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Calcineurin Associated with the Inositol 1,4,5-Trisphosphate Receptor–FKBP12 Complex Modulates Ca^{2+} Flux

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Summary

The immunosuppressant drug FK506 binds to the immunophilin protein FKBP12 and inhibits its prolyl isomerase activity. Immunosuppresive actions, however, are mediated via an FK506–FKBP12 inhibition of the Ca^{2+}-activated phosphatase calcineurin. Physiologic cellular roles for FKBP12 have remained unclear. FKBP12 is physically associated with the RyR and IP_3R Ca^{2+} channels in the absence of FK506, with added FK506 disrupting these complexes. Dissociation of FKBP12 results in alteration of channel Ca^{2+} conductance in both cases. We now report that calcineurin is physiologically associated with the IP_3R–FKBP12 and RyR–FKBP12 receptor complexes and that this interaction can be disrupted by FK506 or rapamycin. Calcineurin anchored to the IP_3R via FKBP12 regulates the phosphorylation status of the receptor, resulting in a dynamic Ca^{2+}-sensitive regulation of IP_3-mediated Ca^{2+} flux.

Introduction

The immunophilin proteins mediate the clinical effects of the immunosuppressant drugs cyclosporin A, FK506, and rapamycin. Cyclophilins, the first class of immunophilins to be identified, bind to cyclosporin A, a cyclic undecapeptide (Handschumacher et al., 1984; for review see Walsh et al., 1992). FK506 and rapamycin, structurally related macrolides, instead bind to the FK506-binding protein (FKBP) class of immunophilins. Although there are a number of members of the immunophilin family, immunosuppressant actions appear largely attributable to cyclophilin A and B and FKBP12 (Bram et al., 1993). The cyclophilins and FKBPs do not share amino acid sequence similarity, but both display peptidylprolyl–*cis–trans* isomerase (rotamase) activity thought to be associated with protein folding (Schmid, 1993). Furthermore, immunophilin rotamase activity is inhibited by their respective immunosuppressant ligands. Immunosuppressant actions, however, do not appear to derive from inhibition of this isomerase activity. Rather, the drug–immunophilin complex of cyclosporin A–cyclophilin or FK506–FKBP12 binds to the protein phosphatase calcineurin to inhibit its activity, thereby augmenting levels of phosphorylated calcineurin substrates such as the transcription factor NFAT (for nuclear factor of activated T cells) (Liu et al., 1991; O'Keefe et al., 1992; Clipstone and Crabtree, 1992; for review of calcineurin, see Klee et al., 1988). In its phosphorylated state, NFAT cannot translocate to the nucleus, where it regulates expression of genes critical for T cell activation such as interleukin-2 (IL-2). Rapamycin acts differently than cyclosporin A and FK506, as it blocks the actions of IL-2 rather than its synthesis (Bierer et al., 1990). Rapamycin binds with high affinity to FKBP12 and inhibits its rotamase activity, but the rapamycin–FKBP12 complex does not interact with calcineurin. Rather, this immunosuppressant–immunophilin complex binds to a recently identified target protein designated RAFT (for rapamycin and FKBP12 target) or FRAP (for FKBP–rapamycin-associated protein) (Sabatini et al., 1994; Brown et al., 1994).

Marks and colleagues have observed an association of FKBP12 with the ryanodine receptor (RyR) of skeletal muscle in the absence of FK506 (Jayaraman et al., 1992; Timerman et al., 1993; Brillantes et al., 1994). RyR is a tetrameric Ca^{2+} channel that mediates Ca^{2+}-induced Ca^{2+} release in muscle, brain, and other tissues (Takeshima et al., 1989; McPherson and Campbell, 1993). We have demonstrated that the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R), a structurally and functionally related tetrameric Ca^{2+} channel that shares up to 40% homology to RyR in some regions, is also a direct target of FKBP12 (Cameron et al., 1995; for review see Furuichi and Mikoshiba, 1995; Snyder and Sabatini, 1995). Whereas FK506 stimulates the binding of FKBP12 to calcineurin, FK506 dissociates FKBP12 from RyR and from IP_3R. When FKBP12 is "stripped" from the RyR–FKBP12 or IP_3R–FKBP12 complex, the Ca^{2+} channels of these two proteins become "leaky." Accordingly, net accumulation of Ca^{2+} into RyR- or IP_3R-gated stores is diminished, and releasing agents such as caffeine or IP_3 cause increased Ca^{2+} flux at lower concentrations. It was assumed that the isomerase activity of FKBP12 was the means by which this protein modulates the RyR and IP_3R. However, recent evidence obtained from mutant FKBP12 proteins lacking isomerase activity suggests that this is not the case (Timerman et al., 1995). Accordingly, we have attempted to investigate alternative means by which FKBP12 modulates IP_3R- and RyR-mediated Ca^{2+} flux.

IP_3R can be phosphorylated at three distinct sites by protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and cyclic AMP–dependent protein kinase (PKA), respectively (Ferris et al., 1991). Whether phosphorylation and dephosphorylation physiologically regulate IP_3R function has not been established. The association of FKBP12 with IP_3R suggested a potential functional link of calcineurin and IP_3R. We now demonstrate a physical association of calcineurin with the IP_3R–FKBP12 and RyR–FKBP12 complexes that is potently disrupted by treatment with FK506 or rapamycin. We also show that complexed calcineurin modulates the phosphorylation status and Ca^{2+} flux properties of IP_3R.

Results

Copurification and Coimmunoprecipitation Demonstrate an FK506-Sensitive Association of Calcineurin with IP_3R and RyR

To look for an association of calcineurin with IP_3R, we purified IP_3R from rat cerebellum to apparent homogeneity by sequential fractionation on heparin and concanavalin A columns as previously described (Ferris et al., 1989). The purified receptor was then subjected to further fractionation on a fast protein liquid chromatography (FPLC) Superose 6 column, which separates proteins according to their molecular mass. Purified IP_3R immunoreactivity is concentrated in fractions 9–12 (Figure 1A), an elution point that reflects the molecular mass of the IP_3R tetramer, which is greater than 1.2×10^6 Da. Subjecting the identical FPLC fractions to Western blot analysis with an antibody specific for calcineurin reveals that calcineurin immunoreactivity closely parallels the distribution of IP_3R (Figure 1B). Immunoreactivity for both IP_3R and calcineurin is blocked by preabsorption with antigen (data not shown). Thus, not only is calcineurin present in a highly purified preparation of IP_3R, but when such a preparation is fractionated by gel filtration calcineurin comigrates with the IP_3R even though its molecular mass is only 10% that of the IP_3R tetramer. In contrast, when pure calcineurin is fractionated by FPLC in an identical fashion, calcineurin immunoreactivity migrates in fractions 18–19, a later elution point more consistent with a protein of molecular mass around 80 kDa (Figure 1C). Treating our preparation of IP_3R with rapamycin, which disrupts the IP_3R–FKBP12 interaction (Cameron et al., 1995), before FPLC fractionation causes calcineurin immunoreactivity to shift back to fractions 18 and 19 (Figure 1D), while the IP_3R continues to run in fractions 9–12 (data not shown).

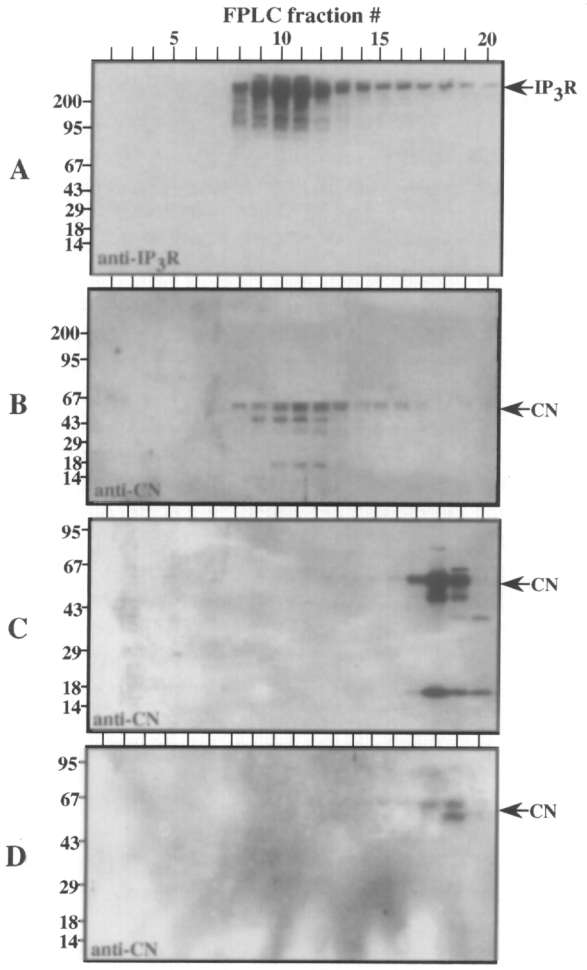

Figure 1. Copurification of Calcineurin with IP_3R Is Disrupted by Treatment with Rapamycin

In (A), (B), and (D), purified preparations of IP_3R were subjected to FPLC gel filtration and analyzed via Western blot analysis.

(A) shows the majority of IP_3R immunoreactivity in FPLC fractions 9–12, as reported previously (Cameron et al., 1995).

(B) shows that calcineurin (CN) immunoreactivity is observed in these same samples and closely parallels the distribution of IP_3R immunoreactivity.

(C) shows that pure calcineurin, fractionated in an identical fashion, elutes in fractions 18 and 19.

(D) shows that IP_3R fractions treated with rapamycin before FPLC fractionation contain calcineurin immunoreactivity in lanes 18 and 19.

We also demonstrate the specific association of calcineurin with IP_3R and RyR by immunoprecipitation experiments. We employed antibodies to IP_3R with crude preparations of rat cerebellar membranes. The immunoprecipitate displays immunoreactive calcineurin as well as IP_3R (Figure 2). Inclusion of increasing concentrations of FK506 in the membrane preparation decreases the amount of calcineurin in the immunoprecipitate. Quantification of band intensity reveals that half-maximal reduction of the presence of calcineurin in the immunoprecipitate occurs between 10 and 100 nM FK506. This range is similar to the EC_{50} obtained for FK506 dissociation of FKBP12 from RyR reported by Timerman and colleagues to be in the range of 120–500 nM (Timerman et al., 1993). Cyclosporin A fails to influence the calcineurin content of the immunoprecipitate, while rapamycin, which like FK506 binds tightly to FKBP12, markedly reduces the calcineurin content of the immunoprecipitate. The association of calcineurin with the IP_3R–FKBP12 complex appears to require Ca^{2+}, as EGTA depletes the calcineurin content of the immunoprecipitate (Figure 2). Control immunoprecipitation experiments were carried out with antibodies against calbindin and microtubule-associated protein 2 (MAP-2), which like IP_3R are proteins of high abundance in cerebellar Purkinje cells. Calcineurin immunoreactivity was not seen in the immunoprecipitate in either case, nor in experiments using the anti-IP_3R antibody preimmune serum (Figure 2).

We have also demonstrated a similar association of calcineurin with RyR (Figure 3A). Immunoprecipitation of cerebellar homogenates with anti-RyR antibodies results in coprecipitation of calcineurin. Both FK506 and EGTA deplete the immunoprecipitate of calcineurin. The association of calcineurin with IP_3R and RyR is further supported by immunoprecipitation experiments conducted with antibodies to calcineurin (Figure 3B). In these experiments, IP_3R and RyR immunoreactivity is apparent in the calcineurin immunoprecipitate. Treatments with either FK506 or EGTA prevent the appearance of IP_3R or RyR in the

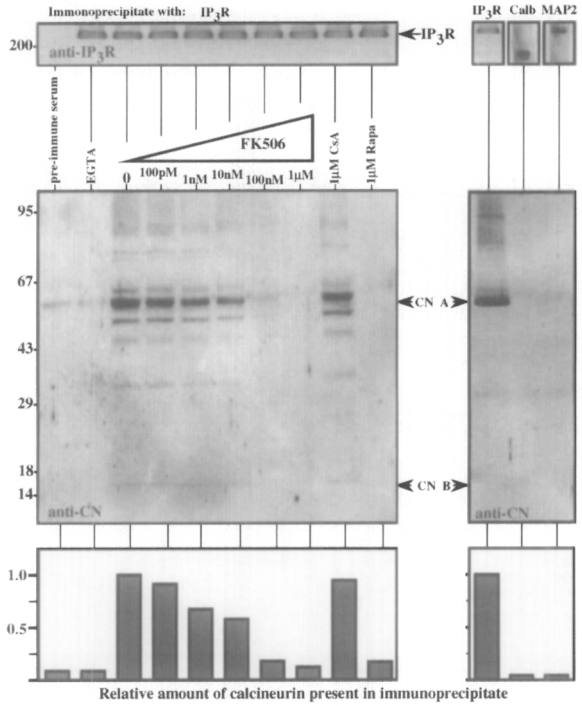

Figure 2. Coimmunoprecipitation of Calcineurin with IP_3R Is Ca^{2+} Dependent and FK506 Sensitive

A crude homogenate was prepared from rat cerebellum and used for coimmunoprecipitation assays. Immunoprecipitations were performed as described and analyzed via Western blot analysis as indicated. Immunoprecipitation with an antibody specific for IP_3R precipitates calcineurin (CN) (middle panel), as well as IP_3R (upper panel). The calcineurin coimmunoprecipitation is blocked by the inclusion of increasing amounts of FK506, rapamycin, or EGTA (20 mM), but not by cyclosporin A (CsA), whereas a constant amount of IP_3R is present in each immunoprecipitate. Relative intensity of immunoreactive calcineurin bands was quantified and is shown in the lower panel for each condition, giving an EC_{50} of dissociation of FKBP12 from IP_3R in the range of 10–100 nM FK506. Control immunoprecipitations using antibodies against calbindin and MAP-2 under identical conditions did not coimmunoprecipitate calcineurin (shown in panel on right).

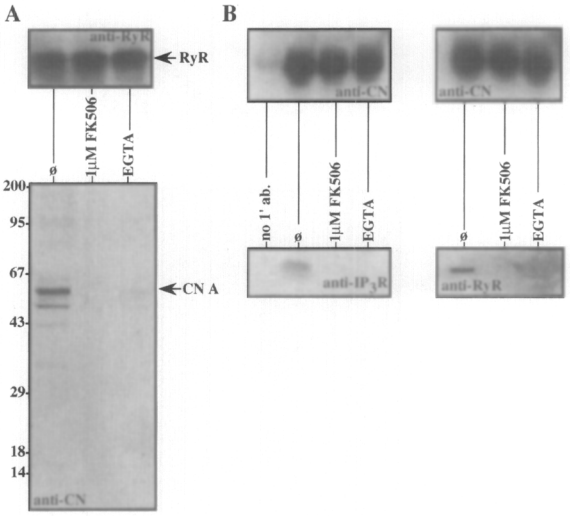

Figure 3. Calcineurin Coimmunoprecipitates with RyR in a Ca^{2+}-Dependent and FK506-Sensitive Manner

(A) Demonstrates an experiment identical to that shown in Figure 2, but performed with an antibody specific for RyR. Calcineurin (CN) coimmunoprecipitation (lower panel) with RyR (upper panel) is blocked by the inclusion of FK506 or EGTA (20 mM), whereas RyR is present in the immunoprecipitate in constant amounts.

(B) Shows an immunoprecipitation experiment using an antibody specific for calcineurin. While the upper panel demonstrates equal calcineurin content in the immunoprecipitates, the lower panel demonstrates that IP_3R or RyR will coimmunoprecipitate unless FK506 or EGTA (20 mM) is added.

immunoprecipitate. Probing the calcineurin immunoprecipitate with anti-MAP-2 or anti-calbindin antibodies via Western blot analysis shows no evidence of either of those two proteins in the calcineurin immunoprecipitate (data not shown).

Calcineurin Activity in IP_3R–FKBP12–Calcineurin Complex Regulates Phosphorylation Status of IP_3R

To ascertain whether calcineurin associated with the IP_3R–FKBP12 complex retains catalytic activity, we developed a substrate-specific assay for calcineurin phosphatase activity. We first used a known calcineurin substrate, MAP-2, phosphorylated by PKA. Addition of purified calcineurin together with Ca^{2+} and calmodulin markedly reduces phosphorylation of MAP-2 (Figure 4A). EGTA prevents this activity. Addition of FK506 and FKBP12 inhibits calcineurin activity, whereas FKBP12 alone has no effect. Likewise, addition of FK506 or rapamycin alone does not alter calcineurin activity (data not shown). This is consistent with previous observations that the FK506–FKBP12 complex, but neither molecule alone, inhibits calcineurin activity (Liu et al., 1991). Whereas rapamycin binds tightly to FKBP12, the complex does not interact with calcineurin (Liu et al., 1991). Addition of rapamycin and FKBP does not inhibit calcineurin activity in our assay. Cyclosporin A added with FKBP12 also does not inhibit calcineurin activity, consistent with the inability of cyclosporin A to bind to FKBP12. Analysis with a phosphorimager permits consistent quantification of the degree of phosphorylation of MAP-2 and thus determination of calcineurin activity against a specific substrate under varying experimental conditions (Figure 4B).

Our purified IP_3R preparations possess calcineurin activity (Figure 5A). In the presence of Ca^{2+} and calmodulin, IP_3R preparations dephosphorylate MAP-2 with the same activity as purified calcineurin (units of phosphatase activity per milligram of calcineurin added or present in preparation). EGTA blocks this dephosphorylation. Addition of cyclosporin A and cyclophilin also inhibits phosphatase activity, as does addition of calcineurin autoinhibitory peptide or deltamethrin, all of which are known to be potent inhibitors of calcineurin activity (Liu et al., 1991; Hashimoto et al., 1990; Enan and Matsumura, 1992). Treatment of calcineurin or IP_3R preparations with permethrin, a noninhibiting analog of deltamethrin (Enan and Matsumura, 1992), or okadaic acid, a protein phosphatase inhibitor not active against calcineurin at low concentrations (Bialojan and Takai, 1988), does not inhibit observed phosphatase activity. Pretreatment of IP_3R preparations with FK506

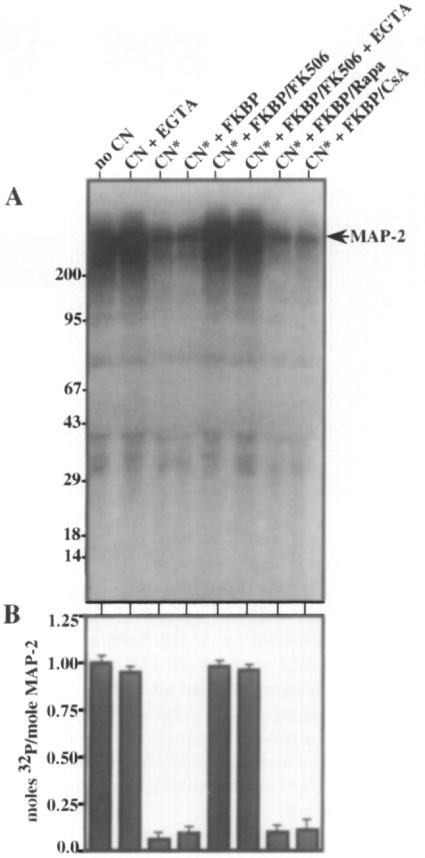

Figure 4. Measurement of Calcineurin Phosphatase Activity Toward a Specific Protein Substrate, Evaluated with MAP-2

MAP-2 was phosphorylated by PKA using radiolabeled ATP as described, and the reaction was stopped by addition of 10 μM Walsh inhibitor. Phosphorylated MAP-2 was then incubated with calcineurin (CN, 2 μM) for 30 min at 37°. Ca^{2+} (100 μM) and calmodulin (4 μM) (indicated by an asterisk), EGTA (20 mM), FKBP12 (1 μM), FK506 (1 μM), cyclosporin A (CsA, 1 μM), or rapamycin (1 μM) were included in the calcineurin reaction as indicated. The reactions were terminated with 5× sample buffer, and SDS–PAGE was performed. Gels were dried down and exposed on a Molecular Dynamics phosphorimager, which allowed identification of radiolabeled MAP-2 as shown in (A) and quantitation of MAP-2 phosphorylation as shown in (B). This experiment was repeated four times with essentially the same results.

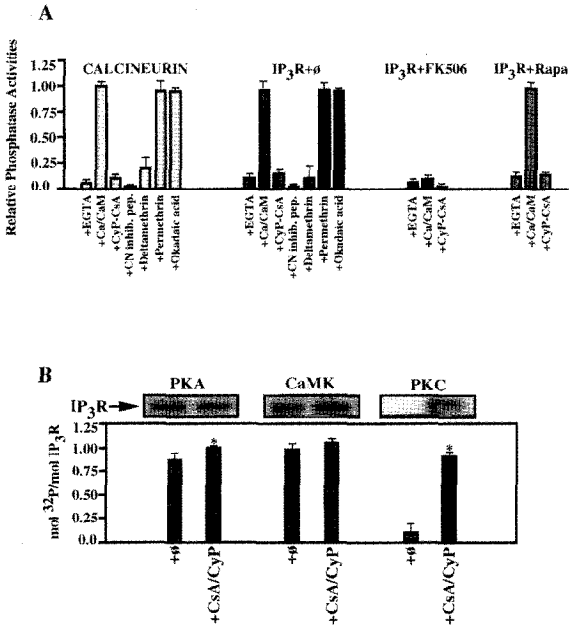

Figure 5. Purified Preparations of IP_3R Display Calcineurin Activity against MAP-2 and against Complexed IP_3R Itself

IP_3R was purified as described and assayed for calcineurin (CN) activity against MAP-2 as described above.

(A) shows that no pretreatment or pretreatment of the preparation with rapamycin did not affect calcineurin activity, indicating that complexed calcineurin is catalytically active. Pretreatment of preparations with FK506 (1 mM) inhibited calcineurin activity. Pure calcineurin, as well as phosphatase activity from IP_3R preparations, was sensitive to cyclosporin A (CsA, 1 mM), calcineurin autoinhibitory peptide (100 mM), and deltamethrin (1 nM). Phosphatase activity was not inhibited by permethrin (1 nM) or okadaic acid (500 nM), both of which are known to be inactive against calcineurin.

(B) shows phosphorylation of purified IP_3R with PKA, CaMKII, or PKC as indicated. The inclusion of a specific calcineurin inhibitor (1 mM cyclosporin A and 1 mM cyclophilin) modestly enhances phosphorylation by PKA and CaMKII and dramatically increases phosphorylation by PKC, demonstrating that IP_3R itself is a substrate for complexed calcineurin. The asterisk indicates a significant difference in the case of PKA and PKC ($p < .005$). This experiment was repeated four times with essentially the same results.

alone blocks the calcineurin activity seen in the complex. This is consistent with FK506 binding to the FKBP12 contained in the IP_3R complex and inhibiting activity of the associated calcineurin. By contrast, pretreatment of IP_3R preparations with rapamycin fails to inhibit the associated calcineurin activity. Treatment of the complex with rapamycin also disrupts the IP_3R–FKBP12 interaction and thus liberates calcineurin from the complex. The retention of calcineurin activity in these preparations is consistent with the failure of the rapamycin–FKBP12 complex to bind to and inhibit calcineurin.

IP_3R can be phosphorylated by PKA, CaMKII, and PKC, with the three enzymes phosphorylating at three distinct sites (Ferris et al., 1991). Purified IP_3R phosphorylated by PKA is a substrate for exogenously added calcineurin (data not shown). Also, purified IP_3R preparations display calcineurin activity toward PKA-phosphorylated IP_3R (data not shown). Accordingly, we directly examined whether complexed calcineurin is catalytically active toward associated IP_3R that has been phosphorylated by PKA, CaMKII, and PKC. Since active calcineurin is already in a complex with purified IP_3R, the effects of its phosphatase activity on IP_3R are difficult to study. Instead, we ascertained whether treatment with cyclosporin A and cyclophilin, which together inhibit calcineurin, would alter the phosphorylation status of phosphorylated IP_3R (Figure 5B). In the absence of cyclosporin A–cyclophilin treatment, the level of phosphorylation of IP_3R is about the same for PKA and CaMKII, but is much lower for PKC. This fits with our earlier studies in which it was very difficult to detect substantial phosphorylation of purified IP_3R with PKC (Supattapone et al., 1988b; Ferris et al., 1991). Treatment with cyclosporin A and cyclophilin increases the final observed

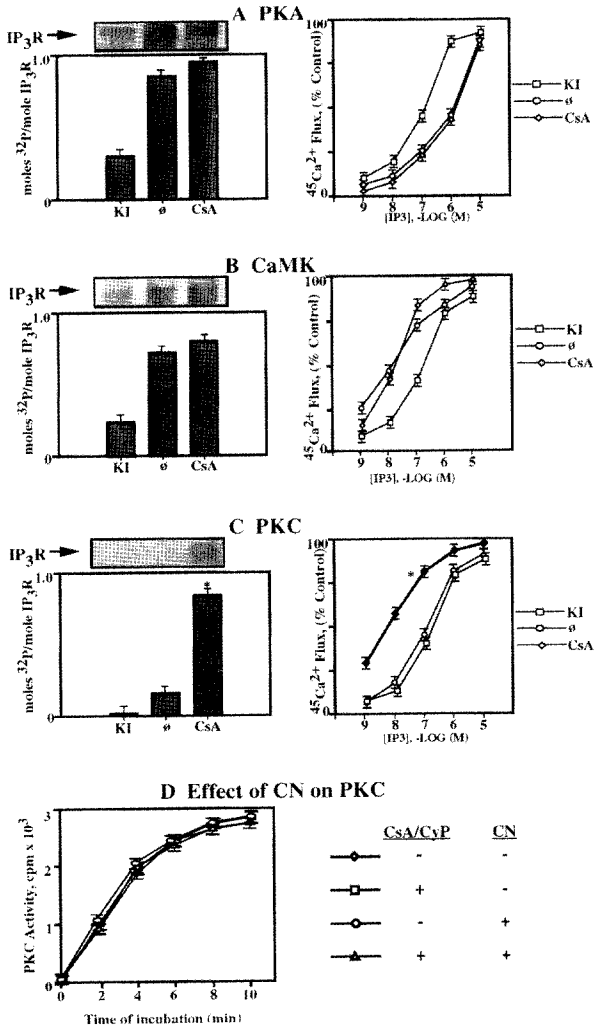

Figure 6. Effects of Calcineurin Inhibition on Enhancement of IP_3R Phosphorylation Parallel Actions on IP_3-Mediated Ca^{2+} Flux

Rat cerebellar microsomes were prepared as described and phosphorylated by PKA, CaMKII, or PKC as indicated. A specific kinase inhibitor, control vehicle, or calcineurin inhibitor (1 mM cyclosporin A [CsA] and 1 mM cyclophilin) was included in the phosphorylation reaction, and the final phosphorylation status of the IP_3R was determined (shown on the left) along with IP_3R-mediated Ca^{2+} flux properties (shown on the right) for each condition.

(A) shows that PKA phosphorylation of IP_3R resulted in a decreased potency of IP_3 in causing Ca^{2+} flux, but neither degree of phosphorylation nor Ca^{2+} flux characteristics were altered by inhibition of calcineurin.

(B) shows that CaMKII-phosphorylated membranes demonstrated an increased potency of IP_3-mediated Ca^{2+} flux, but calcineurin inhibition did not significantly alter phosphorylation status or Ca^{2+} flux characteristics of IP_3R.

(C) shows a pronounced enhancement of PKC phosphorylation of IP_3R by calcineurin inhibition, which was paralleled by a similar increase in the potency of IP_3 in fluxing Ca^{2+} from PKC-phosphorylated membranes in the presence of a calcineurin inhibitor. The asterisk indicates that only for PKC did inhibition of calcineurin result in a significant effect on IP_3R phosphorylation and Ca^{2+} flux. This experiment was repeated four times with essentially the same results.

(D) shows measurement of PKC catalytic activity toward a substrate, myelin basic protein, amino acids 4–14, which is not dephosphorylated by calcineurin. Thus, inclusion of activated calcineurin in the presence or absence of calcineurin inhibitors allows for assessment of the effect of calcineurin phosphatase activity on PKC kinase activity itself. No change in PKC activity was seen either with calcineurin activation or inhibition.

level of phosphorylation by PKC more than 10-fold, while only modestly increasing phosphorylation by PKA and CaMKII.

Phosphorylation and Calcineurin-Mediated Dephosphorylation Regulate IP_3R-Associated Ca^{2+} Flux

We examined the influence of phosphorylation status upon Ca^{2+} flux mediated by IP_3R utilizing cerebellar microsome preparations. We previously demonstrated IP_3-induced Ca^{2+} flux in purified IP_3R reconstituted into lipid vesicles (Ferris et al., 1989). However, the additional manipulations involved in phosphorylating the receptor protein degrade the Ca^{2+} flux system, precluding analysis of phosphorylation influences on Ca^{2+} flux using purified IP_3R in lipid vesicles. Microsome preparations made from rat cerebellum load Ca^{2+} in a time- and ATP-dependent fashion into IP_3-sensitive and IP_3-insensitive stores. IP_3-sensitive stores comprise approximately 60% of the total in these preparations, and addition of varying concentrations of IP_3 during loading will cause a net reduction in Ca^{2+} uptake via an outward flux through IP_3R. This IP_3-sensitive inhibition of loading is consistently and reproducibly observed and allows for assessing potency of submaximal doses of IP_3 in stimulating Ca^{2+} flux via IP_3R under a variety of experimental conditions (for further explanation of $^{45}Ca^{2+}$ flux assays, see Experimental Procedures). The potency of IP_3 in augmenting Ca^{2+} flux from microsomes is diminished in preparations treated with PKA when compared with matched preparations treated with PKA in the presence of Walsh inhibitor of phosphorylation (Figure 6A). These findings confirm our earlier observations (Supattapone et al., 1988b). In the same preparations, the Walsh inhibitor markedly diminishes levels of PKA phosphorylation of IP_3R. Treatment with cyclosporin A and cyclophilin only slightly increases phosphorylation levels and does not significantly affect the potency of IP_3 on Ca^{2+} flux.

Membranes phosphorylated by CaMKII display a greater potency for IP_3 in stimulating Ca^{2+} flux compared with membranes treated with peptide [Ala-286]CaMKII(281–302), a specific inhibitor of CaMKII (Hvalby et al., 1994). Cyclosporin A–cyclophilin only modestly increases CaMKII phosphorylation of IP_3R further and does not significantly alter IP_3 effects on Ca^{2+} flux (Figure 6B).

As observed previously, phosphorylation of purified IP_3R by PKC is much less than what is observed with PKA and CaMKII, although inhibition of this phosphorylation is observed with PKC(19–36), a specific PKC inhibitor (Hvalby et al., 1994). Cyclosporin A–cyclophilin markedly augments the PKC phosphorylation of IP_3R. Inhibition of PKC is associated with a slight but insignificant decrease in IP_3 potency in augmenting Ca^{2+} flux (Figure 6C). In contrast, treatment with cyclosporin A and cyclophilin causes a marked augmentation of IP_3 potency in stimulating Ca^{2+} flux, with the IC_{50} for IP_3 reduced about 20-fold. The control experiment shown in Figure 6D demonstrates that cal-

cineurin activity itself has no effect on the catalytic activity of PKC. Therefore, in our experiments enhancement of IP_3R phosphorylation by PKC following calcineurin inhibition must be due to an inhibition of calcineurin phosphatase activity toward phosphorylated IP_3R.

Thus, phosphorylation by both PKC and CaMKII increases the potency of IP_3 in stimulating Ca^{2+} flux, while PKA phosphorylation decreases potency. Most strikingly, calcineurin complexed with IP_3R appears to be a major regulator of the PKC phosphorylation and Ca^{2+} conductance of IP_3R, as inhibition of calcineurin by cyclosporin A–cyclophilin markedly influences IP_3 effects on Ca^{2+} flux in PKC-phosphorylated preparations.

Discussion

The major finding of the present study is that calcineurin is physiologically associated in a physical and functional complex with IP_3R and FKBP12. Both copurification and coimmunoprecipitation experiments support this linkage. The association is selectively disrupted by FK506 and rapamycin, but not by cyclosporin A. These data imply that calcineurin is anchored to IP_3R via FKBP12 (as is shown in Figure 7). However, we have not ruled out interactions of calcineurin with IP_3R at a site removed from that of FKBP12 binding. In the latter model, the dissociation of calcineurin from the IP_3R complex caused by rapamycin would be secondary to allosteric changes initiated by FKBP12 removal. The linkage appears to be Ca^{2+} dependent, being inhibited by Ca^{2+}-chelating agents. RyR also occurs in a complex with calcineurin and FKBP12, which is rapamycin and Ca^{2+} sensitive. RyR-mediated Ca^{2+} flux activity is regulated via phosphorylation by Ca^{2+}-activated kinases (Hain et al., 1995), and it will be interesting to determine whether complexed calcineurin regulates RyR function as it does for IP_3R.

In the IP_3R complex, calcineurin retains its catalytic activity toward known substrates such as MAP-2, and, more importantly, it regulates the phosphorylation status of IP_3R itself. This is most evident for phosphorylation of IP_3R by PKC, which is barely demonstrable in the absence of calcineurin inhibition.

Interestingly, phosphorylation by the different kinases varies in effects on Ca^{2+} flux, with PKC and CaMKII augmenting IP_3 potency and PKA phosphorylation decreasing potency. Zhang et al. (1993) observed that low concentrations of Ca^{2+} augment the stimulation by IP_3 of Ca^{2+} flux in permeabilized fibroblasts, an effect abolished by CaMKII inhibitors. This is consistent with our findings that CaMKII phosphorylation increases IP_3 potency in stimulating Ca^{2+} flux. Zhang et al. (1993) also observed that high concentrations of Ca^{2+} inhibit IP_3 stimulation of Ca^{2+} flux and that this effect is reversed by FK506, suggesting that with higher Ca^{2+} concentrations calcineurin activity decreases IP_3 effects. Since PKC and CaMKII are the two kinases that stimulate IP_3 actions, presumably the calcineurin inhibition of flux reflects dephosphorylation of PKC- or CaMKII-phosphorylated sites on IP_3R (or both). Our findings that the PKC site is much more sensitive to calcineurin than the CaMKII site strongly imply that FK506 primarily blocks dephosphorylation of a PKC site.

The IP_3R displays a bell-shaped Ca^{2+}-sensitive response to IP_3, with low levels of Ca^{2+} (<300 nM) enhancing IP_3-stimulated Ca^{2+} release and higher concentrations (>300 nM) inhibiting Ca^{2+} release (Iino, 1990). This biphasic response allows for both a positive and negative feedback of Ca^{2+} levels on IP_3R-mediated Ca^{2+} flux and is known to be critical for Ca^{2+} wave propagation that is observed in many cell types when stimulated by diverse agonists (Bezprozvanny et al., 1991; Finch et al., 1991; Miyazaki et al., 1992; for review of Ca^{2+} oscillations, see Berridge and Galione, 1988; Clapham, 1995). From their results, Muellam and colleagues have suggested that the opposite effects of low and high Ca^{2+} concentrations on IP_3-stimulated Ca^{2+} flux might result from differential activation of Ca^{2+}-activated kinases and phosphatases (Zhao and Muallem, 1990; Zhang et al., 1993). Our observations that Ca^{2+}-regulated kinases and phosphatases modulate IP_3R in a

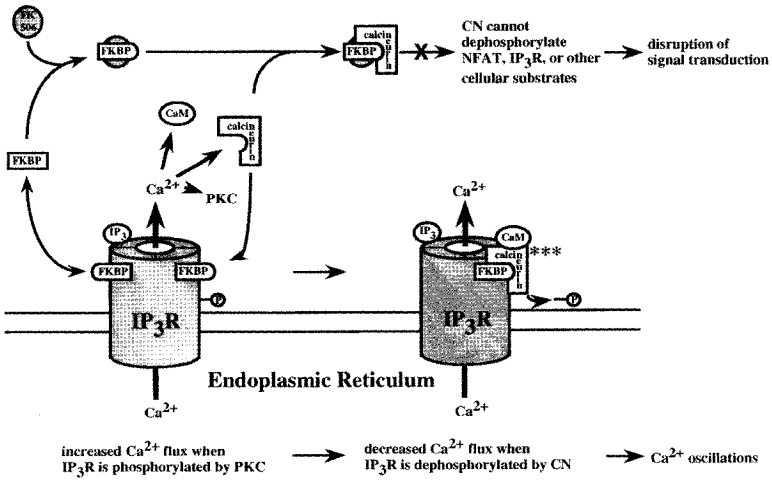

Figure 7. Calcineurin Is Associated with IP_3R via an FKBP12 Anchor in a Ca^{2+}-Sensitive Manner

The figure shows the IP_3R–FKBP12 complex in the endoplasmic reticulum membrane. When the receptor is activated by IP_3, Ca^{2+} is liberated from the endoplasmic reticulum lumen via the IP_3R channel, and the local cytoplasmic $[Ca^{2+}]$ increases. This increase results in activation of PKC, which phosphorylates the IP_3R and further increases IP_3R-mediated Ca^{2+} flux. Increased Ca^{2+} levels also activate calmodulin and calcineurin (CN), causing an association of calcineurin with the IP_3R–FKBP12 complex via FKBP12 and activation of the phosphatase activity of calcineurin. This activation results in dephosphorylation of the PKC-phosphorylated site on IP_3R and a decrease in IP_3R-mediated Ca^{2+} flux. The cyclic increases and decreases generated by this macromolecular complex may mediate Ca^{2+} oscillations.

protein complex provide a potential molecular basis for Ca^{2+} regulation of IP_3R and for some aspects of the generation of Ca^{2+} oscillations. Besides phosphorylation by PKA, CaMKII, and PKC, IP_3R can autophosphorylate as well as phosphorylate exogenous substrates (Ferris et al., 1992). The existence of kinase and phosphatase in a single molecular complex with IP_3R provides a means for exquisitely modulated, rapid phosphorylation and dephosphorylation of IP_3R, which regulates its Ca^{2+} conductance. We propose that such influences participate in the fine-tuning of intracellular Ca^{2+} dynamics. Whether PKC or other kinases occur bound to the same complex as IP_3R, FKBP12, and calcineurin remains to be determined.

Ca^{2+} effects on IP_3R function had also been implicated by the inhibition of $[^3H]IP_3$ binding to IP_3R in brain membranes by low Ca^{2+} concentrations (Supattapone et al., 1988a). The failure of Ca^{2+} to inhibit $[^3H]IP_3$ binding to purified IP_3R implied the existence of a tissue constituent, designated "calmedin," that conferred Ca^{2+} effects on IP_3R and was lost during receptor purification (Danoff et al., 1988). Other studies suggested that Ca^{2+} inhibits $[^3H]IP_3$ binding to IP_3R in membranes by activating phospholipase C to generate IP_3, which dilutes added $[^3H]IP_3$ (Mignery et al., 1992). While those studies may explain some aspects of observed Ca^{2+} effects on IP_3-mediated Ca^{2+} release, the bell-shaped response of IP_3R to Ca^{2+} is still poorly understood and is perhaps clarified by the observed association of FKBP and calcineurin with IP_3R.

The influences of phosphorylation of membranes on IP_3-stimulated Ca^{2+} flux cannot be ascribed definitively to phosphorylation of IP_3R. Conceivably, phosphorylation of other proteins could indirectly affect IP_3R function. However, there is a close relationship between the differential effects of phosphorylation of IP_3R by the three kinases and dephosphorylation by calcineurin with actions on Ca^{2+} flux, which strongly implies that the observed changes in Ca^{2+} flux reflect alterations in the phosphorylation status of IP_3R.

Our findings provide a molecular mechanism for a closely linked phosphorylation–dephosphorylation regulation of IP_3R and presumably of RyR. Dynamic regulation of protein function by phosphorylation and dephosphorylation is a ubiquitous phenomenon (Hunter, 1995), and in other instances the kinase and phosphatase occur in a molecular complex with their substrates to ensure fidelity of protein phosphorylation (Hubbard and Cohen, 1993). Calcineurin can be localized to key neuronal substrates at postsynaptic densities by association with the A kinase anchoring protein, AKAP79 (Coghlan et al., 1995). Interestingly, the calcineurin-binding domain identified within AKAP79 resembles the domain within FKBP12 known to participate in the interaction with calcineurin in the presence of FK506 (Aldape et al., 1992). For the calcineurin–IP_3R–FKBP12 complex, FKBP12 may function analogously to AKAP79, anchoring a phosphatase, in this case calcineurin, to an appropriate substrate, in this case IP_3R (Figure 7).

The high affinity and specificity of FK506–FKBP12 interactions and the role of the drug as "molecular glue" between FKBP12 and calcineurin suggests that FK506 mimics an endogenous protein ligand that physiologically links FKBP12 and calcineurin. Consistent with these notions, extensive modeling of FK506 has revealed structural similarities of the drug to the transition state structures for *cis–trans* isomerization of leucylprolyl and valylprolyl bonds, which are also the optimal rotamase substrates for FKBP12 (Ikeda et al., 1994; Albers et al., 1990). Our findings that calcineurin interacts with FKBP12 in a physiologic way via the IP_3R–FKBP12 and RyR–FKBP12 complexes suggest that IP_3R and RyR have such "FK506-like" peptide domains within their structure, presumably in a region of high shared homology between the two receptors. Using the yeast two-hybrid system, we have recently identified the FKBP-binding domain within IP_3R and have characterized residues critical for the IP_3R–FKBP interaction (A. M. C. and S. H. S., unpublished data). Fleischer and colleagues (Timerman et al., 1995) showed that FKBP12 mutants lacking rotamase activity modulate Ca^{2+} conductance of RyR as effectively as native FKBP12. This suggests that some activity of FKBP12 other than prolyl isomerization accounts for its FK506-sensitive effects on RyR Ca^{2+} conductance. The anchoring of calcineurin to IP_3R and RyR by FKBP12, which enables the phosphatase activity of calcineurin to modulate channel Ca^{2+} conductance, may be the means whereby FK506 influences Ca^{2+} conductance. Thus, the proposed physiologic role of FKBP12 in its complex with calcineurin and the IP_3R or RyR would be to facilitate the cycle of receptor phosphorylation–dephosphorylation, which in turn would modulate Ca^{2+} conductance and participate in the generation of Ca^{2+} oscillations (Figure 7).

FKBP12 was first identified in T cells to explain the immunosuppressant actions of FK506. Might our present findings account for aspects of T cell physiology? Antigen stimulation of T cells activates phospholipase C to generate IP_3. This results in an IP_3R-mediated Ca^{2+} elevation, which enhances calcineurin phosphatase activity toward NFAT, permitting its entry into the nucleus and initiation of IL-2 transcription (Jayaraman et al., 1995). This elevation in cytoplasmic Ca^{2+} (which at the cell population level can be observed to persist for hours) is thought to reflect the summation of asynchronous Ca^{2+} oscillations in individual cells (Lewis and Cahalan, 1989). The persistent oscillatory response seems to be necessary for induction of IL-2 transcription (Goldsmith and Weiss, 1988). When FKBP12 was first identified, it was proposed as a modulator of ion channels involved in the signal transduction pathway of T cell activation (Siekierka et al., 1989; Harding et al., 1989). Upon stimulation of T cells calcineurin moves from a freely cytosolic to a membrane-bound form (Chantler, 1985). Our data imply that FKBP12 functionally links calcineurin activity with modulation of IP_3R, so that even the low levels of FK506 given therapeutically, which are sufficient for calcineurin inhibition, would alter IP_3R Ca^{2+} conductance as well as inhibit calcineurin activity toward other cellular substrates such as NFAT (Figure 7). Furthermore, the recent demonstration of the presence and participation of RyR in T cell activation (Hakamata et al., 1994)

is consistent with the notion of physically associated and spatially restricted Ca^{2+}-activated kinases and phosphatases modulating IP_3R–FKBP12 and RyR–FKBP12 complexes to generate Ca^{2+} oscillations and resultant physiologic responses.

Experimental Procedures

FPLC

IP_3R was purified as described previously (Ferris et al., 1989) and concentrated in Amicon Centriprep-100 concentrators. Concentrated IP_3R was run on an FPLC Superose 6 gel filtration column in a buffer of 250 mM NaCl, 1% CHAPS, 50 mM Tris (pH 7.4) at a flow rate of 0.2 ml/min, and 1 ml fractions were collected as described previously (Cameron et al., 1995). Purified calcineurin was obtained from Sigma, resuspended in running buffer as above, and fractionated by FPLC identically. Treatment of IP_3R with rapamycin during preparation involved addition of 1 μM final concentration rapamycin to IP_3R before concentration and inclusion of 1 μM rapamycin in FPLC running buffer. Samples were then loaded for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized by Western blot analysis.

Immunoprecipitations

Crude cerebellar membrane homogenates were prepared as follows: rat cerebella were removed and homogenized in a buffer of 50 mM Tris (pH 7.4), 1 mM EDTA, 1 mM BME, 0.1 mg/ml leupeptin, 1 mg/ml pepstatin, and 0.1 mg/ml PMSF. The homogenate was centrifuged at 45,000 × g for 10 min. The pellet was resuspended in buffer without BME, solubilized in 1% CHAPS for 20 min, and recentrifuged at 45,000 × g for 20 min. The supernatant (1 ml) was then used as starting material for immunoprecipitation reactions and was treated with final concentrations of 100 μM Ca^{2+}, 20 mM EGTA, varying concentrations of FK506, rapamycin, or ethanol control vehicle for 4 hr at room temperature and then primary antibody or preimmune serum for 1 hr at 4° as indicated. Where indicated, IP_3R antibody was included at 1 μg/ml, RyR antibody (Affinity Bioreagents) was included at 1 μg/ml, calbindin antibody (SWant) was included at 1 μg/ml, MAP-2 antibody (Sternberger Monoclonals) was included at 1 μg/ml, and calcineurin antibody (Upstate Biotechnology) was included at 1 μg/ml. Protein G–Sepharose (Pharmacia) was used to precipitate IP_3R antibody and protein A–Sepharose (Oncogene Science) was used to precipitate RyR, MAP-2, calbindin, and calcineurin antibodies. Immunoprecipitates were washed two times each with PBS plus 1% CHAPS, PBS plus 1% CHAPS plus 0.5 M NaCl, and PBS, loaded for SDS–PAGE and subjected to Western blot analysis. Quantification of immunoreactive calcineurin band was performed by Adobe Photoshop analysis of mean pixel intensity within a standard area containing each band. The value of the intensity of the calcineurin band obtained with no FK506 added was arbitrarily set at 1.0 and the intensity of the other bands are reported relative to that.

Western Blotting Analysis

Fractions obtained from FPLC or immunoprecipitation were analyzed by conventional Western blot analysis using either an antibody specific for IP_3R (Sharp et al., 1993) at a dilution of 1:10,000, an antibody specific for calcineurin (Upstate Biotechnology) at a dilution of 1:4,000, or an antibody specific for RyR (Affinity Bioreagents) at a dilution of 1:4,000, as recommended. For Western blot analysis, anti-MAP-2 (Sternberger Monoclonals) and anti-calbindin (SWant) antibodies were both used at a dilution of 1:1,000.

Calcineurin Assay

Approximately 10 μg of purified MAP-2 was phosphorylated with 50 U of PKA (Sigma) and 10 μM [^{32}P]ATP (Amersham) at 6000 Ci/mmol. The phosphorylation reaction was allowed to proceed for 1 hr at 37°C and then was stopped by inclusion of 10 μM Walsh inhibitor peptide. Phosphorylated MAP-2 was then used as a substrate in a calcineurin assay, which typically involved a reaction volume of 100 μl and contained either 20 mM EGTA, 100 μM Ca^{2+}, 4 μM calmodulin (Sigma), 1 μM human recombinant FKBP (Sigma), 1 μM FK506, 1 μM rapamycin, 1 μM cyclosporin A, or 1 μM human recombinant cyclophilin (Sigma) as indicated in a buffer of 100 mM NaCl, 50 mM Tris (pH 7.4), 5 mM DTT, and 10 mM $MgCl_2$. We included 2 μM calcineurin (Sigma) or 10 μg of purified IP_3R pretreated for 1 hr at room temperature with 1 μM immunosuppressant or control vehicle, as indicated. Calcineurin autoinhibitory peptide (100 μM), deltamethrin (1 nM), permethrin (1 nM), or okadaic acid (500 nM; all from Biomol) were included as indicated. Reactions were allowed to proceed for 30 min at 37°C and then were stopped with SDS–PAGE sample buffer and loaded for PAGE. Gels were dried down and then analyzed by phosphorimager. Quantification of MAP-2 phosphorylation status was determined by the ImageQuant program and reported as shown.

Phosphorylation Reactions

Purified IP_3R or microsomal preparations were phosphorylated as described previously (Ferris et al., 1991), except that PKC was obtained from Promega and CaMKII was provided by J. P. S. Reactions included either 10 μM peptide inhibitor, control vehicle, or 1 μM cyclophilin (Sigma) and 1 μM cyclosporin A.

IP_3R Ca^{2+} Flux Assays

Rat cerebellar microsomes were prepared as described previously (Verma et al., 1992), phosphorylated with cold ATP under conditions described above, and assayed for IP_3-sensitive $^{45}Ca^{2+}$ flux as described previously (Verma et al., 1992). Cerebellar microsomes take up $^{45}Ca^{2+}$ in a time- and ATP-dependent way, and in a typical assay Ca^{2+} loading plateaus at 250 nmol of Ca^{2+} per milligram of protein. Free Ca^{2+} levels are determined by using a Ca^{2+}-sensitive electrode (Orion) and are titrated to 100 nM by addition of EGTA. Free Ca^{2+} is spiked with $^{45}Ca^{2+}$ to give 100,000 cpm per assay. Total microsome loading is typically around 20,000 cpm after an A23187 (LC Services) blank is subtracted, which is typically around 200 cpm. Treatment with the ionophore A23187 allows for complete equilibration of Ca^{2+} and establishment of background counts per minute in the absence of loading. This net uptake is inhibited in these preparations by releasing $^{45}Ca^{2+}$ via the IP_3R with the addition of increasing concentrations of IP_3. Maximal concentrations of IP_3 (10 μM) typically inhibit this total Ca^{2+} loading 60%, giving a typical signal of 12,000 cpm for IP_3-sensitive stores. Treatment of microsomes with submaximal doses of IP_3 reliably and reproducibly inhibits Ca^{2+} loading, from which an IC_{50} for IP_3 inhibition of Ca^{2+} loading can be calculated under a variety of experimental conditions. Thus, in these experiments, IP_3-mediated inhibition of microsome $^{45}Ca^{2+}$ uptake is reported as $^{45}Ca^{2+}$ flux. Inclusion of 10 μM IP_3 results in approximately 60% inhibition of total $^{45}Ca^{2+}$ uptake (the remaining 40% is loaded into IP_3-insensitive pools), and no further inhibition of loading is seen with higher concentrations of IP_3. This maximal release value is reported as 100% Ca^{2+} flux, and the inhibition seen with submaximal concentrations of IP_3 is reported as a percentage of that maximum control. The IP_3 concentration at which a 50% inhibition of loading of this IP_3-sensitive Ca^{2+} pool is observed in replicates of three and is reported as IC_{50} and can be compared under varying conditions of phosphorylation as shown in Figure 6.

PKC Activity Assay

A standard assay of PKC catalytic activity was performed as described previously (Yasuda et al., 1990), which employed a PKC substrate (myelin basic protein, amino acids 4–14; Upstate Biotechnology) that is not a substrate for calcineurin phosphatase activity. Calmodulin (Sigma) was included at 4 μM, and where indicated calcineurin (Sigma) was included at 2 μM, cyclosporin A at 1 μM, and human recombinant cyclophilin (Sigma) at 1 μM. In this way the effect of activated or inhibited calcineurin on PKC activity could be independently assessed. PKC activity was assayed under four conditions as listed that examined the effect of both activated and inhibited calcineurin, as well as controlled for the presence of a calcineurin inhibitor.

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