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Inhibitory Role for Calcineurin in Stimulus-Secretion Coupling Revealed by FK506 and Cyclosporin A in Pituitary Corticotrope Tumor Cells

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The properties of the calcium/calmodulin-dependent protein phosphatase calcineurin and its potential role in stimulus-secretion coupling were examined in AtT20 mouse pituitary corticotrope tumor cells. Protein phosphatase activity was assayed by measuring the liberation of ^{32}P from ^{32}P-casein, adrenocorticotropin secretion was measured by radioimmunoassay. About 60% of the total phosphatase activity was inhibited by 500 nM okadaic acid, suggesting the presence of protein phosphatases 1 and/or 2A. A further 25-30% reduction of phosphatase activity was achieved by chelating free calcium. Addition of the EF-hand protein blocker trifluoperazine or a calcineurin autoinhibitory peptide fragment markedly reduced okadaic acid resistant and calcium-dependent protein phosphatase activity indicating that calcium-dependent ^{32}P release is largely due to calcineurin (protein phosphatase 2B). The remaining 10-15% of total activity was Mg^{2+} dependent and blocked by NaF, hence possibly due to protein phosphatase 2C. Calcineurin activity was inhibited by the immunosuppressants FK506 and cyclosporin A, either when added to the cell lysates or after preincubation of intact cells with the drugs for 30 min at 37°C. When added to lysates, cyclosporin A inhibited calcium/calmodulin-dependent phosphatase more effectively than FK506. However, when tested on intact cells, FK506 proved 10-fold more potent than cyclosporin A. Both immunosuppressive agents enhanced the calcium-dependent release of adrenocorticotropic hormone into the medium, once more, FK506 was 10-fold more potent than cyclosporin A. Taken together, these data suggest that calcineurin is an inhibitory element in the signal transduction pathway controling exocytotic secretion in pituitary cells that express voltage-operated calcium channels. This is in direct contrast with leukocytes where voltage-operated calcium channels are not found, and calcineurin is an important element for agonist-induced activation.

Recent advances in the mechanism of action of the immunosuppressive drugs FK506 and cyclosporin A suggest that these compounds inhibit cellular activation in lymphocytes by blocking the calcium/calmodulin-dependent protein phosphatase calcineurin (protein phosphatase 2B) [1]. Typically, FK506 and cyclosporin A are taken up into cells through an active transport process [2] and then bound by immunophilin proteins: FKBP for FK506 and cyclophilin for

Abbreviations: Protein phosphatase - PPase; Adrenocorticotropic hormone - ACTH.

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cyclosporin A [3]. The protein-drug complexes then bind to calcineurin and strongly inhibit its protein phosphatase activity [3, 4]. As immunophilin proteins are not confined to cells of the immune system [3, 5], FK-506 and cyclosporin A may be useful tools to study the role of calcineurin in a variety of cells.

Overall, studies in leukocytes (lymphocytes [1, 6], basophilic leukemia cells [7] and neutrophil granulocytes [8]) as well as kidney tubule cells [9] suggest that calcineurin is a component of agonist pathways that lead to cellular activation usually accompanied and causally related to a rise in intracellular free calcium [3, 9, 10]. However, since calcineurin is activated by calcium, it is also potentially an enzyme involved in the termination of agonist-induced activation, as proposed previously for voltage-operated calcium channels [11].

This study reports the presence of calcineurin phosphatase activity in the mouse pituitary tumor cell line AtT20 and that this activity is inhibited by FK506 as well as cyclosporin A. Furthermore, both of these drugs enhance the calcium-dependent release of ACTH, suggesting that calcineurin is primarily an inhibitory component of signal transduction in cells that express voltage-operated calcium channels.

Materials and Methods

Reagents: Unless otherwise specified all reagents were from Sigma Ltd, Poole, Dorset, U.K. sources of other materials were as follows: Purified bovine brain calmodulin and okadaic acid : Novabiochem-Calbiochem, Nottingham U.K; calcineurin autoinhibitory fragment [6]: Bachem U.K. (Saffron Walden, Essex, U.K.); trifluoperazine : Aldrich, Gillingham, Dorset; nimodipine: Semat Ltd, (St Albans, Herts, U.K.); γ-^{32}P-ATP (S.A. 10 Ci/mmol) was from NEN-Dupont (Stevenage, Herts,U.K.).

Cells: Mouse pituitary corticotrope tumor (AtT20 D16:16) cells (passage 18-35) were maintained as previously described [12]. For phosphatase activity studies cells grown in 10% fetal bovine serum and Dulbecco's Modified Eagle's Medium were washed twice with ice-cold Hank's salt solution and then detached from the culture vessels by Hank's salt solution containing 0.1 % EDTA and no divalent cations. The number of cells was counted using a hematocytometer and aliquots were removed for the determination of protein content by the Coomassie blue method [13]. Subsequently the cells were pelleted by centrifugation for 10 min at 4°C at 800 x *g* and resuspended in hypotonic lysis buffer [6] (0.05M Tris-HCl, pH7 containing , 1mM EDTA, 0.1mM EGTA, Trasylol (Bayer) 0.15% (v/v), leupeptin 5µg/ml, lima bean trypsin inhibitor, 50µg/ml, 0.5mM dithiotreitol) at $2x10^7$ cells/ml. After three cycles of freeze-thawing in liquid N_2 and a 30°C water bath, the lysates were centrifuged for 5 min at 12,000 x *g* at 4°C, and the pellets as well as supernatants were saved for the measurement of protein phosphatase activity.

Measurement of protein phosphatase activity: Dephosphorylated bovine casein was labelled with ^{32}P with the catalytic subunit of protein kinase A according to the method of Tallant and Cheung [14] to a specific activity of 50-65 Ci/mol.

Cell-lysate (1.3-10 µl) was incubated in a total volume of 70µl at 30°C in 0.1M Tris-HCl pH7 containing Trasylol (Bayer) 0.15% (v/v), leupeptin 5µg/ml, lima bean trypsin inhibitor, 50µg/ml, 0.5mM dithiotreitol [6]. (Inhibitors of proteases were included in the reaction buffer because of the known susceptibility of calcineurin to proteolytic cleavage [15]). Divalent cations, EGTA and phosphatase inhibitors (okadaic acid, NaF) were added in varying amounts as indicated in the figure legends and the text. The reaction mixture was preincubated for 10min at 30°C after which the phosphatase reaction was initiated by adding ^{32}P-casein to 12µM final

concentration and the incubation was continued for 5min. The reaction was stopped by adding 100µl each of 25% (w/v) trichloroacetic acid and 1% (w/v) bovine serum albumin and placing the samples on ice for 5min. The tubes were vortexed and centrifuged at 12,000 x g. Two hundred µl of the supernatant were removed and the amount of radioactivity present quantified by liquid scintillation counting.

FK506 and cyclosporin A were dissolved in ethanol at 1mM and added directly to the reaction mixture prior to preincubation. Alternatively, intact cells adhering to the tissue culture vessel were preincubated for 30 min in Hepes buffered Dulbecco's Modified Eagle's Medium containing 0.25% bovine serum albumin [12] at 37°C and cell lysates were prepared as described above.

Measurement of ACTH secretion: Cells were treated as described previously [12]. Various concentrations FK506 and cyclosporin A were added to the incubation medium for 30 min at 37°C to allow uptake into the cells [2]. Subsequently, the cells were incubated in fresh medium containing drugs for a further 30 min. The medium from this period was collected and processed for radioimmunoassay of ACTH as reported earlier [12]. Data were evaluated by analysis of variance followed by Dunnett's test for multiple comparisons to a single control group.

Results

Properties of protein phosphatase activity in AtT20 cell lysates: Approximately 70% of total PPase activity was found in the supernatant fraction of the cell lysate. Unless stated otherwise, all results presented below refer to PPase activity in the supernatant fraction. About 60-70% of PPase activity was inhibited by

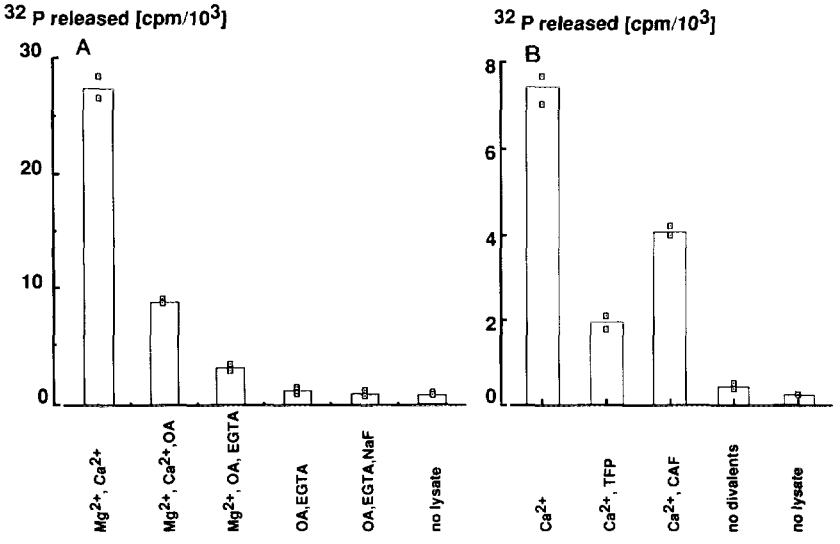

Figure 1. A) Effect of various agents on protein phosphatase activity as measured by the release of ^{32}P into a trichloroacetic acid soluble fraction from ^{32}P-labeled casein. AtT20 cells lysates were incubated with 12µM ^{32}P-casein for 5min at 30°C. Data are representative of 5 similar experiments. The total PPase activity (first column) was 1.439 nmoles(mg protein x min)$^{-1}$. B) Effect of calcium (100µM), trifluoperazine (TFP,100 µM) and calcineurin autoinhibitory fragment (CAF, 50µM) on protein phosphatase activity in the presence of 500 nM okadaic acid. Data are representative of 3 similar experiments. The Ca^{2+}-stimulated PPase activity (first column) in this experiment was 0.264 nmoles(mg protein x min)$^{-1}$.

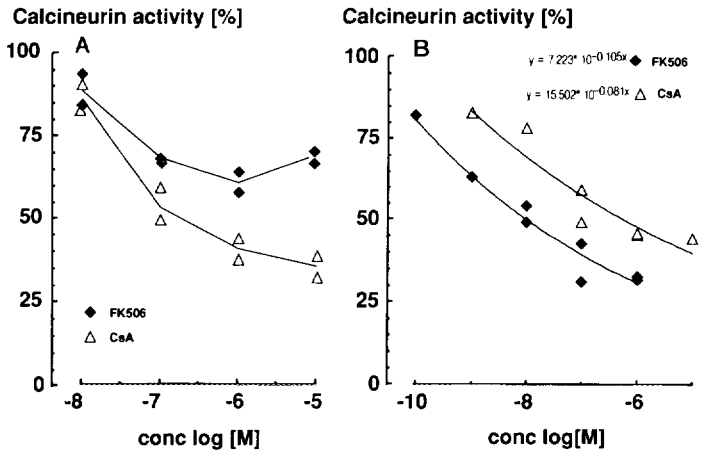

Figure 2. Inhibition by FK506 and cyclosporin A of calcineurin phosphatase activity in AtT20 cell lysates: (A) drugs added to cell lysates and incubated at 30°C for 10 min prior to the addition of substrate and (B) after preincubation of intact cells for 30 min at 37°C and assayed for protein phosphatase activity. Data are means of triplicates from two separate experiments.

500 nM okadaic acid (Fig 1A), increasing the concentration of this drug to 1 μM produced no further reduction of ^{32}P release (not shown).

Okadaic acid resistant PPase activity was analysed further by manipulating the concentrations of divalent cations and established blockers of calcineurin [8, 16]. Omission of $Ca^{2+}$and Mg^{2+} completely blocked okadaic acid resistant PPase activity (Fig 2B). Addition of 0.1mM Ca^{2+} caused a marked stimulation of ^{32}P release (Fig 2B), which could be inhibited by 100 μM trifluoperazine and the calcineurin autoinhibitory fragment peptide (50μM). Mg^{2+} and Ca^{2+} had apparently synergistic effects on Ca^{2+}-dependent PPase activity, in that the amount of activity measured in the presence of Mg^{2+} and Ca^{2+} (290±28) was greater than the sum of the activities measured in the presence of Mg^{2+} plus EGTA (50±4.5) and Ca^{2+} (188±10) (data are phosphate released pmol(mg protein x min)$^{-1}$ mean±SEM, n=3).

All subsequent experiments were carried out in the presence of 500nM okadaic acid. Calcineurin activity is defined as the difference in the amount of radioactivity released into the medium in the presence of 500nM okadaic acid, 1mM Mg^{2+}, 0.1mM Ca^{2+} and okadaic acid plus 1mM Mg^{2+} and 5mM EGTA. These conditions were chosen as 1mM Mg^{2+} approximates the average concentration of Mg^{2+} in the cytoplasm and maximal stimulation of calcineurin activity was observed with 0.01-0.1mM Ca^{2+} (not shown). Under these conditions calcineurin activity was a linear function of both the time of reaction and the amount of cell-lysate added, furthermore, none of the treatments examined altered phosphatase activity measured in the presence of Mg^{2+}, EGTA and okadaic acid.

Effects of FK506 and cyclosporin A on calcineurin activity: Preincubation for 10 min at 30°C of the cell-lysates in reaction mixture containing 1mM Mg^{2+}, 0.1 mM

Ca^{2+} and various amounts of FK506 or cyclosporin A produced a concentration dependent inhibition of calcineurin activity (Fig 2A). Note that at lower concentrations the immunosuppressants appeared equipotent, whilst at higher levels the effect of FK506 was maximal at 40% inhibition, whereas up to 70% of inhibition could be achieved with cyclosporin A. Parallel experiments with the particulate fraction of the same lysates showed that there was no inhibition at all by FK506 and a small but detectable (25%) suppression by cyclosporin A (not shown).

Preincubation of intact cells for 30min at 37°C with various concentrations of FK506 or cyclosporin A produced radically different results, in that FK506 proved to be 10-fold more potent than cyclosporin A as an inhibitor of calcineurin activity (Fig2B). No additional enhancement of the inhibitory effect of either agent was observed if the time of preincubation was increased to 60 min (not shown).

Stimulation of hormone secretion by FK506 and cyclosporin A: Both FK506 and cyclosporin A stimulated the secretion of ACTH (Fig 3). Cyclosporin A was 10-fold less potent than FK506, which is similar to the relative potency to block calcineurin activity when applied to intact cells. The secretagogue effect of FK506 as well as cyclosporin A was blocked by the dihydropyridine blocker of L-channels nimodipine (Fig 3 insert), indicating that hormone secretion was triggered *via* the physiologically relevant secretory pathway and not by non-specific perturbation of the plasma membrane. When FK506 was applied without preincubation the apparent EC_{50} was about 10-fold higher than with preincubation suggesting that a significant amount of time is required for the stimulatory effect to develop (data not shown).

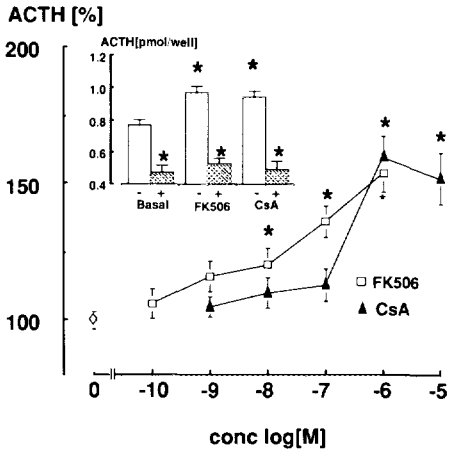

Figure 3. Stimulation of ACTH secretion by FK506 and cyclosporin A in AtT20 cells. Data are means ± SEM, n=4-12/group. Analysis of variance: F:10;114, P<0.0001, post-hoc analysis with Dunnett's two-tailed test. * P<0.05 compared with the control group. *Inset:* Effect of the dihydropyridine calcium channel antagonist nimodipine (striped columns) on basal, FK506 and cyclosporin A evoked ACTH secretion. Data are means ± SEM, n=6/group, * P<0.05 vs control basal group.

Discussion

The present data show that the potencies of FK506 and cyclosporin A to inhibit calcineurin activity in AtT20 cells and to stimulate the secretion of ACTH are closely correlated indicating that the effect on hormone secretion is due to the inhibition of calcineurin.

The properties of PPase activities in AtT20 cells extracts indicate that at least 3 types of PPase are present in the cells. The bulk of PPase activity is blocked by okadaic acid suggesting that it is due to PPases 1 and/or 2A [16, 17]. Significant amounts of calcium/EF-hand protein-dependent PPAse could be identified, and currently the only known such PPase is calcineurin [6]. Finally, the presence of Mg^{2+} dependent PPAse that is not blocked by okadaic acid and trifluoperazine is suggestive of PPase 2C [16].

The potential functional significance of calcineurin was examined using FK506 and cyclosporin A, which have been used extensively to examine the role of calcineurin in signal transduction in lymphocytes [2, 18]. Both compounds inhibited calcineurin activity in AtT20 cell lysates, indicating that the prolyl-isomerase immunophilin proteins FKBP and cyclophilin [3], or closely related molecules, that are the intracellular receptors for these drugs, are present in AtT20 cells. When the drugs were added to cell lysates FK506 was considerably less effective than cyclosporin A at inhibiting calcineurin activity. This is in contrast to intact cells preincubated with FK506 or cyclosporin A where the relative potencies of the compounds to block calcineurin activity in cell lysates prepared from the pretreated cells conformed with previous data in lymphocytes, showing that FK506 is about 10-fold more effective than cyclosporin A. Furthermore, in experiments with intact cells up to 80 % inhibition of calcineurin activity was observed as opposed to studies with cell lysates where the maximum was 40%. As no functional FKBP immunophilin seems to be retained in the particulate fraction of AtT20 cells, the plausible explanation of these findings is that the intracellular topography of FKBP is an important determinant of its potency to combine with FK506 and suppress calcineurin activity. Alternatively, FK506 may be taken up more effectively into the cells than cyclosporin A, but current evidence obtained in a kidney cell derived cell line [19] does not appear to support this notion

Importantly, the ability of FK506 and cyclosporin A to inhibit calcineurin activity in intact cells correlated closely with the potency to enhance the secretion of ACTH. The action of both immunosuppressants was completely blocked by nimodipine, indicating a dependence on L-type Ca^{2+} channels, the main Ca^{2+} channel involved in the secretory response to most secretagogues in AtT20 cells [12, 20]. In this system the maximum stimulation of ACTH with the neurohormone corticotropin-releasing factor is 2.5-fold [12], hence, the 1.5-fold stimulation seen with FK506 and cyclosporin A is by no means insignificant.

With respect to the possible mechanism of action of FK506 and cyclosporin A, it is of note that AtT20 cells fire spontaneous action potentials [21, 22] and

intracellular free Ca^{2+} transients [23]. Hence the "basal" state of the cells is characterized by a significant degree of calcium channel activity as reflected by the effects of nimodipine on "basal" hormone secretion. The consequence of calcineurin inhibition may the prolongation of L-channel opening [11] resulting in a rise in intracellular free Ca^{2+} that is sufficient to enhance hormone secretion.

When compared with lymphocytes [6, 24] the apparent half-effective concentration of FK506 to inhibit calcineurin secretion and to stimulate ACTH release is relatively high. However, the efficacy of these drugs depends primarily on the amount [3, 25], and possibly the cellular topography of immunophilins present in the cells under study, and it may well be that these factors are different in leukocytes relative to other tissues.

In conclusion, the present findings support the notion that calcineurin is involved in the control of calcium-(L)-channel activity in anterior pituitary cells and neurons [11]. Despite the well-known abundance of calcineurin in brain [26], this important hypothesis has been highly controversial in the past largely because of the lack of inhibitors of calcineurin. The demonstration in the present paper of the suitability of established immunosuppressants to probe the role of calcineurin in excitable cells expressing voltage-operated calcium channels should greatly facilitate future studies of this problem.

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