Molecular Neurobiology

ISSN0893-7648/97/15(3): 285–306/$13.50

FK506 and the Role of Immunophilins in Nerve Regeneration

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Abstract

FK506 is a new FDA-approved immunosuppressant used for prevention of allograft rejection in, for example, liver and kidney transplantations. FK506 is inactive by itself and requires binding to an FK506 binding protein-12 (FKBP-12), or immunophilin, for activation. In this regard, FK506 is analogous to cyclosporin A, which must bind to its immunophilin (cyclophilin A) to display activity. This FK506-FKBP complex inhibits the activity of the serine/threonine protein phosphatase 2B (calcineurin), the basis for the immunosuppressant action of FK506. The discovery that immunophilins are also present in the nervous system introduces a new level of complexity in the regulation of neuronal function. Two important calcineurin targets in brain are the growth-associated protein GAP-43 and nitric oxide (NO) synthase (NOS).

This review focuses on studies showing that systemic administration of FK506 dose-dependently speeds nerve regeneration and functional recovery in rats following a sciatic-nerve crush injury. The effect appears to result from an increased rate of axonal regeneration. The nerve regenerative property of this class of agents is separate from their immunosuppressant action because FK506-related compounds that bind to FKBP-12 but do not inhibit calcineurin are also able to increase nerve regeneration. Thus, FK506's ability to increase nerve regeneration arises via a calcineurin-independent mechanism (i.e., one not involving an increase in GAP-43 phosphorylation). Possible mechanisms of action are discussed in relation to known actions of FKBPs: the interaction of FKBP-12 with two Ca^{2+} release-channels (the ryanodine and inositol 1,4,5-triphosphate receptors) which is disrupted by FK506, thereby increasing Ca^{2+} flux; the type 1 receptor for the transforming growth factor-β (TGF-β1), which stimulates nerve growth factor (NGF) synthesis by glial cells, and is a natural ligand for FKBP-12; and the immunophilin FKBP-52/FKBP-59, which has also been identified as a heat-shock protein (HSP-56) and is a component of the nontransformed glucocorticoid receptor.

Taken together, studies of FK506 indicate broad functional roles for the immunophilins in the nervous system. Both calcineurin-dependent (e.g., neuroprotection via reduced NO formation) and calcineurin-independent mechanisms (i.e., nerve regeneration) need to be invoked to explain the many different neuronal effects of FK506. This suggests that multiple immunophilins mediate FK506's neuronal effects. Novel, nonimmunosuppressant ligands for FKBPs may represent important new drugs for the treatment of a variety of neurological disorders.

Index Entries: Calcineurin; cyclosporin A; FK506; FKBP-12; glucocorticoid receptor; immunophilin; immunosuppressant; nerve regeneration.

The Current Status of Drugs for Nerve Regeneration

The development of new therapeutics to augment nerve regeneration is an area of intense research activity. Some of the agents that have been experimentally examined are the melanocortins, adrenocorticotrophic hormone (ACTH) and α-melanocyte-stimulating hormone (α-MSH) (Bijlsma et al., 1981; 1984; De Koning and Gispen, 1987a; Edwards et al., 1985; Sporel-Özakat et al., 1990; Strand and Kung, 1980; Strand and Smith, 1986), the trisubstituted ACTH analog Org 2766 (Sporel-Özakat et al., 1990; De Koning and Gispen, 1987b), testosterone (Jones, 1993), uridine in experimental diabetic neuropathy (Gallai et al., 1992), gangliosides in diabetic animals (Ekström and Tomlinson, 1990), insulin and insulinlike growth factor (Ekström et al., 1989; Kange et al., 1989; Roth et al., 1995), isaxonine (Sebille et al., 1982), and the long chain fatty alcohol *n*-hexacosanol (Starr et al., 1996). Limited clinical trials have been reported with several of these agents. The most widely lauded of these are the gangliosides. However, in those countries where clinical trials have been undertaken (e.g., Britain, Italy, but not the United States), the use of gangliosides has been discouraged or banned because of the development of Guillain-Barré syndrome in a small percentage of patients (Schonhofer, 1991; Figueras et al., 1992; Landi et al., 1993). Similarly, isaxonine (Nerfactor), was withdrawn from clinical trials because of hepatotoxicity (Letteron et al., 1984). Org 2766 has been tried in a limited number of patients, the results to-date being equivocal (Gorio et al., 1993). One agent currently in clinical use that has been shown to speed functional recovery following a sciatic-nerve crush lesion is nimodipine (Gispen et al., 1991), a dihydropyridine calcium antagonist. However, results obtained from animal studies on its regenerative properties have not been impressive; although regeneration begins slightly earlier, ultimately, there was no difference from untreated animals in the time needed to achieve full recovery (Angelov et al., 1996). Furthermore, the use of this agent for the treatment of nerve regeneration could be limited by possible untoward cardiovascular effects (Murad, 1990). Thus, the search continues for an agent that can be used to enhance nerve regeneration in humans without adverse side effects.

FK506: Historical Background

FK506 is a new FDA-approved immunosuppressant macrolide drug isolated from *Streptomyces tsukubaensis* (Kino et al., 1987a,b) and used for organ transplantations (Hoffman et al., 1990; Starzl et al., 1987, 1989). The drug was discovered and isolated in 1984 from a soil sample obtained from Tsukuba, Japan, upon screening microbial fermentation broths using a mixed lymphocyte reaction (Kino and Goto, 1993). The first experimental study of FK506 on cardiac transplantation in rats was reported in 1987 by Ochiai and coworkers (Ochiai et al., 1987). Clinical trials, pioneered by Thomas Starzl's group at the University of Pittsburgh began in February 1989 (Starzl et al., 1987). Over the years, FK506 has been shown to possess two important properties that make it superior to cyclosporin A, currently the most widely employed drug for preventing allograft rejection. First, FK506 is a more potent immunosuppressant (by approx 10X) than cyclosporin A in vitro (Kino et al., 1987a,b; Tocci et al., 1989), in animals (Ochiai et al., 1987; Murase et al., 1987; Ochiai et al., 1988; Sakai et al., 1991; Todo et al., 1987, 1988, 1989), and in humans, leading to fewer instances of rejection and retransplantation (Hoffman et al., 1990; Starzl et al., 1989; McDiarmid et al., 1995). Second, initial reports indicated that its toxicity in humans was far less than that associated with cyclosporin A (Starzl et al., 1989; Shapiro et al., 1990). Whereas the incidence of toxicity has subsequently been found to vary among transplant centers (Klintmalm, 1994; Neuhaus et al., 1994), including the development of moderate-to-severe neurotoxicity (including cortical blindness, tremor, seizures,

and encephalopathy) in anywhere from 3–21% of patients (Lopez et al., 1991; Mueller et al., 1994; Wijdicks et al., 1994; Bronster et al., 1995; Vincenti et al., 1996), this discrepancy has been attributed to the tendency to overdose with FK506 (Fung et al., 1996). Consequently, FK506 is beginning to replace cyclosporin A as the drug of choice in the treatment of allograft rejection.

FK506: Overview of Neuronal Properties

Whereas its immunosuppressive effects alone make this an important new clinical drug, FK506 has also been found to possess a variety of neuronal properties, including protection against ischemic brain injury (Sharkey and Butcher, 1994; Ide et al., 1996; Tokime et al., 1996; Butcher et al., 1997) and glutamate neurotoxicity in vitro (Dawson et al., 1993) (although a very recent study [Butcher et al., 1997] did not substantiate protection against glutamate toxicity in vivo), prevention of *N*-methyl-D-aspartate (NMDA)-receptor desensitization (Tong et al., 1995), prevention of kindling (Moriwake et al., 1996) blockade of long-term potentiation (LTP) and long-term depression (LTD) in the visual cortex (Torii et al., 1995; Funauchi et al., 1994), facilitation of LTP (Ikegami et al., 1996) and blockade of LTD in the rat hippocampus (Hodgkiss and Kelly, 1995), and alteration in neurotransmitter release (Steiner et al., 1996) and endocytosis (Kuromi et al., 1997). In regard to the latter, whereas LTP and LTD have not been proven to underlie learning and memory; it may be relevant that cyclosporin A also inhibits memory formation in day-old chicks (Bennett et al., 1996). It is likely that all these FK506 neuronal properties are mediated by calcineurin inhibition. For example, calcineurin is known to regulate NMDA-receptor desensitization (*see* Tong et al., 1995). Furthermore, by preventing calcineurin-dependent NOS dephosphorylation, FK506 would inhibit NOS activity thereby reducing formation of NO that has been implicated in mediating neurotoxicity (Dawson et al., 1993; Snyder and Sabatini, 1995), and the generation of both LTP (Schuman and Madison, 1991; O'Dell et al., 1991; Kantor et al., 1996) and LTD (Shibuki and Okada, 1996; Malen and Chapman, 1997). The apparent contradictory findings that FK506 elicits both an inhibition of NMDA-induced neurotransmitter release (Steiner et al., 1996) and an augmentation of depolarization-induced neurotransmitter release have been attributed to a similar underlying mechanism; i.e., altered phosphorylation of the calcineurin substrates NOS and synapsin I, respectively (Steiner et al., 1996). It is possible that any one of these alterations may play a role in the development of some of the neurological sequelae observed in the occasional patient undergoing FK506 therapy (Lopez et al., 1991; Mueller et al., 1994; Wijdicks et al., 1994; Bronster et al., 1995; Vincenti et al., 1996).

In addition to these interesting neuronal properties, FK506 speeds nerve regeneration in the peripheral nervous system of the rat with a focal crush lesion of the sciatic nerve (Gold et al., 1994, 1995). The nerve regenerative property of FK506 is the focus of this review. *A priori*, it would not be expected that an immunosuppressant drug (FK506) would alter axonal regeneration since Wallerian degeneration is not an immune-mediated event (Griffin et al., 1993). Furthermore, even if a reduction in macrophage infiltration (Brück and Friede, 1990) occurs following FK506 administration, such an alteration would be expected to impair nerve regeneration by delaying the removal of products of Wallerian degeneration from the distal stump (Beuche and Friede, 1984; Friede and Brück, 1993) studies utilizing the Ola (Wld) mouse mutant (Perry et al., 1990a,b), for example, show that a delay in Wallerian degeneration and macrophage infiltration leads to an impairment in axonal regeneration of sensory (Ludwin and Bisby, 1992) and motor (Chen and Bisby, 1992) neurons. Why, therefore, should FK506 alter nerve regeneration? To answer this question, it is first

necessary to understand FK506's mechanism of immunosuppression.

The Immunophilins and Immunosuppression

Immunosuppressant drugs FK506 and cyclos-porin A are prodrugs that are activated when bound to their respective binding proteins (immunophilins), FK506-binding-protein (FKBP) and cyclophilin, respectively (Schreiber and Crabtree, 1992). The function of the immunophilins as mediators of immunosuppressant drugs has recently been reviewed (Schreiber, 1991). FKBPs are a family of immunophilin proteins named according to their size (in kD): for example, FKBP-12, -13, -25, -51, -52, and -59 (Schreiber and Crabtree, 1992; Alnemri et al., 1993; Sigal and Dumont, 1992). Initially, it was thought that the immunosuppressant actions of FK506 and cyclosporin A were caused by the ability of their respective immunophilins to prevent the interconversion of the *cis-* and *trans-* isomers of prolyl residues of proteins (Walshet al., 1992). However, subsequent studies proved that peptidyl prolyl isomerase (PPI) activity does not play a role in immunosuppression (Dumont et al., 1992; Wiederrecht et al., 1992), since not all immunosuppressant analogs of these two agents inhibit isomerase activity (Sigal et al., 1991) and, conversely, not all isomerase inhibitors are immunosuppressants (Tocci et al., 1989; Dumont et al., 1992; Sigal et al., 1991; Bierer et al., 1990; for review, *see* Wiederrect and Etzkorn, 1995). [The rotamase activity of the immunophilins has been shown to have physiological functions, such as inhibition of collagen assembly by cyclosporin A (Lewin, 1995; Compton et al., 1992; Bächinger et al., 1993) and FK506 (Hans Peter Bachinger, personal communication).] It is widely accepted that the most likely mechanism for the action of these agents involves inhibition of the activity of *calcineurin* (Freman et al., 1992), a calcium/calmodulin-dependent phosphoserine/phosphothreonine protein phosphatase, also known as PP-2B (Walsh et al., 1992; Dumont et al., 1992; Liu et al., 1991). By inhibiting the calcineurin-induced dephosphorylation of a nuclear factor of activated T-cells (NFAT), FK506 and cyclosporin A prevent NFAT translocation into the nucleus where it induces interleukin-2 (IL-2) secretion, thereby preventing T-cell proliferation (Tocci et al., 1989; Schreiber and Crabtree, 1992; Sigal and Dumont, 1992; Freman et al., 1992; Terada et al., 1992). Of the variety of FKBPs, it has been demonstrated (Sigal and Dumont, 1992; Liu et al., 1991) that FKBP-12 mediates FK506's immunosuppressant activity in T-cells. Whether the inhibition of the expression of the proto-oncogenes *c-myc* and *c-fos* (Sigal and Dumont, 1992) is involved in their immunosuppressant action is unclear. Recently, a second FKBP (FKBP-51), which is specifically expressed in T-lymphocytes (T-cells), has been demonstrated to also inhibit calcineurin (Baughman et al., 1995) suggesting that multiple immunophilins may participate in mediating FK506's immunosuppressant action.

The Immunophilins in the Nervous System

Immunophilins are enriched in neurons throughout the central (Steiner et al., 1992) and peripheral (Lyons et al., 1995) nervous systems. Their distributions and putative actions in regulating neuronal function have also been reviewed (*see* Snyder and Sabatini, 1995; Wiederrect and Etzkorn, 1995; Sánchez and Ning, 1996). Most studied is FKBP-12 (Harding et al., 1989; Siekierka et al., 1989), a ubiquitous protein that has been highly conserved throughout evolution (Siekierka et al., 1990). Snyder and coworkers, who first reported that FKBP-12 is present in neuronal tissue, showed that the mRNA levels for FKBP-12 increase in motor (facial) neurons following peripheral nerve axotomy (Lyons et al., 1992). In addition, calcineurin is present not only in T-cells but also in brain (Steiner

et al., 1992) where it comprises up to 1% of total protein in some brain regions (Klee, 1991). Furthermore, the high levels (relative to other tissues) of this protein in brain (Mukai et al., 1993) correspond regionally with the presence of high levels of FKBP (Steiner et al., 1992). Interestingly, one of the major targets for calcineurin in neurons is the growth-associated protein GAP-43 (Skene, 1990). Both GAP-43 (Skene, 1989; Skene and Willard, 1981a,b) and neuronal phosphatases (Bixby and Jhabvala, 1993) are concentrated in growth cones where they are believed to play an important role in nerve regeneration. Most importantly, a preliminary report (Steiner et al., 1991) indicates that FK506 increases phosphorylation of GAP-43. Based upon this information, FK506 (and, if this model is correct, cyclosporin A) should alter nerve regeneration, perhaps via phosphorylation-dependent activation of GAP-43 (Liu and Storm, 1990). Although this hypothesis initially appeared tenable, the most recent findings from my laboratory (summarized in the next three sections) do not support a calcineurin-dependent mechanism for how FK506 increases the rate of axonal regeneration.

FK506 Increases the Rate of Axonal Regeneration in the Rat Sciatic Nerve

We reported preliminarily in 1993 (Gold et al., 1993) and formally in 1994 (Gold et al., 1994), that FK506 increases functional recovery and nerve regeneration in young adult Sprague-Dawley rats (160–225 g) given a focal sciatic nerve crush (axotomy). A second paper, in 1995, showed that FK506 increases the rate of axonal regeneration in this model (Gold et al., 1995). Axotomy was performed by crushing the nerve twice (for a total of 30 s using a No. 7 Dumont jeweler's forceps) at the level of the hip; the crush site was marked by a sterile 9-0 suture inserted in the epineurial sheath (Gold et al., 1994; 1995). Daily subcutaneous injections of FK506 (1 mg/kg) reduced (by approx 1.5 d) the number of days until the onset of an ability to right the foot and move the toes ("onset"), and the number of days until the animal demonstrated an ability to walk on its hind feet and toes ("walking") (Fig. 1); animals were observed by at least two independent investigators in a double-blinded study (i.e., the treatment regimen was unknown to both the presenter and the observers of the animals). Control (saline- or vehicle-treated) animals first demonstrated an ability to right the foot between 17 and 18 d. In contrast, three of the five FK506-treated animals walked almost normally by 18 days. These differences in functional recovery were reflected in the morphological appearance of the sciatic nerve and its branches distal to the nerve crush at 18 d after axotomy. Axonal calibers (determined by electron microscopy in plastic-embedded sections from glutaraldehyde-perfused animals) for the largest 30% of regenerating axons were increased by 93% in the tibial nerve branch to the soleus muscle from FK506-treated animals (*see* Fig. 3 in Gold et al., 1994). Axons were found to have advanced further toward their targets and reinnervation of intrafusal fibers was evident in the most distal (interosseus) muscles. The maximal distance of axonal elongation from the crush site was measured at 10 and 15 d following axotomy using radiolabeling techniques (Gold et al., 1995), the rate of regeneration being estimated from the slope of the resultant line determined between these two-time points. We found that a 1 mg/kg daily dosage of FK506 significantly ($p < 0.05$) increased (by 16%) the axonal regeneration rate from 3.8 mm/d in saline-treated rats to 4.4 mm/d in FK506-treated animals (*see* Fig. 7 in Gold et al., 1994); regeneration rate was determined using radiolabeling techniques that provide a more accurate and less biased assessment of regenerative distance than the more commonly used pinch test. Taken together, these initial studies demonstrated that FK506 speeds functional recovery by increasing the rate of axonal regeneration in the rat sciatic nerve.

We have recently extended these studies by examining the dose-dependency for FK506's

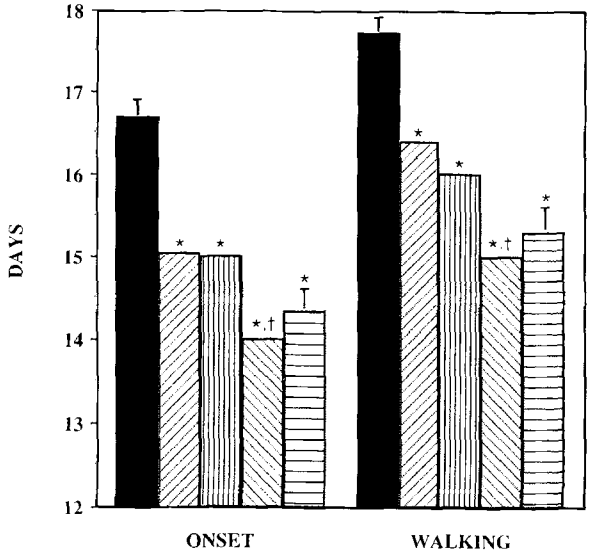

Fig. 1. Bar graphs showing number of days from axotomy until onset of toe movement and an ability to right the foot (left), and an ability to walk on the hind feet (right) are present in saline-treated and FK506-treated rats. Both signs of functional recovery appear earlier in all FK506-treated groups compared to saline-treated controls, being present earliest in the animals given the 5 mg/kg dose. $*p < 0.0001$, compared to saline-treated controls (by one-way ANOVA and Fisher's post-hoc test); $\dagger p < 0.01$, compared to 1 and 2 mg/kg groups (by one-way ANOVA and Fisher's post-hoc test); $\dagger\dagger p < 0.001$, compared to 1 and 2 mg/kg groups (by one-way ANOVA and Fisher's post-hoc test).

nerve regenerative effect (Wang et al., 1997). For these studies, we employed daily subcutaneous injections of FK506 at dosages of 2, 5, or 10 mg/kg. In all our analyses, the best results were obtained in animals given the 5 mg/kg dose. In terms of functional recovery, the 5 mg/kg FK506-treated group demonstrated the earliest recovery of function in the hindfeet (Fig. 1); for example, the number of days until the onset of an ability to right the foot and move the toes ("onset"), and the number of days until the animal demonstrated an ability to walk on its hind feet and toes ("walking") were reduced from 16.8 ± 0.20 d to 14 ± 0 d and 17.8 ± 0.2 d to 15 ± 0 d, respectively, in the saline-treated animals (n = 5) and in the

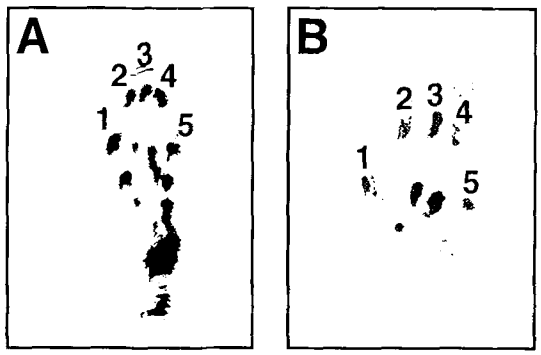

Fig. 2. Representative footprints at 18 d following axotomy from a saline-treated rat **(A)** and an animal given 5 mg/kg FK506 **(B)**. Each image was generated by scanning the original footprint using MacImage (Xerox Imaging Systems, Stamford, CT). The foot and all toes (numbered) are clumped together in the footprint from the saline-treated rat. In contrast, the footprint from the FK506-treated animal exhibits toe spread for all five digits; the lack of an imprint by the heel shows that the animal was able to support its weight on its toes and the front of its foot during walking.

5 mg/kg group (n = 3), respectively. Representative footprints at 18 d following axotomy (corresponding to the time of morphological analysis; see below) are shown in Fig. 2. Whereas the footprint from the saline-treated, axotomized control animal shows a continued deficit (Fig. 2A), the footprint from the animal given the 5 mg/kg dose (Fig. 2B) appears virtually normal; this rat demonstrates an ability to walk on toes and the front of its foot, as shown by the lack of a heel imprint. Electron microscopy, performed at 18 d, confirmed the behavioral findings. Nerves from FK506-treated animals contained larger, more advanced regenerating axons, representing the morphological correlate of the earlier functional recovery in these animals. The percent increase in mean axonal areas from control values demonstrated a bell-shaped dose-dependency (Fig. 3); mean axonal areas in FK506-treated animals (including our earlier data from rats treated with 1 mg/kg/d) were increased by 67, 83, 120, and 100% in the 1, 2, 5, and 10 mg/kg groups, respectively. Measurement of axonal regenera-

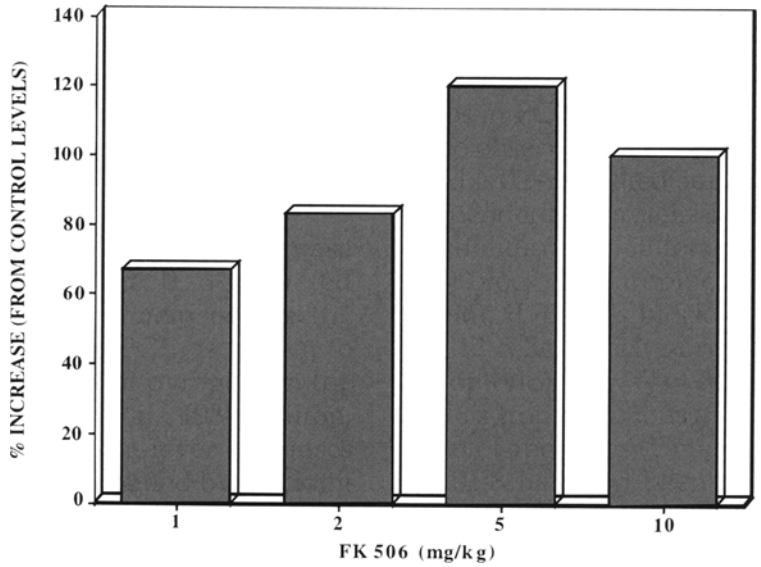

Fig. 3. FK506 dose-dependently increases mean axonal area in the soleus nerve. The percent increase in axonal areas from control values increases with dose between 1, 2, and 5 mg/kg, declining slightly in the 10 mg/kg group.

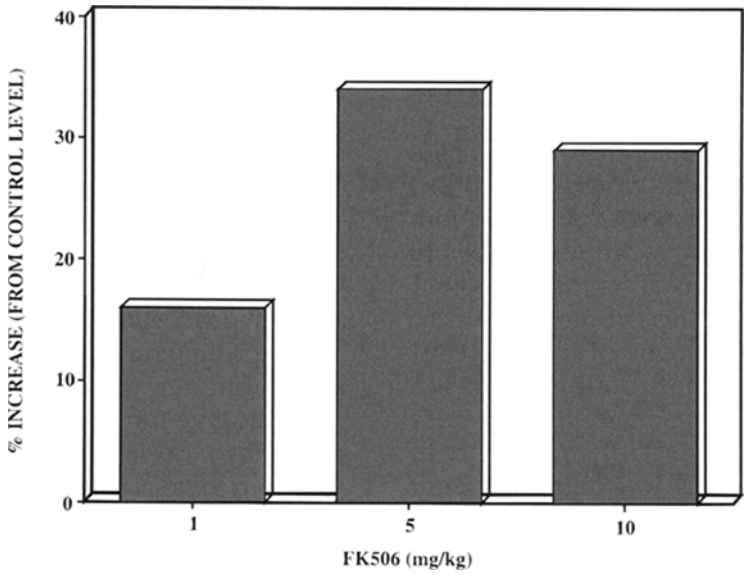

Fig. 4. FK506 dose-dependently increases the rate of axonal regeneration in the sciatic nerve. The percent increase in regeneration rate from control values increases with dose between 1 and 5 mg/kg, declining slightly in the 10 mg/kg group.

tion using radiolabeling techniques also exhibited a bell-shaped dose-response (Fig. 4); the percent increase in rate of axonal regeneration from control values was 16, 34, and 29% in the 1, 5, and 10 mg/kg groups, respectively. Taken together, these data establish the dose-dependency for the ability of FK506 to increase nerve regeneration in vivo.

Cyclosporin A Does Not Increase Axonal Regeneration Rate

To determine if calcineurin is involved in the ability of FK506 to increase the rate of axonal regeneration, we tested cyclosporin A to see if the drug shares FK506's regenerative properties (Wang et al., 1997). Since the drug–immunophilin complexes inhibit calcineurin activity to suppress T-cell function, it would be expected that equivalent doses of cyclosporin A would also increase the rate of axonal regeneration in a dose-dependent manner; i.e., if calcineurin inhibition is the underlying mechanism for FK506's nerve regenerative effect. Dosages (10 and 50 mg/kg/d) of cyclosporin A chosen for study were based upon its relative potency for immunosuppression and PPI (with cyclosporin A being approx 1/10 as potent as FK506) and our finding that a daily dose of 5 mg/kg of FK506 maximally increases the axon regeneration rate; the 50 mg/kg/d dosage approaches the maximal tolerated dose in rats and is sufficient to prevent allograft rejection in rodents (Cosenza et al., 1994). Neither dose of cyclosporin A altered the rate of axonal regeneration (as determined by radiolabeling techniques) nor did the drug speed functional recovery in the sciatic nerve. These findings indicate that cyclophilin A-mediated calcineurin inhibition does not mediate the ability of FK506 to increase nerve regeneration.

FKBP-12 Ligands Not Inhibiting Calcineurin Increase Nerve Regeneration

To determine whether FK506-FKBP-12 ligands increase nerve regeneration via a calcineurin-independent mechanism, studies have recently been conducted to test the regenerative potential of potent FKBP-12 inhibitors that do not inhibit calcineurin. Snyder and coworkers (Steiner et al., 1997) reported that *topical* administration of L-685,818 (18-OH, 21-ethyl-FK506) to the nerve-crush site accelerates functional recovery and increases axonal calibers (using paraffin-embedded nerves fixed by immersion in 10% formalin) distal to the crush lesion. Unfortunately, any correlation between functional recovery and morphological changes in these animals is problematic since the investigators limited their assessment of the axons to a distance of only 2 mm from the crush injury at 18 d following the lesion. It is therefore unclear whether the presence of more myelinated fibers is because of an effect of the drug on axonal sprouting as opposed to axonal elongation *per se*, an interpretation supported by their in vitro data (Steiner et al., 1997). Furthermore, the recent finding that L-685,818-FKBP-12 is able to inhibit calcineurin in *C. neoformans* (Odom et al., 1997) makes this compound of questionable value for definitively ruling out calcineurin activity in the nervous system.

Based upon the structural domains of FK506 (Fig. 5), two groups have synthesized novel small molecules that retain the FKBP-12 binding domain but *lack* the structural components of the effector domain. Snyder and coworkers reported (Steiner et al., 1997a,b) that *systemic* administration (i.e., subcutaneous injections for 18 d) of two FKBP-12 ligands (with binding affinities of 25- and 250-n*M*, respectively) but that do not inhibit calcineurin activity (and are not immunosuppressants) increase the size of myelinated fibers (at 2 mm distal to the crush site). As in the case of the L-685,818 compound, it is difficult to rule out an effect of the compounds on axonal sprouting, a possibility apparently supported by their findings in the striatum from *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice (Steiner et al., 1997b) (*see* FK506: Regeneration of CNS Neurons). This concern is exacerbated by their failure to assess functional recovery in animals given these compounds. These investigators also report an increase in myelination that may merely reflect the increase in axonal calibers, as opposed to a direct effect on myelination. Morphometric analysis of glutaraldehyde-fixed tissue is needed to differentiate between these two possibilities (e.g., calculating the G-ratio: diameter of the axon/diameter of the nerve fiber).

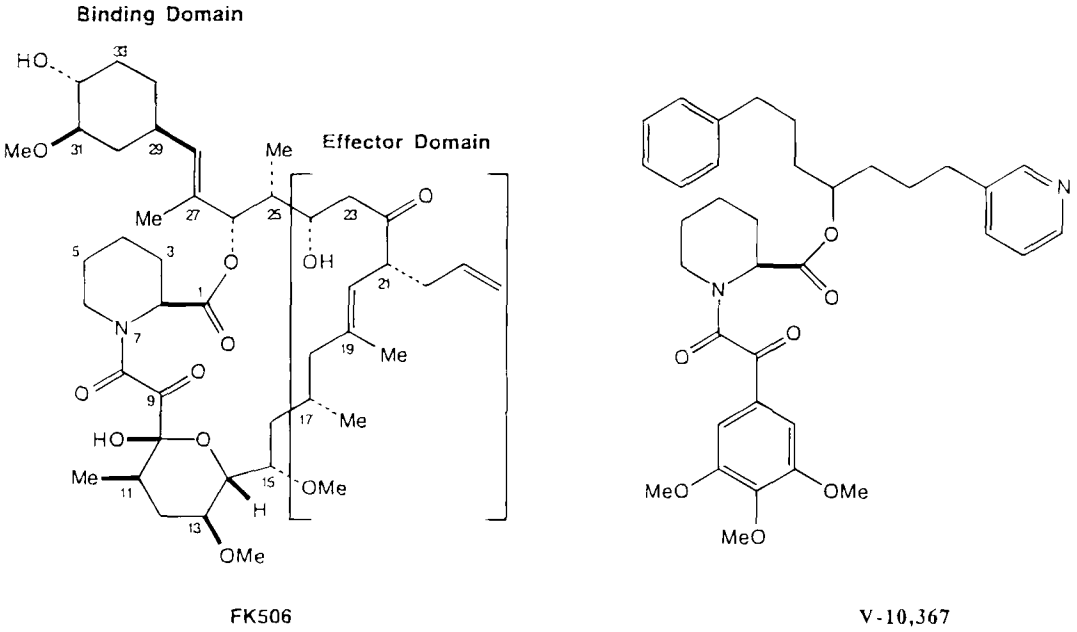

Fig. 5. Comparison of the structures of FK506 (left) and V-10,367 (right). The bracketed portion of FK506 represents the calcineurin-binding domain which is absent in V-10,367.

We have tested (Gold et al., 1997) by *systemic* administration (i.e., subcutaneous injections for 18 d), the nerve-regenerative property of V-10,367 (Fig. 5), a small molecule that also lacks the structural components of the effector domain necessary for calcineurin binding but that binds to FKBP-12 with much greater (<1 n*M*) affinity (Armistead et al., 1995) than the compounds used by Snyder and coworkers (Steiner et al., 1997a,b). As expected, this compound does not inhibit calcineurin activity (D. R. Armistead, personal communication). Rats given subcutaneous injections of V-10,367 (400 mg/kg/d) showed a more rapid (by approx 2 d) functional recovery following a focal sciatic nerve crush compared to vehicle-treated control animals. The morphological correlate of this earlier return of function was the presence of larger-sized regenerating axons in the sciatic nerve. By electron microscopy, mean axonal areas in the soleus nerve were 50% larger in the V-10,367-treated animals compared to control values. We found no evidence for an increase in myelination, as determined from the G-ratio (B.G. Gold and M.-S. Wang, unpublished observation). Whereas the increased size of regenerating axons was somewhat less than with FK506, further studies are needed to determine the optimal dosage of this compound. The compound is also effective by oral administration at dosages as low as 5 mg/kg (B. G. Gold, M. Zeleny-Pooley, and M.-S. Wang, unpublished observation). Thus, our study is the first to demonstrate that systemic administration of a potent FKBP-12 ligand that lacks the structural components necessary for calcineurin inhibition speeds functional recovery by accelerating the growth of regenerating axons to the distal musculature following a sciatic nerve-crush lesion. Future studies should address whether the compound also accelerates nerve regeneration in older animals as well as in other species.

FK506: Effects on Neurite Outgrowth in Culture

In the seminal 1994 study, Snyder and coworkers (Lyons et al., 1994) showed that FK506 potently (as low as 0.1 n*M*) increases

neuritic outgrowth from PC12 cells and DRG explant cultures in a concentration-dependent fashion. In PC12 cells, neurite outgrowth was assessed by determining the percentage of cells with processes greater than 5 μm. The ability of FK506 to enhance neurite outgrowth in PC12 cells was dependent upon the concentration of NGF. Maximal efficacy was found in the presence of relatively low (1–10 ng/mL) concentrations of NGF, since at higher concentrations of NGF (>10 ng/mL) the maximal response (in terms of % of cells bearing processes) was already demonstrated. Half-maximal response was obtained at 0.1 n*M* in the presence of submaximal (1 ng/mL) concentrations of NGF. The finding that similar results were obtained in DRG cultures indicates that FK506 activity is not dependent upon exogenously supplied neurotrophins (e.g., NGF) (*see* Putative Mechanisms for FK506's Ability to Increase Nerve Regeneration).

Recently, these investigators reported that cyclosporin A also increases neurite outgrowth in these two systems. We have used human neuroblastoma SH-SY5Y cells to study the concentration-dependency of FKBP-12 ligands for increasing neurite outgrowth. We measured neurite process length at 96 and 168 h after addition of the text agent. Maximal efficacy was observed between 1 and 10 n*M* in the presence of 10 ng/mL NGF. In contrast to Snyder and coworkers (Steiner et al., 1997a), and in accordance with our in vivo data (*see* Cyclosporin A Does Not Increase Axonal Regeneration Rate), we found that cyclosporin A (1–1000 n*M*) did not significantly alter neurite outgrowth in SH-SY5Y cells (B. G. Gold and M. Zeleny-Pooley, unpublished observations).

The results of these studies stand in contrast to those reported by Jay and coworkers (Chang et al., 1995) who found that FK506 *reduces* neurite outgrowth in cell culture. These studies employed a much higher (50 μ*M*) concentration of FK506 in the presence of very high levels of NGF (100 ng/mL). In this context, we have found that 1 μ*M* FK506 actually inhibits NGF-induced neurite outgrowth in SH-SY5Y cells (B. G. Gold and M. Zeleny-Pooley, unpublished results). Thus, in the context of the broad range of concentrations of FK506 we have examined, our results are, in fact, consistent with their findings.

FK506: Regeneration of CNS Neurons

Cell culture systems provide a simple means to examine whether FK506 also increases nerve regeneration in the CNS. We have conducted in vitro studies to examine whether FK506 also promotes axonal regeneration in cells not derived from the PNS. Our studies indicate that FK506 (1–100 n*M*), in the absence of exogenously supplied neurotrophins (e.g., neurotrophin-3; NT-3) also increased neuritic outgrowth in rat cortical neurons and hippocampal neurons (Fig. 6) in a concentration-dependent manner (B. G. Gold and M. Zeleny-Pooley, unpublished results).

An important question is whether FK506 is also effective in vivo in aiding nerve regeneration in the CNS. One preliminary report has examined the ability of daily subcutaneous injections of FK506 (0.5 mg/kg) to increase regeneration of dorsal-root axons into the spinal cord (Sugawara et al., 1995). This study was restricted to the examination of unmyelinated axons immunoreactive for calcitonin gene-related peptide (CGRP). Whether these axons originated in the DRG or represented abnormal sprouting of axons intrinsic to the spinal cord is unclear (Goldberger et al., 1993). Moreover, it is possible that in this model FK506 may have inhibited an autoimmune response leading to increased regeneration via its immunosuppression action.

A recent report (Steiner et al., 1997b) indicates that a nonimmunosuppressant FKBP-12 ligand also enhances the density of striatal dopaminergic innervation (as determined by immunocytochemical staining for tyrosine hydroxylase) in MPTP-intoxicated mice. Importantly, the FKBP-12 ligand increased the density of staining when given after the toxic agent,

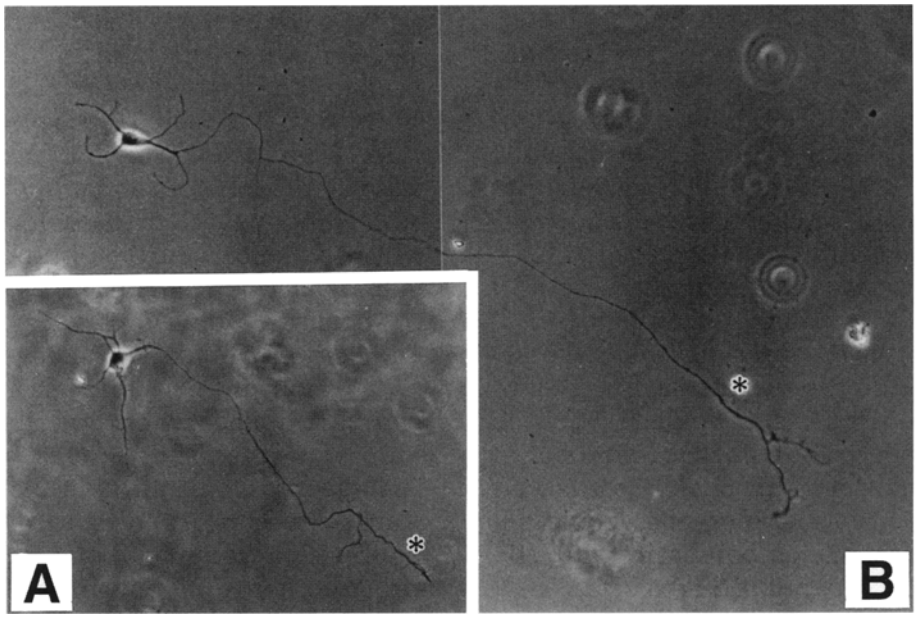

Fig. 6. Hippocampal neurons 86 h in culture either untreated **(A)** or treated with 100 n*M* FK506 **(B)**. The axonal processes (*) are clearly discernible and are markedly elongated in the neuron given FK506; neuritic length was 82 and 166 μm in **(A)** and **(B)**, respectively. Neurons were grown on coverslips that were inverted onto 24-well plates precoated with a monolayer of cortical astrocytes, according to Banker and Cowan (1977). Magnification 680X.

suggesting that the compounds can increase innervation following denervation. The increase in fiber density does not necessarily indicate regeneration of damaged fibers but may actually arise from collateral and/or terminal sprouting of intact axons. The alternative possibility that the increase in immunoreactivity merely reflects an increase in axonal transport of tyrosine hydroxylase cannot be ruled out. In addition, the possibility of inappropriate innervation of adjacent regions needs to be examined in detail. Whereas functional studies are needed to support the morphological data, a similar study in 6-hydroxydopamine-lesioned rats showed that the FKBP-12 ligand reduced motor disturbance (i.e., amphetamine-induced rotations). Further studies on the ability of FKBP-12 ligands to increase regeneration of CNS axons are warranted to assess their potential for the treatment of human neurodegenerative diseases.

FK506 Neuronal Actions: Calcineurin-Dependent and Calcineurin-Independent Mechanisms

Taken together, studies employing cyclosporin A and FK506-like compounds demonstrate that calcineurin inhibition is not necessary for FKBP-12 ligands to speed nerve regeneration and implicate a FKBP-pathway distinct from immunophilin-mediated inhibition of calcineurin in the ability of FK506 to increase axonal elongation. Thus, these findings do not support the hypothesis (Gold et al., 1995) that FK506 speeds axonal regeneration by increasing the phosphorylation state of GAP-43 secondary to calcineurin inhibition. However, it is possible that this pathway, by leading to a loss in the dynamics of GAP-43 phosphorylation (Meiri et al., 1991), could

negatively alter nerve regeneration, an explanation for the decreased drug efficacy observed at high (10 mg/kg) daily dosages (*see* FK506 Increases the Rate of Axonal Regeneration in the Rat Sciatic Nerve). Other calcineurin targets can also be discounted as playing a role in FK506's nerve regenerative effect. For example, inhibition of the calcineurin target NOS would lead to a reduction in NO formation (Dawson et al., 1993). Conflicting results have been reported on NO regulation of neurite outgrowth. A preliminary report using sensory neurons indicates that NOS inhibitors increase and, conversely, increased NO formation decreases neurite outgrowth in vitro (Wayne and Skene, 1995). In contrast, a recent paper (Hindley et al., 1997) shows that NO donors increase neurite outgrowth from PC12 cells and primary hippocampal neurons, a result contrary to that expected if NOS inhibition were to play a role in FK506's ability to increase nerve regeneration. Regardless of whether NO positively or negatively impacts neurite outgrowth, a role for the calcineurin target NOS in nerve regeneration is ruled out by studies using FKBP-12 ligands lacking calcineurin inhibition (Steiner et al., 1997a,b; Gold et al., 1997). However, it should be noted that calcineurin inhibition may play a role in mediating other important neuronal properties of FK506. For example, FK506's neuroprotective action against glutamate toxicity in vitro may utilize this pathway (Dawson et al., 1993; Snyder and Sabatini, 1995). In addition, the demonstration that systemic injections of FK506 (Kitamura et al., 1994) and cyclosporin A (Matsuura et al., 1996) protect striatal neurons against depletion of dopamine by MPTP or 6-hydroxydopamine, respectively, strongly suggests that calcineurin inhibition may also mediate this neuroprotective action. Together with the finding that a FKBP-12 ligand increases regeneration of dopaminergic neurons in MPTP-treated mice, these exciting findings suggest that FK506 (or related compounds) may be beneficial in the treatment of Parkinson's disease.

Putative Mechanisms for FK506's Ability to Increase Nerve Regeneration

The mechanism by which FK506 increases the rate of peripheral nerve regeneration is unknown. Whereas our studies (Wang et al., in press) (*see* FKBP-12 Ligand Not Inhibiting Calcineurin Increases Nerve Regeneration) appear to rule out a calcineurin-dependent mechanism, it is possible that FKBP-12 may still be involved, albeit via a different pathway. For example, the FKBP-12 has also been found to demonstrate stable interactions with the two Ca^{2+} channels release Ca^{2+} from internal stores: the ryanodine (Timerman et al., 1993; Brillantes et al., 1994; Giannini et al., 1995) and the inositol 1,4,5-triphosphate (IP_3) receptors (Cameron et al., 1995a,b); FKBP and calcineurin interact under physiological conditions to modulate Ca^{2+} flux in these channels and FK506, by inhibiting calcineurin and preventing receptor dephosphorylation, increases Ca^{2+} transport through these channels. The possibility that FK506's ability to stabilize these channels and alter Ca^{2+} release (Brillantes et al., 1994; Cameron et al., 1995b) is involved in the drug's regenerative effects (*see* Snyder and Sabatini, 1995) appears to be supported by a very recent preliminary report (Takei et al., 1996) showing that inactivation of the type 1 IP_3 receptor in chick DRG growth cones inhibits neuritic growth. However, the somewhat higher concentrations of FK506 (10–100 n*M*) necessary to disrupt association of FKBP-12 from the IP_3 receptor (Cameron et al., 1995a), compared to concentrations that enhance neurite outgrowth in vitro (Lyons et al., 1994; B. G. Gold and M. Zeleny-Pooley, unpublished observation), makes this possibility less attractive.

An alternative mechanism by which FK506 could alter nerve regeneration via FKBP-12 is suggested by the finding that FKBP-12 is a natural ligand for the type 1 receptor for transforming growth factor-β (TGF-β1), where it functions as an inhibitor of TGF-β1 receptors (Wang et al., 1994, 1996). Since TGF-β1 stimulates nerve

growth factor (NGF) synthesis by glial cells (Lindholm et al., 1990), and NGF has been suggested to play a role in axonal elongation (Taniuchi et al., 1988), FK506 could increase regeneration indirectly via an increase in NGF. Such a mechanism may be supported by the findings that FK506 appears to increase the sensitivity of PC-12 cells to NGF (Lyons et al., 1994) and by the demonstration that TGF-β1, at similarly low concentrations (i.e., 1 ng/mL), promotes regrowth of injured neurites in vitro (Abe et al., 1996). However, this hypothesis is unattractive given that a role for NGF in axonal regeneration is not supported by in vivo studies (Diamond et al., 1992); in fact, we have shown recently that delivery of NGF to the neuronal cell body (by intrathecal infusion) delays regeneration (Gold, 1997). Furthermore, FKBP-12 has been shown to be dispensable for TGF-β signaling (Charng et al., 1996). In addition, as in the case of the IP_3 receptor (*see* preceding paragraph), much higher concentrations of FK506 (μ*M*) are needed to disrupt association of FKBP-12 from the TGF-β1 receptor (Wang et al., 1994) relative to those that stimulate neurite outgrowth in vitro (Lyons et al., 1994); B. G. Gold and M. Zeleny-Pooley, unpublished observation.

An unexplored area is the possible involvement of FKBPs besides FKBP-12 in mediating FK506's nerve regenerative effect. Most interesting is the FK506–FKBP-52 complex, which does not inhibit calcineurin (*see* Snyder and Sabatini, 1995). Instead, human FKBP-52 (rabbit FKBP-59) (human FKBP-52) (Tai et al., 1992) has been identified as a heat-shock protein (hsp-56) (Sanchez, 1990) and, together with the hsp-90 and hsp-70, this novel immunophilin comprises a component of a subclass of glucocorticoid receptor complexes (Owens-Grillo, et al., 1995; Perdew and Whitelaw, 1991; Lebeau et al., 1992; Czar et al., 1994); FK506 does not alter glucocorticoid signaling pathways but may produce conformational changes in the complex (Ratajczak and Carrello, 1996). In this context, we have found that subcutaneous injection of FK506 (10 mg/kg/d, for 2 wk) increases expression of hsp-70 (as shown by immunocytochemistry) in selected neurons in the brain (including the cortex, hippocampus, and amygdala), and in the spinal cord and dorsal root ganglion (DRG) (Goto and Singer, 1994). It may therefore be relevant that the glucocorticoid dexamethasone has been found to increase GAP-43 mRNA levels in regenerating hypoglossal neurons (Yao and Kiyama, 1995). Moreover, it has recently been shown that FK506 potentiates the potency of steroid hormones (Kralli and Yamamoto, 1996). Thus, our (B. G. Gold, J. Y. Yew, and M. Zeleny-Pooley, unpublished observations) recent demonstration that FK506 increases GAP-43 mRNA levels in DRG neurons may support an involvement of this pathway in FK506's ability to increase nerve regeneration.

Consistent with our nerve regenerative studies (*see* Cyclosporin A Does Not Increase Axonal Regeneration Rate), the ability of FK506 to protect hippocampal CA1 and cerebral neurons against transient global ischemia is not shared by cyclosporin A (Yagita et al., 1996). This suggests that a calcineurin-independent mechanism also underlies FK506's ability to provide neuroprotection against ischemic injury. The recent demonstration that global ischemia induces an alteration in the ryanodine receptor (Nozaki et al., 1996) suggests that FK506 via the FKBP-12 may protect against damage via binding to the FKBP-12. However, the necessity of using repeated drug administration (Drake et al., 1996) suggests that simple inhibition of NO may not fully explain the neuroprotective actions of FK506. FK506's ability to protect against focal cerebral ischemia (Sharkey and Butcher, 1994; Ide et al., 1996; Tokime et al., 1996) may therefore involve additional FKBP pathways, perhaps mediated by hsp-70 induction via glucocorticoid receptors. Whether this pathway also plays a role in the ability of FK506 to protect against ischemic damage is an important area for future work.

Why FK506 exhibits a reduction in efficacy for axonal regeneration at high (10 mg/kg) dosages is unknown. Interestingly, a bell-shaped dose-response has been observed for other agents that increase regeneration. For example, the melanocortins (α-MSH), insulin-like growth factor-I, and ACTH demonstrate a

similar decrease in efficacy beyond a certain optimal dosage (Bijlsma et al., 1981; Contreras et al., 1995; Bijlsma et al., 1983); however, for the latter two agents, hypoglycemia and corticosteroid activity, respectively, may be responsible for the reduced regenerative efficacy of high dosages (Bijlsma et al., 1981; Contreras et al., 1995). For the melanocortins, it has been suggested (Joosten et al., 1996) that the bell-shaped dose-response is caused by the action of multiple melanocortin receptors (Hol et al., 1995). Whereas alternative mechanisms are indeed possible, the reduced efficacy of higher (10 mg/kg) dosages of FK506 suggests that multiple FKBPs (Snyder and Sabatini, 1995; Sigal and Dumont, 1992; Cardenas et al., 1995; Wiederrecht et al., 1993) or FKBP-mediated pathways (acting in opposition) underlie the ability of FK506 to alter nerve regeneration. For example, whereas the results presented in this review indicate that FK506 speeds regeneration via a calcineurin-independent pathway, calcineurin inhibition (which may be less sensitive) would be expected to impair regeneration (Chang et al., 1995) leading to a reduction in efficacy at higher (10 mg/kg) dosages of FK506.

Summary and Future Directions

Taken together, it appears that distinct mechanisms underlie the immunosuppressant (calcineurin-dependent) and nerve regenerative (calcineurin-independent) properties of FK506. Based upon structural analysis of FK506-FKBP interactions (Griffith et al., 1995; Itoh et al., 1995a,b), it has been possible to separate these properties and design new FKBP ligands (Armistead et al., 1995; Batchelor et al., 1994; Shuker et al., 1996) that do not inhibit calcineurin. Of equal importance for drug development is the possibility that different pathways underlie FK506's nerve regenerative (Wang et al., 1997; Steiner et al., 1997a,b; Gold et al., 1997) and neurotoxic properties (Lopez et al., 1991; Mueller et al., 1994; Wijdicks et al., 1995; Bronster et al., 1995; Vincenti et al., 1996). The latter have been linked mechanistically to the immunosuppressant properties of FK506 via calcineurin inhibition (Dumont et al., 1992); calcineurin inhibition also has been shown to underlie development of some pathological hallmarks of Alzheimer's disease (Ladner et al., 1996). Thus, it is possible that FK506's neurotoxic properties will not be shared by these new (noncalcineurin-binding) FKBP ligands.

It should be apparent, however, that our knowledge is critically lacking in regard to the underlying molecular mechanism(s) by which FKBP ligands produce their neuronal effects. Of paramount importance will be identification of the molecular targets for FKBP ligand-FKBP complexes. This information should become available in the near future given the recent development of innovative new techniques such as the three-hybrid system (Licitra and Liu, 1996) to "fish" for such targets. It will then be necessary to sort out which of these targets are physiologically relevant for promoting nerve regeneration. These studies should also shed new light on the neuron's intrinsic mechanism(s) regulating axonal elongation. Regardless of the underlying mechanism involved, the development of novel FKBP ligands (Navia and Chaturvedi, 1996) may lead to the generation of new drugs for the treatment of human nerve injuries.

Acknowledgments

I thank Michelle Zeleny-Pooley and Dan Austin for preparation of figures, Fujisawa Pharmaceuticals, Osaka, Japan for the generous gift of FK506, and Vertex Pharmaceuticals, Cambridge, MA for providing compound V-10,367. I especially would like to thank David Armistead (Vertex Pharmaceuticals) for sharing information on the chemistry of V-10,367 and for helpful discussions concerning the manuscript, and Solomon Snyder (Johns Hopkins University) for providing advanced copies of two manuscripts. I am indebted to Peter S. Spencer for his insightful and critical review of the manuscript. This paper is dedicated to my brother, Michael S. Gold (University of New Mexico).

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