg protein loadings (the 10-µg densities being presumed to have saturated) gave $F_{1,12}$ (protein concentration) = 26, $P < 0.001$; $F_{2,12}$ (FBS) = 1.9, $P > 0.1$, $F_{4,12}$ (protein concentration × FBS) = 0.97, $P > 0.4$.

Protein-loading experiments were also undertaken with lysates of C6 cells cultured in 10% FBS (Fig. 3D). A good linearity with protein concentration of up to 6 µg/well was seen, after which the signal intensity was saturated (Fig. 3E). When C6 cells cultured in 0, 0.4, or 10% FBS were assayed for calmodulin immunoreactivity at protein loadings of 3, 5, and 10 µg/well, no significant differences were found (Fig. 3, F and G). The relatively small increase in pixel intensity for the bands as the protein content/well is increased from 5 to 10 µg presumably reflects saturation of the signal.

DISCUSSION

In the present study, the effects of cannabinoids and tamoxifen upon C6 glioma cell viability have been investigated at different FBS concentrations. Three such concentrations were used: 10%, the concentration generally used for culturing; 0.4%, a concentration reported to arrest glioma cell growth in Go ([15], although in our hands there was still cell growth as indicated by a time-dependent increase in the rate of MTT reduction in the wells); and serum-free. In agreement with the human glioblastoma study of Pollack *et al.* [5], the toxic potency of tamoxifen increased as the FBS content of the culturing medium was reduced. This would suggest that this phenomenon is a general property of glioma cells and not purely restricted to human glioblastoma cells.

The mechanism behind the increased sensitivity of C6 cells to tamoxifen as the FBS concentration is reduced is not known. Tamoxifen is by no means a specific estrogen receptor antagonist, and it has been suggested that its actions as a PKC inhibitor may be involved in its cytotoxicity [5, 8, 16–18]. Tamoxifen is also a calmodulin antag-

onist [17–19]. One possible explanation for the FBS concentration dependence of the toxicity is that there is a change in the concentration of one of these target proteins for tamoxifen. To investigate this possibility, the expression of estrogen receptors, calmodulin, and PKC α in C6 glioma cells cultured in different FBS was investigated. Estrogen receptors were not detected in the C6 glioma cells. In a study of the estrogen receptor expression in different brain tumours, only three of twenty gliomas investigated expressed estrogen receptors and all three were medulloblastomas [20]. In another study [21], three different human glioblastoma cell lines were examined and estrogen receptor expression was only found in one (U138MG), although other authors have reported this cell line to be estrogen receptor-negative [5, 8]. Thus, in C6 cells, tamoxifen produces cell toxicity via an estrogen receptor-independent pathway.

Expression of calmodulin and PKC α was investigated in the C6 cells cultured at the different FBS concentrations. There was no significant change in the concentration of calmodulin as the FBS concentration was reduced. PKC α was found to be expressed in the cells, but we were not able to demonstrate consistent changes as the FBS concentration was changed. However, Soma *et al.* [15] reported that PKC activity was high in C6 glioma cells arrested in Go by reduction of the serum concentration to 0.4% and was reduced upon addition of serum. Taken together, these data would suggest that changes in PKC activity rather than calmodulin content might underlie the changes in sensitivity to tamoxifen as the FBS concentration is changed.

In contrast to the robust effects of tamoxifen upon C6 cancer cell proliferation, the effects of the cannabinoids were rather modest. Consistent with the study of Sanchez *et al.* [9], Δ^9-THC reduced C6 glioma cell viability in serum-free medium by 6 days of incubation. This reduction was also seen with cannabidiol, but not with the endogenous cannabinoid AEA. The effect was dependent upon the FBS concentration of the culture medium, and no effects of any of the cannabinoids were seen at 10% FBS. Thus, the effect of Δ^9-THC upon cell viability, which has been suggested to be due to a cannabinoid CB1 receptor-independent stimulation of sphingomyelin breakdown [9], shares with tamoxifen the property that it is dependent upon the FBS present in the culture medium. The relatively weak effect of Δ^9-THC would at first sight argue against the possible use of cannabinoids as a treatment for glioma. However, a recent study has reported that intratumoural injection of Δ^9-THC for 7 days via osmotic pumps increased survival time in 9/15 rats and eradicated the tumour in a further 3/15 rats inoculated with C6 glioma cells [22]. This finding, together with reports that Δ^9-THC causes apoptosis in human prostate cancer cells [23], that activation of cannabinoid receptors prevents the mitogenic effects of prolactin and nerve growth factor in human breast and prostate cancer cells [24–26], and that cannabinoids have effects towards nausea induced by chemotherapy [27] underlines the need for further investigation into the possible therapeutic usefulness of these compounds.

Tamoxifen, in addition to affecting glioma cell viability, has been reported to affect the sensitivity of glioma cells to the deleterious effects of other treatments [6–8]. Thus, for example, in human glioblastoma cells, tamoxifen increases the sensitivity of the cells to γ-radiation and to BCNU (1,3-bis (2-chloroethyl)-1-nitrosourea), whereas the sensitivity to etoposide is unchanged [8]. In the present study, our aim was to determine whether the combination of tamoxifen and cannabinoids could reduce C6 glioma cell viability more effectively than either treatment per se. The rather dramatic "all or nothing" effect of tamoxifen, however, made assessment of synergistic effects with cannabinoids difficult. Nevertheless, there was no evidence that a threshold concentration of tamoxifen either increased the effects of the cannabinoids upon cell viability or caused these effects to appear earlier.

In conclusion, this study has demonstrated that the FBS-dependent effects of tamoxifen are also found for rat glioma cells and are seen with Δ^9-THC; that the effects of Δ^9-THC are also seen with cannabidiol but not with AEA; and that no synergy between tamoxifen and cannabinoids can be observed.

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NOTE ADDED IN PROOF

In a newly published study, Maccarrone *et al.* (*J Biol Chem* **275:** 31938–31945, 2000) reported that anandamide produces apoptotic body formation and DNA fragmentation in human CHP100 neuroblastoma and U937 lymphoma cells. This effect, which they did not see in C6 glioma cells, was potentiated by inhibtors of anandamide uptake and metabolism, and was mediated at least in part by ranilloid rather than cannabinoid receptors.

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