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Taurine Might Be Acting as a Trophic Factor in the Retina by Modulating Phosphorylation of Cellular Proteins

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Protein phosphorylation is involved in the regeneration of the nervous system. Taurine modulates the phosphorylation of specific proteins in the retina, and also increases outgrowth from ganglion cells. In order to test the possible role of protein phosphorylation on the outgrowth from the retina and on the trophic effect of taurine, in vitro studies were performed in the presence of phorbol and nonphorbol protein kinase C activators, the protein kinase C inhibitor tamoxifen, and phosphatase inhibitors. After crush of the optic nerve, explants of the goldfish retina were cultured and the outgrowth was evaluated by measuring the length and the density of neurites. The activation of protein kinase C decreased the outgrowth from the explants and impaired the stimulatory effect of taurine. Phosphatase inhibitors produced a similar effect on basal and taurine-modulated outgrowth. In certain concentrations, some of these drugs did not affect the emission of neurites in the absence of taurine, but decreased the effect of the amino acid. Tamoxifen also reduced the outgrowth, probably acting at other cellular levels or indicating that the regulation of outgrowth by phosphorylation is a complex and dual process. The response to the drugs, evaluated by length or density of fibers, was not the same, since rate of outgrowth was more affected than density, which suggests that both parameters are modulated at differential stages or sensitivities to the tested agents. J. Neurosci. Res. 53:377–384, 1998. © 1998 Wiley-Liss, Inc.

Key words: outgrowth; phosphatases; protein kinase C; retina; taurine

INTRODUCTION

Taurine affects the phosphorylation of specific proteins in rat retina, brain, and heart (Lombardini and Props, 1996). This amino acid primarily inhibits the phosphorylation of a ≈20-kDa protein in the retina (Lombaridini, 1993), but could be a stimulator of protein phosphorylation in the mitochondrial fraction of this structure (Lombardini, 1992). It has been recently demonstrated that this protein could correspond to histone H2B (Lombardini, 1997). In addition, other groups have shown the effect of taurine on protein phosphorylation in some tissues, such as the cat cortex (Sturman and Gargano, 1990) and the rat heart (Schaffer et al., 1990). Taurine plays a role as a trophic agent in the postcrush retina (Lima et al., 1988; Matus et al., 1997), an effect partially mediated by increases in calcium flux (Lima et al., 1993). It could be that this amino acid affects retinal outgrowth in relation to or by direct modifications of protein phosphorylation.

Protein phosphorylation has been shown to be involved in the regeneration of the nervous system. The developmentally downregulated phosphorylated isoform of microtubule-associated protein 1B is present in the regenerating sciatic nerve of the mouse, but not of the rat, probably indicating a relation with a continual capacity for growth and remodelling in mice (Busch et al., 1996). Moreover, there is a correlation between axonal growth and expression of this protein in spinal nerves of the mouse in vitro (Tonge et al., 1996). Although protein kinase C (PKC) has been related to initiation of outgrowth (Cambray-Deakin and Burgoyne, 1990), and to distal influence on neurites from ganglion cells (Jian et al., 1994) probably the general effect, as demonstrated by the use of the activator of PKC, tetradecanoyl phorbol acetate (TPA), in the goldfish retina (Jian et al., 1994), could be a proximal negative influence on the regeneration of the optic nerve. Other examples concerning the relevance of the balance of phosphoproteins in regeneration are that the phosphorylation of neurofilaments might be related to the stiffness of the neurite tip promoting its advance (Pijak et al., 1996); the protein kinase inhibitor

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KT5926 prevents the nerve growth factor-promoted responses in PC12 cells (Teng and Greene, 1994), the upregulation of PKC β-isoform taking place in mouse sciatic nerve after injury (Wiklund et al., 1996); the inhibition of PKC results in regeneration of the filamentous network which accumulates in neurodegenerative disease (Carter et al., 1996), and the protein phosphatase (PP) inhibitor, okadaic acid, inhibits the in vitro regeneration of adult frog sciatic nerve (Svensson et al., 1995). Although specific substrates are not identified in most of the studies, the concentration and the phosphorylation of growth-associated protein, GAP-43, by PKC increase after wounding of the rabbit corneal epithelium (Lin and Bazán, 1995).

Twelve identified PKC isoenzymes have been shown in mammals, and up to eight are present in the vertebrate retina (Wood et al., 1997), displaying variable functions (Pagh-Roehl et al., 1995; Wood et al., 1997). PKC, α-isoform, has been localized in the rod bipolar cells of the mouse and the goldfish (Vaquero et al., 1996), and in the rabbit (Casini et al., 1996). The subspecies α, β, and γ (Osborne et al., 1992), and ϵ, δ, and ζ (Osborne et al., 1994) are differentially distributed in mammalian and nonmammalian retina. Interestingly, the density of PKC-immunoreactive cells of rabbit retina shows a peak at postnatal day 11 followed by a drastic decrease up to adulthood (Casini et al., 1996), and chelerythrine, a specific PKC inhibitor, blocks the antiproliferative effects of transforming growth factor β-2 and of a metabotropic glutamate receptor agonist on human Müller cells (Ikeda and Puro, 1995).

The modulation of the phosphorylation of specific proteins by taurine (Lombardini and Props, 1996) together with the quoted and considerable reports on the role of PKC in regeneration, evaluates the relevance of the phosphorylation stage in the mechanism of action of taurine as a trophic agent. The aims of this study were: (1) to explore the effects of PKC activators or inhibitor, and PP inhibitors on the basal outgrowth from the postcrush goldfish retina; (2) to determine the possible effect of inhibitors of calcium-calmodulin kinase II (CCMK II) in this process; and (3) to better understand the mechanism of action of taurine as a regenerating substance in the retina.

MATERIALS AND METHODS

Animals

Goldfish (*Carassius auratus*), 4–6 cm in length were adapted to darkness for 30 min and anesthetized in 0.05% tricaine (Sigma, St. Louis, MO) prior to dissection of the retina. The lesion of the optic nerve was performed 10 days before explantation, by pulling the eye forward of the orbit and crushing the nerve with fine forceps (Lima et al., 1988).

Preparation of Explants and Culture Conditions

The retina of fish was dissected, placed in a small volume of culture medium, and chopped in a McIlwain tissue chopper into 500-μm squares (Lima et al., 1988). Explants were placed (10–15 per dish) in poly-L-lysine (Sigma) precoated culture flasks. Nutrient medium (3 ml per dish) was Leibovitz (L-15, Gibco, Grand Island, NY), 20 mM HEPES, 10% fetal calf serum, and 0.1% w/v gentamicin (Sigma). Cytosine arabinose (Sigma, 2 mM) was added 24 hr after plating.

Culture in the Presence of Protein Kinase Activators or Inhibitor and of Phosphatase Inhibitors

The postcrush explants were cultured in the absence or in the presence of 4 mM taurine (Sigma), and the PKC activators: phorbol 12,13-dibutyrate, PDBu, 0.05–50 μM (Hortelano et al., 1992); N-n-heptyl-5-chloro-1-naphthalenesulfonamide, SC-10, 0.05–10 μM (Coderre, 1992); (−)-7-octylindolactam, OCL, 1–50 nM (Lin and Chuang, 1993), and the negative control 4-α-phorbol 12-myristate 13-acetate, 4-α-PMA, 0.01–10 μM; the PKC inhibitor tamoxifen, 1–250 μM (Issandou et al., 1990); or the PP inhibitors, okadaic acid, OKA, 0.01–100 nM, and calyculin A, CAL, 0.1–2 nM (Cohen, 1989). Also, the inhibitor of CCMK II, (2-N-(4-methoxybenzenesulfonyl)hydroxyamino-N-(4-chlorocinnamyl)-N-methylbenzyl-amine), KN93, 1 μM (Mamiya et al., 1993) and its negative control (2-N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzyl-amine, phosphate), KN92, 1 μM were tested. All were purchased from Research Biochemicals International (Natick, MA), except PDBu, OCL, KN93, and KN92, obtained from Calbiochem (La Jolla, CA). The drugs were dissolved in dimethyl-sulfoxide, final maximal concentration 0.07%. Viability was determined by exclusion of Trypan blue in control and taurine-added cells after 5 days in culture, as well as in the presence of PDBu, 0.1 μM; SC-10, 0.1 μM; OCL, 5 nM; tamoxifen, 5 μM; OKA, 0.1 nM; and CAL, A 1 nM. Control explants, those cultured in the absence of taurine, and explants in the presence of taurine, without any additional drug were included in each group of experiments in order to make the adequate comparison, since basal length of neurites and percentage of stimulation with taurine could vary between group of animals.

Evaluation of Outgrowth

The outgrowth of postcrush retinal explants was determined after 5 and 10 days in culture. The length of the neurites (20–30 per explant or less, if the number of neurites was lower) was measured in μm with an ocular micrometer. The neurite to be measured was evaluated from the border of the explant and was followed up to the

maximal length reached, which could include the fusion of fibers also emitted by the evaluated explant. The number of fibers per explants was defined as the density of neurites in a scale of 0–4.

Statistical Analysis

Each value is expressed as mean ± standard error. Two-tailed analysis of variance (ANOVA) was used for comparison. The probabilities of the differences between means were derived from the ANOVA (Barlow, 1983).

RESULTS

Effect of Protein Kinase C Activators

In Figure 1 are shown the results with the activators of PKC. The length of the neurites from the explants was increased by the addition of taurine to the medium; also, the density of fibers was elevated by the amino acid (Table I). Five days after plating, the phorbol ester PKC activator, PDBu, did not change the length of neurites in the absence or in the presence of taurine at low concentration, but higher concentration decreased both (Fig. 1A). The nonphorbol activator of PKC, SC-10, did not alter the length of neurites in the range of concentrations used, and significantly decreased the trophic effect of taurine (Fig. 1B). The other nonphorbol activator of PKC used, OCL, did decrease the elevation of neurite length produced by taurine at low concentration without affecting basal outgrowth, and diminished the rate of emission of neurites at higher concentrations in both cases (Fig. 1C). The inactive phorbol ester 4-α-PMA at low concentration did not modify the outgrowth from control or taurine-supplemented explants. At 10 days in culture, PDBu and OCL at the higher concentration tested, did not have a significant effect on the basal outgrowth, and all three PKC activators inhibited the trophic effect of taurine (Fig. 4). Concerning the density of fibers in control or taurine-supplemented explants, none of the PKC activators modified it at 5 days in culture, but SC-10 reduced it in control explants at 10 days (Table I). Only OCL inhibited the effect of taurine at 10 days after plating (Table I). Viability (80–87%) did not significantly differ between control, taurine- or drug-added cells after 5 days in culture. The results of explants cultured in the absence or in the presence of taurine were pooled from several experiments: length of neurites in μm, control 55.71 ± 1.30 (n = 430), and taurine 68.33 ± 1.50 (n = 409), $P < 0.001$; density of neurites, control 2.69 ± 0.09 (n = 88), and taurine 3.22 ± 0.08 (n = 82), $P < 0.001$.

Effect of the Protein Kinase C Inhibitor Tamoxifen

Tamoxifen did not modify the emission of neurites in concentrations of 1 and 2 μM, but decreased the effect of taurine (Fig. 2). At 10 μM, it also decreased the basal outgrowth. Concentrations higher than 50 μM completely inhibited the outgrowth from the explants.

TABLE I. Density of Neurites From Goldfish Retinal Explants Cultured in the Presence or in the Absence of Taurine†

Culture condition	Time in culture (days)	
	5	10
CTR	2.60 ± 0.24 (81)	2.96 ± 0.22 (72)
Tau	3.34 ± 0.21 (70)*	3.30 ± 0.20 (60)
PDBu CTR	2.50 ± 0.32 (12)	2.13 ± 0.25 (15)
Tau	3.20 ± 0.22 (15)	3.77 ± 0.25 (13)
SC-10 CTR	2.19 ± 0.24 (16)	1.93 ± 0.15 (15)*
Tau	2.93 ± 0.26 (15)	2.86 ± 0.20 (14)
OCL CTR	2.86 ± 0.27 (14)	2.57 ± 0.26 (14)
Tau	2.50 ± 0.26 (14)	1.79 ± 0.18 (14)**
OKA CTR	2.19 ± 0.16 (16)	1.90 ± 0.20 (10)
Tau	2.31 ± 0.15 (16)**	2.90 ± 0.22 (10)
CAL CTR	2.82 ± 0.28 (11)	3.26 ± 0.15 (19)
Tau	1.79 ± 0.15 (14)**	2.38 ± 0.25 (16)

†Each value is the mean ± S.E.M. Number in parentheses = n. CTR, explants in the absence of taurine. Tau, explants in the presence of 4 mM taurine. PKC activators, 5 μM PDBu, 10 μM SC-10, and 50 nM OCL; phosphatase inhibitors, 0.5 nM OKA, and 2 nM CAL, as indicated in Materials and Methods.
*$P < 0.05$ with respect to CTR.
**$P < 0.05$ with respect to Tau.

Effect of Phosphatase Inhibitors

The effect of the two PP inhibitors tested, OKA and CAL, is shown in Figure 3. OKA, in a concentration range mainly affecting PP-2A, did not produce any significant modification in neurite length; however, it decreased the stimulatory effect of taurine at the two higher concentrations employed (Fig. 3A). By contrast, CAL, in a range inhibiting PP-1 and -2A, diminished the outgrowth of the explants in the absence and in the presence of taurine (Fig. 3B). After 10 days in culture, the length of neurites was reduced by both inhibitors of PPs in the presence or in the absence of taurine (Fig. 4). Neither OKA nor CAL affected the density of fibers at 5 or 10 days in culture, but both decreased the effect of taurine at 5 days after plating (Table I).

Effect of the Inhibition of Calcium-Calmodulin Kinase II

The inhibitor of CCMK II, KN93, did not reduce the outgrowth either in the presence or in the absence of taurine when compared to control or taurine alone, but explants supplemented with the amino acid and KN93 were not significantly different from those in the presence of only KN93 (Fig. 5). The analog KN92, employed as a negative control, did not have any effect on the emission of neurites from the explants.

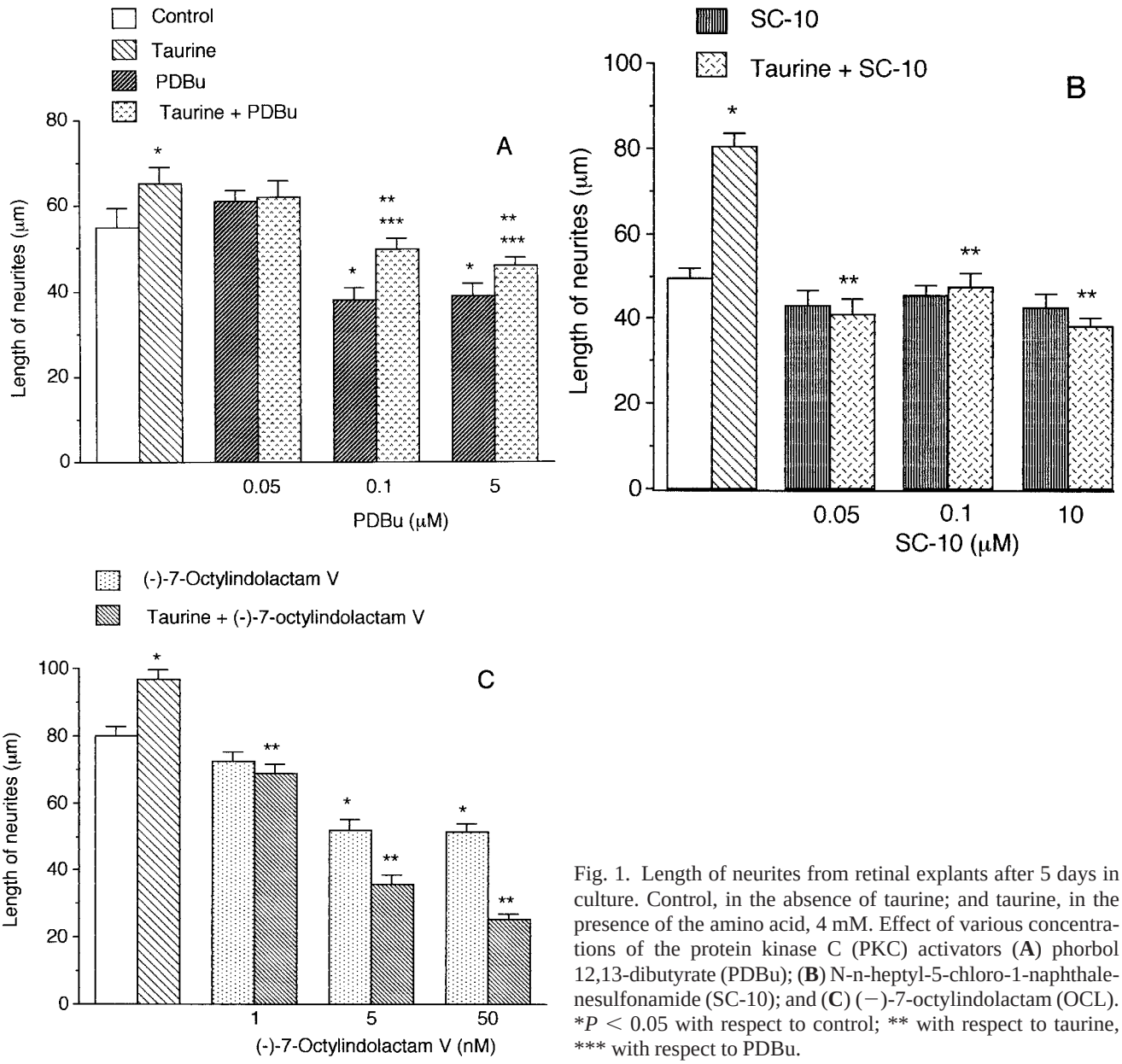

Fig. 1. Length of neurites from retinal explants after 5 days in culture. Control, in the absence of taurine; and taurine, in the presence of the amino acid, 4 mM. Effect of various concentrations of the protein kinase C (PKC) activators (**A**) phorbol 12,13-dibutyrate (PDBu); (**B**) N-n-heptyl-5-chloro-1-naphthalenesulfonamide (SC-10); and (**C**) (−)-7-octylindolactam (OCL). *$P < 0.05$ with respect to control; ** with respect to taurine, *** with respect to PDBu.

DISCUSSION

The activation of PKC by the phorbol ester PDBu and by other nonphorbol stimulators of the enzyme indicate the relevance of phosphorylation for outgrowth from the goldfish retina. It seems that an increase in the phosphorylated stage of certain unidentified substrates impairs the rate of emission of neurites. In addition, the trophic effect of taurine was reduced by the activator PDBu. Interestingly, SC-10 did not modify the outgrowth in the absence of taurine, but it reduced the effect of taurine. This might be an indication of the role of phosphorylation in the trophic effect of the amino acid, since basal outgrowth could be maintained but further increase was not allowed. OCL also reduced the basal outgrowth when used at high concentration, but low concentration only affected the outgrowth in the presence of taurine. The magnitude of the effects obtained with these activators was greater in taurine-stimulated than in control explants, which might be evidence of multiple steps of modulation in basal outgrowth, and a probable specific cascade activated by the amino acid. The role of taurine in protein phosphorylation has been extensively studied by Lombardini (1992, 1997), although there are some phosphoproteins in which phosphorylation increases with taurine (Lombardini, 1992); the most documented example concerns the ≈20-kDa phosphoprotein

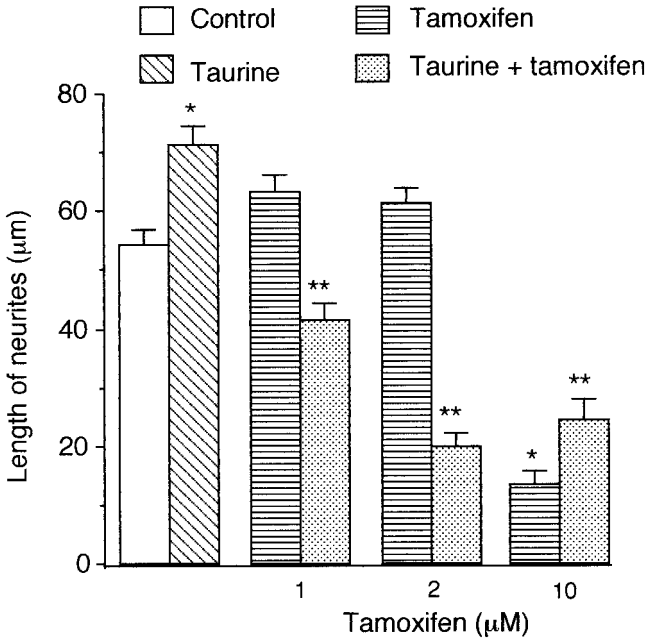

Fig. 2. Length of neurites from retinal explants after 5 days in culture. Control, in the absence of taurine; and taurine, in the presence of the amino acid, 4 mM. Effect of various concentrations of the PKC inhibitor tamoxifen. *$P < 0.05$ with respect to control; ** with respect to taurine.

in which phosphorylation is reduced by this amino acid or is increased by the treatment with taurine-reducing agents, such as guanidinoethane sulfonate (Lombardini, 1997).

Several examples in the literature indicate that the effect of PKC activators of a different nature is dependent not only on the concentration used, but also on the time of exposure. For instance, the activator of PKC 12-myristate 13-acetate (PMA), downregulates serotonin transporter in choriocarcinoma cells exposed to the drug for short periods of time, and increases it when incubation time is longer (Ramamoorthy et al., 1995). Thus, some reports contemplate controversial effects of PKC activators or inhibitors related to different steps in cellular processes.

The balance in phosphorylating-dephosphorylating reactions is critical for regeneration of peripheral nerves. The inhibition of protein kinases prevents induced outgrowth by nerve growth factor, without affecting the basal outgrowth in PC12 (Teng and Greene, 1994); however, OKA in a moderate concentration increases phosphorylation levels and decreases the in vitro regeneration of adult frog sciatic nerve (Svensson et al., 1995). Besides the increasing proof of the relevance of phosphoproteins in regeneration of the nervous system, the identification of those molecules playing specific roles in neurite growth and elongation is still a problem to be solved. The characterization of substrates involved in taurine effect and PKC could be histones, since taurine

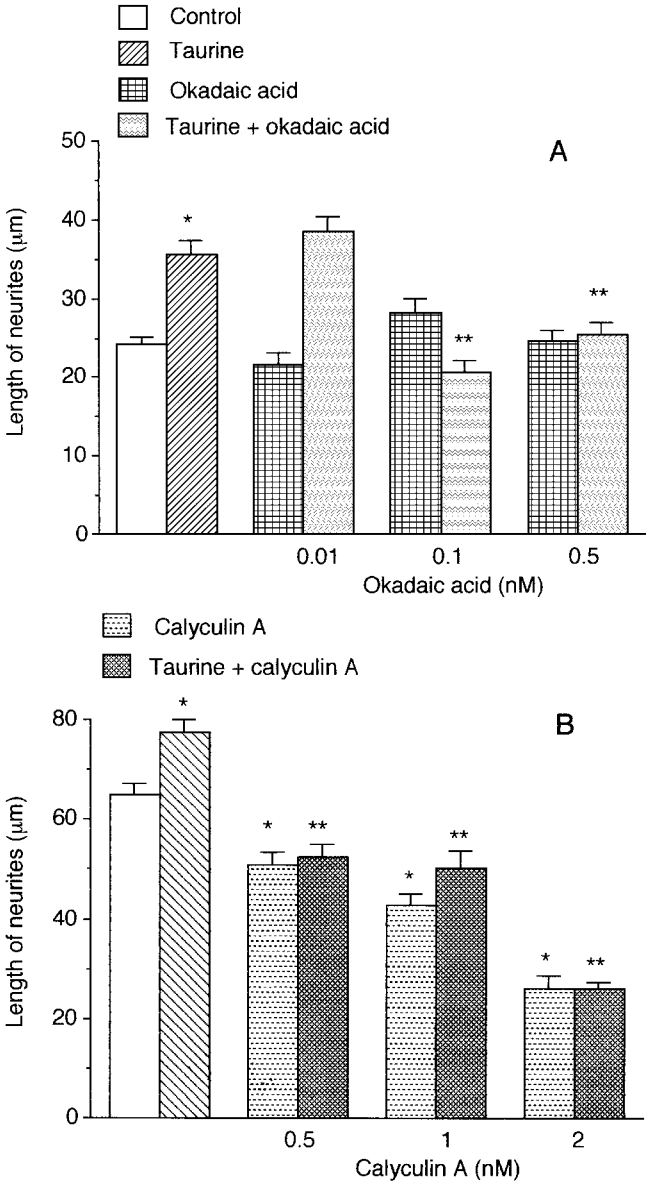

Fig. 3. Length of neurites from retinal explants after 5 days in culture. Control, in the absence of taurine; and taurine, in the presence of the amino acid, 4 mM. Effect of various concentrations of the phosphatase inhibitors (**A**) okadaic acid (OKA), and (**B**) calyculin A (CAL). *$P < 0.05$ with respect to control; ** with respect to taurine.

apparently reduces the phosphorylation of histone H2B (Lombardini, 1997). In the periphery, for instance, the nuclear histone-H1 kinase activity and the amount of phosphate bound to it biphasically increase after partial hepatectomy (Takada et al., 1994).

The results with the PP inhibitor OKA, according to the range of variation in the IC_{50} for PP-1 (10–60 nM) and for PP-2A (0.1–1 nM; Cohen, 1989; Cohen et al., 1990), indicate that PP-2A does not seem to be involved in the

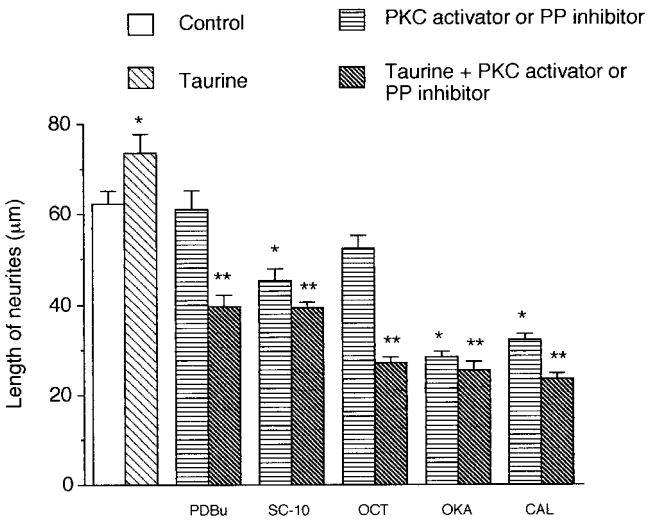

Fig. 4. Length of neurites from retinal explants after 10 days in culture. Control, in the absence of taurine; and taurine, in the presence of the amino acid, 4 mM. Effect of the PKC activators phorbol 12,13-dibutyrate (PDBu) 1 μM, N-n-heptyl-5-chloro-1-naphthalenesulfonamide (SC-10) 10 μM, and (−)-7-octylindolactam (OCL) 50 nM, and of the phosphatase inhibitors okadaic acid (OKA) 0.5 nM, and calyculin A (CAL) 2 nM. *$P < 0.05$ with respect to control; ** with respect to taurine.

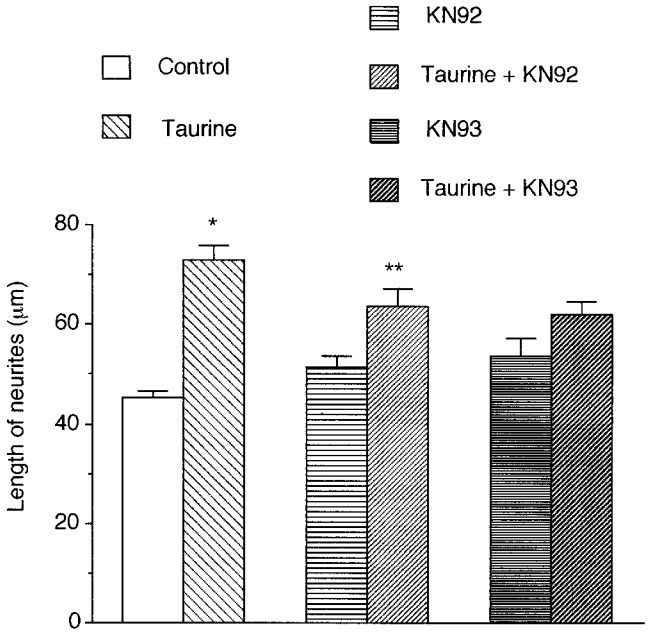

Fig. 5. Length of neurites from retinal explants after 10 days in culture. Control, in the absence of taurine; and taurine, in the presence of the amino acid, 4 mM. Effect of the CCMK II inhibitor 2-N-(4-methoxybenzenesulfonyl)hydroxy-amino-N-(4-chlorocinnamyl)-N- methylbenzyl-amine (KN93) 1 μM, and the analogue negative control KN92 1 μM. *$P < 0.05$ with respect to control; ** with respect to taurine.

dephosphorylation of substrates related to basal outgrowth, but could play a role in the trophic effect of taurine. According to this observation are the results with CAL, in which ranges of selectivity for PP-1 and PP-2A are closer to each other than those of OKA. This inhibitor, probably affecting both PP, inhibited control and taurine-supplemented explant outgrowth. PP-1 and -2A are known to be present in bovine retina (Bugnon et al., 1995) and also a number of different PPs are expressed in rat Müller cells (Shock et al., 1995). In teleost, the elongation of rod inner-outer segments is modulated by PP-1 and PP-2A (Pagh-Roehl et al., 1995).

Outgrowth of control explants occurred between 5 and 10 days in the presence of PKC activators or PP inhibitors, but emission of neurites from explants cultured in the presence of taurine was arrested. This might indicate that there is a critical period of time during which taurine affects outgrowth, which was previously shown by time course studies (Lima et al., 1988). In addition, it seems that PPs play a more determinant role than PKC activity in vitro, since there was no further increase of sprouting at longer periods of time either in control or in taurine-supplemented explants.

Controversial results on the effect of PP activity modifications have been reported. OKA is a potent tumor promoter, acting as a phosphatase inhibitor (Haystead et al., 1989); however, OKA and CAL inhibit the glucocorticoid-induced apoptosis in murine T cell hybridomas (Ohoka et al., 1993) and that induced in human leukemia cells by diverse agents (Song et al., 1992). In other types of leukemia cell lines, such as IPC, OKA triggers apoptosis (Gjertsen et al., 1994) and in a line of Burkitt's lymphoma cells, CAL prevents it (Song and Lavin, 1993). Some of these effects are produced with doses ranging from 50 nM to 1 μM, and for this reason could be mediated through mechanisms independent of inhibition of PPs. For instance, OKA and CAL can also produce translocation of PKC from cytosol to membrane in doses higher than those required for inhibition of PPs (Gopalakrishna et al., 1992).

Tamoxifen, 10 μM, has been also shown to be cytocidal in gliomas, by inducing apoptosis (Couldwell et al., 1994). This antiestrogen drug posseses growth-inhibitory properties at μM concentration and might be related in part to its effect on PKC (O'Brian et al., 1985). However, other authors indicate that the antiproliferative activity of tamoxifen could not be attributed to inhibition of PKC, and that it could stimulate the phosphorylation of a specific protein in MCF-7 cells (Issandou et al., 1990). The results with tamoxifen were not expected, but since it is a hormone analog its effect could be exerted by affecting other pathways. Similarly, paradoxical effect of chelerythrine has been reported in the rat retina, which

stimulates phosphorylation instead of reducing it (Lombardini, 1995). More specific PKC inhibitors must be used in the future.

As was shown for Jian et al. (1994) with the CCMK II inhibitor KN62, which did not modify the growth cone from goldfish retinal explants, the similar inhibitor, KN93, only produced a slight reduction in the stimulatory proximal effect of taurine, indicating a small influence if any of CCKM II in this system.

When the role of PKC or PPs are questions of interest in defining a mechanism of action, more than one of the valuable available drugs with different structures must be tested; also, care should be taken in relation to doses and time of exposure. The present results support the necessity of a proper balance of phosphorylation-dephosphorylation during optic nerve outgrowth in vitro; also length of neurites and density seem to be regulated separately, responding differentially to the drugs tested. Moreover, the modifications of taurine trophic effect suggests that taurine might be acting as a stimulator of outgrowth by reducing the phosphorylation of specific proteins to be identified.

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