Phosphorylation of calmodulin on Tyr⁹⁹ selectively attenuates the action of calmodulin antagonists on type-I cyclic nucleotide phosphodiesterase activity

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Tyr⁹⁹ phosphorylation of calmodulin appears to induce a distinct conformational change as is evident from the profound attenuation of the Ca²⁺-induced enhancement of calmodulin's mobility seen during SDS/PAGE. The effect of this conformational change appears to be localized, in that both calmodulin and P-Tyr⁹⁹-calmodulin show identical dose-dependent activation profiles for stimulation of a physiological effector, type-I (Ca²⁺/calmodulin-stimulated) cyclic nucleotide phosphodiesterase (PDE) activity and their presence engenders similar dose-dependent PDE activation by Ca²⁺. In marked contrast with this, with P-Tyr⁹⁹-calmodulin there were 3–4-fold increases in the IC₅₀ values for inhibition of type-I PDE activity by the calmodulin antagonists TFP and W7, together with increased

values for Hill coefficients for inhibition. The polybasic compound poly(L-lysine) potentially augmented the action of calmodulin as a PDE activator, causing an approx. 7-fold decrease in the EC₅₀ value for activation of PDE. It is suggested (i) that the Tyr⁹⁹ phosphorylation of calmodulin, which occurs within a high-affinity Ca²⁺-binding domain, induces a localized conformational change in this peptide which can selectively attenuate the action of calmodulin antagonists on type-I PDE activity while leaving unaffected Ca²⁺-dependent activation, and (ii) that polybasic substances on complexing with calmodulin may serve to enhance the sensitivity of type-I PDE to activation by this regulatory peptide.

INTRODUCTION

Cyclic AMP is a ubiquitous second messenger which plays a pivotal role in controlling the functioning and development of many cells. Synthesis of cyclic AMP is achieved by a family of adenylate cyclases of which up to eight members have so far been identified (Krupinski et al., 1992). The activity of these enzymes is regulated by both stimulatory and inhibitory G-proteins, which are coupled to appropriate receptors (Birnbaumer et al., 1990; Houslay, 1990a,b; Strosberg, 1991). The only known means of degrading the second messenger cyclic AMP is through the action of cyclic AMP phosphodiesterases (PDEs) (Beavo, 1990; Houslay and Kilgour, 1990). However, in some cells, the extrusion of cyclic AMP can play a role also in signal termination (Brunton and Mayer, 1979). PDEs can be categorized into a number of distinct families based upon sequence similarity and biochemical properties (Beavo, 1990). One important family is the so-called type-I or Ca²⁺/calmodulin-activated PDE (Wang et al., 1990). This activity, along with that of a number of other PDEs, is expressed in most, if not all, cell types where it plays a significant role in determining signalling via cyclic nucleotides (Wang et al., 1990; Houslay and Kilgour, 1990; Houslay, 1990a).

Calmodulin is a highly acidic species composed of four structurally related Ca²⁺-binding domains (Goodman, 1981; Means et al., 1982; Babu et al., 1988; Klee, 1988). This ubiquitous protein serves to mediate the actions of Ca²⁺ within cells. A number of studies have shown, however, that calmodulin can be tyrosyl phosphorylated in various cell types after transformation with *src* (Fukami et al., 1986) and also that insulin can increase its tyrosyl phosphorylation in both adipocytes and hepatocytes (Colca et al., 1987; Sacks et al., 1992). Furthermore, solubilized

insulin receptor preparations can elicit the tyrosyl phosphorylation of calmodulin (Graves et al., 1986; Fujita-Yamaguchi, 1989a,b; Sacks et al., 1989; Sacks and McDonald, 1989) in an insulin-stimulated fashion through a process which is dependent on the presence of polybasic compounds (Sacks et al., 1989) able to form an appropriate complex with calmodulin (Saville and Houslay, 1993). Such an action leads to the modification of one of the two tyrosine residues present in calmodulin, namely Tyr⁹⁹ (Laurino et al., 1988; Saville and Houslay, 1993), which lies in the third Ca²⁺-binding domain of calmodulin (Babu et al., 1988). The significance of tyrosyl phosphorylation of calmodulin in intact cells has been questioned (Blackshear and Haupt, 1989) due to its apparently low occurrence (Fukami et al., 1986; Colca et al., 1987), although the specific requirements for a polybasic substance to be present for it to occur (Sacks et al., 1989; Saville and Houslay, 1993) may indicate that tyrosyl phosphorylation of calmodulin is an extremely localized, rather than a widespread event.

To our knowledge, no attempt has been made to determine whether such a specific covalent modification exerts any action upon a calmodulin-sensitive target protein. Here we examine the ability of P-Tyr⁹⁹-calmodulin to regulate the activity of a type-I, Ca²⁺/calmodulin-activated cyclic AMP phosphodiesterase (type-I PDE).

MATERIALS AND METHODS

Materials

Insulin and calcium antagonists were obtained from Sigma, U.K. and radiochemicals were from Amersham, U.K. All other biochemicals were from Boehringer Ltd., U.K. and other chemi-

Abbreviations used: PDE, cyclic nucleotide phosphodiesterase; type-I PDE, Ca²⁺/calmodulin-stimulated cyclic nucleotide phosphodiesterase; TFP, trifluoroperazine; P-Tyr⁹⁹-calmodulin, monophosphorylated calmodulin on Tyr⁹⁹.

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cals were of AR grade from BDH Ltd., U.K. Kodak XR 320 film was from Kodak, Liverpool, U.K. 'Ecoscint' scintillation fluid was obtained from National Diagnostics, Aylesbury, Bucks., U.K.

Preparation of purified insulin receptor

Human placental membranes were used as a source of insulin receptor. The membranes were prepared and the Triton X-100-solubilized receptor subsequently purified as described by Soos et al. (1986) and O'Brien et al. (1986, 1987a,b). This was done by the specific immunoabsorption of the insulin receptor using a monoclonal antibody which binds to a specific epitope on the cytosolic surface of the receptor β -subunit (a kind gift from Professor K. Siddle, Department of Clinical Biochemistry, University of Cambridge, Cambridge, U.K.).

Phosphorylation assay

Partially purified insulin receptors (approx. 100 units of insulin-binding activity; approx. 200 μ g of protein) and an equivalent amount (in terms of the weight of cellulose) of immobilized control IgG, which had been incubated with the same placental extract, were pre-incubated separately for 30 min at 22 °C. Pre-incubations were carried out in a final volume of 2.4 ml of 50 mM Hepes, pH 7.6/0.1% (w/v) Triton X-100/1.3 mM EGTA/133.3 nM insulin/0.3 μ M poly(L-lysine) (molecular mass 65 kDa). Samples (600 μ l) of both reaction mixtures were then removed and added to 200 μ l of Mg^{2+} /ATP containing 30–60 μ Ci of [γ - 32 P]ATP in 100 mM Hepes, pH 7.6, to give final concentrations of 5 mM Mg^{2+} and 250 μ M ATP. The remainder of the pre-incubation mixture was then made up to the same Mg^{2+} and ATP concentrations by addition of 600 μ l of 20 mM Mg^{2+} and 1 mM unlabelled ATP. All of the mixes were incubated in parallel for 4 h at 30 °C. Reactions were terminated, on ice, by addition of 600 μ l of 2 M NaCl to unlabelled incubations [the resultant solution in the absence of insulin-receptor poly(L-lysine) and calmodulin is hereafter referred to as high-salt phosphorylation buffer] and 200 μ l of 2 M NaCl to incubations spiked with [γ - 32 P]ATP. NaCl disrupts the interaction between calmodulin and poly(L-lysine) (Fujita-Yamaguchi et al., 1989b; Saville and Houslay, 1993). The samples were shaken for 20 min at 4 °C, followed by centrifugation at 12000 *g* for 15 min at 4 °C to pellet the immobilized insulin receptor. To remove poly(L-lysine), Dowex-50W was added to the sample [10 μ l of Dowex-50W, 1:1 slurry Dowex/high-salt phosphorylation buffer, for every 100 μ l of calmodulin/poly(L-lysine)]. The mixture was vortexed and the Dowex allowed to settle five times. The sample was then centrifuged for 2.5 min at 14000 *g* to precipitate the resin and the supernatant used to assay PDE activity. The supernatants could be stored as 50–100 μ l aliquots at –80 °C.

When the stoichiometry of substrate phosphorylation was to be determined at least three aliquots of the [32 P]ATP/ Mg^{2+} mix were diluted 5-fold for storage and 10 μ l of each of these dilutions was then counted at the same time as the gel chips. This enabled phosphate incorporation into the substrate to be quantified.

Phosphoamino acid analysis and thrombin digestion of calmodulin

The phosphorylated calmodulin was analysed by the method of Cooper et al. (1983) and shown to be due entirely to modification of tyrosine residues. Thrombin digestion of phosphorylated calmodulin was performed by the method of Wall et al. (1981) to generate two fragments, each of which contained one unique

tyrosine residue. Only one of these was phosphorylated, showing that monotyrosyl phosphorylation of Tyr⁹⁹ only occurred.

SDS/PAGE

SDS/PAGE was performed as described by Laemmli (1970) using a 15% (w/v) acrylamide resolving gel and gels stained using Coomassie Blue. The biotinylated pre-stained proteins used as markers were myosin H-chains (200 kDa), phosphorylase b (97.4 kDa), BSA (68 kDa), ovalbumin (43 kDa), α -chymotrypsinogen (25 kDa), β -lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa). In experiments done to assess the action of Ca^{2+} on calmodulin mobility stacking and resolving gels and running buffer were made to a concentration of 0.1 mM in EDTA or Ca^{2+} as indicated, as described by Burgess et al. (1980).

Protein assay

This was carried out according to the method of Peterson (1977).

2,4-[3,5- 3 H]Dinitrofluorobenzene (DNFB) assay

This was used as an assay for poly(L-lysine) and calmodulin in poly(L-lysine)/calmodulin mixtures. The sample volume was made up to 1 ml with water and 10 μ l of deoxycholate added to each tube, followed by 300 μ l of 24% (w/v) trichloroacetic acid. After 10 min the mixture was centrifuged for 20 min at 12000 *g* and the supernatant was removed. Sodium bicarbonate, pH 9.8, (40 μ l; 0.5 M) containing 14% (w/v) SDS was added to the pellet followed by 10 μ l of [3 H]DNFB (2 μ Ci dissolved in ethanol, made immediately before use) and vortexed immediately before incubation for 40 min at 37 °C. Reactions were terminated by addition of 5 μ l of undiluted 2-mercaptoethanol and samples were incubated for a further 40 min at 37 °C. An aliquot (30 μ l) of the final mixture was spotted on to Whatman 3MM filter paper, which had been soaked in 10% (w/v) trichloroacetic acid and allowed to dry. The filters were immersed in 200 ml of cold 10% (w/v) trichloroacetic acid and shaken for 30 min at room temperature, followed by a second 30 min wash in 200 ml of 10% (w/v) trichloroacetic acid and two 30 min washes in 200 ml of ethanol. The filters were allowed to dry and 12 ml of Ecoscint was added to each, followed by scintillation counting.

Preparation of type-I PDE activity

This was done using rat hepatocytes as a source, as described by Lavan et al. (1989). Such a procedure leads to a partially purified enzyme preparation being isolated, which can hydrolyse both cyclic AMP and cyclic GMP, although cyclic GMP appears to be the preferred substrate, and whose hydrolysis of these cyclic nucleotides is stimulated approx. 4-fold by the addition of 50 μ M Ca^{2+} /10 nM calmodulin. This activity is free of any other contaminating PDE activity (Lavan et al., 1989). In hepatocytes this type-I PDE is found (Lavan et al., 1989) exclusively in the cytosol together with various cyclic AMP-specific type-IV PDEs (Houslay, 1990a). No such activity is found to be membrane-associated, although further type-IV PDEs, as well as cyclic GMP-stimulated type-II and cyclic GMP-inhibited type-III enzymes, are localized to various cellular membranes (Houslay and Kilgour, 1990). The relative importance of such isoforms has been examined using specific inhibitors and computer simulation studies (Houslay, 1990a).

Assay of PDE activity, for which similar results were obtained using either cyclic AMP or cyclic GMP as substrates, was carried out by a modification (Rutten et al., 1973; Marchmont and Houslay, 1980) of the method of Thompson and Appleman

(1971). Unless stated otherwise, before assay of calmodulin-stimulated PDE activity, poly(L-lysine) was separated from calmodulin, which had been subjected to the phosphorylation treatment, using Dowex-50W ion-exchange resin as described above. In all experiments less than 10% of the substrate was hydrolysed, under which conditions linear initial rates were observed. Unless otherwise indicated, final concentrations in the assays were 50 mM Hepes, final pH 7.45, 1 μ M cyclic GMP/cyclic AMP, 10 mM EDTA, 0.19 mM EGTA, 100 μ M free Ca²⁺, 3.5 mM free Mg²⁺, 40 μ M ATP, 125 mM NaCl, 2.5% (v/v) glycerol and the indicated calmodulin concentration. For studies examining the inhibition of type-I PDE activity by calmodulin antagonists, then the final concentration of calmodulin was 3.53 nM. As the affinity of calmodulin for Ca²⁺ is in the micromolar range and Ca²⁺ contamination of buffers is likely to be approx. 20 μ M (Fabiato and Fabiato, 1979; Miller and Smith, 1984), this necessitates the use of a Ca²⁺ chelator, and here HEDTA was used. The free Ca²⁺ concentration was calculated after taking into account the affinity of Ca²⁺, Mg²⁺ and H⁺ for HEDTA, the pH, ionic strength and temperature of the assay (Fabiato and Fabiato, 1979). The incubation mixture consisted of final concentrations of 50 mM Hepes, pH 7.45, 1 μ M cyclic nucleotide, 1.8 mM calmodulin, 10 mM HEDTA, 0.19 mM EGTA, 3.5 mM free Mg²⁺, 40 μ M ATP, 125 mM NaCl, 2.5% (v/v) glycerol and the indicated Ca²⁺ concentration. The maximal fold stimulation of type-I PDE activity in the presence of saturating calmodulin (30 nM) and Ca²⁺ (100 μ M, free) was 3.8–4.2-fold (range, $n = 8$).

Errors shown are S.D. for separate experiments. Assays of PDE activity were routinely done in triplicate.

RESULTS AND DISCUSSION

The tyrosyl kinase activity associated with the insulin receptor plays a pivotal role in signal transmission (Houslay, 1989; Houslay and Siddle, 1989). The major functional substrates for this are the insulin receptor β -subunit itself and a plasma membrane protein called IRS-1 (Sun et al., 1991). The central route of signal transduction then appears to proceed through a kinase cascade which is shared by related tyrosyl kinases (Marshall, 1993). However, the insulin-specific characteristics of signal transduction are likely to relate to the differences in the ability of the insulin receptor kinase to either phosphorylate substrates outside this cascade, of which IRS-1 is one example (Sun et al., 1991) or to activate other species by direct interaction. Relatively few proteins appear to serve as intracellular targets for tyrosyl phosphorylation by the insulin receptor. One possibility, however, is the peptide calmodulin (Fukami et al., 1986; Colca et al., 1987; Sacks et al., 1992). This is an observation of some potential importance as many of the actions of the key messenger Ca²⁺ are mediated by this peptide. While it serves as a good substrate *in vitro*, in intact cells the level of modification of the total pool of calmodulin appears to be small. However, as there is good evidence for the compartmentalization of Ca²⁺ in cells (Berridge, 1993), the modification of a discrete pool of this peptide may have profound local effects. Indeed, that calmodulin needs to form a cross-linked complex with polybasic substances in order to serve as a substrate for the insulin receptor tyrosyl kinase (Saville and Houslay, 1993) implies that such a reaction may well be compartmentalized. Nevertheless, it needs to be shown that the tyrosyl phosphorylation of calmodulin occurring exclusively on Tyr⁹⁹ (Laurino et al., 1988; Saville and Houslay, 1993), a residue occurring in a high-affinity Ca²⁺-binding domain of this peptide (Babu et al., 1988), can elicit any change in the function and properties of this key regulatory peptide. To determine

whether any change induced by this specific tyrosyl phosphorylation has any functional consequences as regards the ability of Ca²⁺-calmodulin to affect the functioning of a biological system we chose to investigate its action on the first system shown to be regulated by this peptide (Cheung, 1970), namely type-I (Ca²⁺-calmodulin-stimulated) PDE activity.

First, however, we analysed whether our P-Tyr⁹⁹-calmodulin preparation could undergo a conformational change in response to Ca²⁺, as does native calmodulin. To do this we exploited the fact that Ca²⁺ elicits a profound increase in the mobility of calmodulin as seen upon SDS/PAGE (Burgess et al., 1980). Thus, calmodulin migrated with an apparent size of 18.5 kDa on gels run in the absence of added Ca²⁺ (Figure 1). However, in gels run with Ca²⁺ present then a reduction in apparent size was observed, to around 15 kDa; this was due to the enhanced mobility caused by calmodulin forming a more compact structure in the presence of this bivalent cation. However, the ability of the addition of Ca²⁺ to the gel system to induce enhanced mobility of calmodulin was profoundly attenuated when P-Tyr⁹⁹-calmodulin was analysed (Figure 1). Thus in the absence of Ca²⁺ addition to the gels, the P-Tyr⁹⁹-calmodulin migrated identically

Figure 1 P-Tyr⁹⁹-calmodulin shows an ablated Ca²⁺-induced mobility change upon SDS/PAGE

SDS/PAGE electrophoresis of (a) native (track 2) and P-Tyr⁹⁹-calmodulin (track 1) in gels and running buffers which contain 0.1 mM EDTA in order to chelate any Ca²⁺ and (b) Native (track 2) and P-Tyr⁹⁹-calmodulin (track 1) with 0.1 mM Ca²⁺ present in gels and running buffers instead of EDTA. The P-Tyr⁹⁹-calmodulin used in this particular experiment was phosphorylated to a stoichiometry of 0.9 mol of phosphate/mol of calmodulin; thus 90% of the applied calmodulin was P-Tyr⁹⁹-calmodulin and 10% of it remained as native calmodulin. In the absence of free Ca²⁺ then both native and P-Tyr⁹⁹-calmodulin migrated identically yielding the single bands shown in tracks 1 and 2 of (a). However, in the presence of free Ca²⁺ (b) then the minor band in track 1, representing native calmodulin [also shown in track 2], migrated with a higher mobility than that of P-Tyr⁹⁹-calmodulin, represented by the major band seen in track 1. The experiment shown is representative of the three performed.

Figure 2 Poly(L-lysine) modifies similarly the stimulation of type-I PDE activity by both calmodulin and P-Tyr⁹⁹-calmodulin

Assays were done in the presence of Ca^{2+} ($100 \mu\text{M}$, total) using native (■, □) or P-Tyr⁹⁹-calmodulin (○, ●) in the presence (□, ●) or absence (■, ○) of poly(L-lysine) (1:5 poly(L-lysine)/calmodulin). PDE activity is expressed as a percentage of the maximum stimulation obtained using either native calmodulin or P-Tyr⁹⁹-calmodulin. The experiment shown is typical of one done five times with triplicate assays of PDE activity. Here, P-Tyr⁹⁹-calmodulin was phosphorylated to a stoichiometry of 0.9 mol of phosphate/mol of calmodulin.

to native calmodulin (18.5 kDa) but, with the addition of Ca^{2+} , a very much smaller increase in mobility was seen compared with that for native calmodulin, with an apparent size of 17 kDa being noted (Figure 1). In order to emphasize this dramatic effect that tyrosyl phosphorylation has in attenuating the conformational change elicited by Ca^{2+} we show here data (Figure 1) for a preparation of calmodulin which was phosphorylated to a stoichiometry of approx. 0.9 mol of ^{32}P /mol of calmodulin. This, in the absence of Ca^{2+} , migrated as a single band, showing that both calmodulin and P-Tyr⁹⁹-calmodulin have intrinsically similar mobilities on SDS/PAGE and thus, unlike with Ca^{2+} , the tyrosyl phosphorylation of this peptide does not cause any gross change which allows it to attain a more compact conformation. However, when gels were run in the presence of Ca^{2+} , then this preparation migrated as a doublet. This was because the major band (90%) of the doublet reflected P-Tyr⁹⁹-calmodulin which now (in the presence of Ca^{2+}) migrated more slowly than the minor band, which reflected that of the residual (10%) native calmodulin. Such data indicate that the tyrosyl phosphorylation of calmodulin can profoundly affect the way in which this peptide responds to Ca^{2+} .

In analysing the effects of tyrosyl phosphorylation on type-I PDE activity we looked at three key parameters, namely the dose-dependency and maximal effects associated with the action of calmodulin itself, Ca^{2+} and also calmodulin antagonists. Figure 2 delineates the dose-dependent activation of PDE activity

by calmodulin, showing an EC_{50} value of $2.1 \pm 0.2 \text{ nM}$, which is typical for this enzyme activity (Lavan et al., 1989; Wang et al., 1990), together with an increase in activity of 4.2 ± 0.2 -fold ($n = 5$ separate experiments).

We have shown (Saville and Houslay, 1993) that poly(L-lysine) can form a complex with calmodulin which appears to be necessary for the insulin receptor to be able to effect its phosphorylation. As our normal preparation of P-Tyr⁹⁹-calmodulin contained this species at a constant ratio of 1:5 poly(L-lysine)/calmodulin, we determined whether this polybasic substance could affect the ability of type-I PDE activity to be stimulated by calmodulin. When poly(L-lysine) was present in PDE assays (in the above ratio), we noted that while calmodulin remained able to activate PDE activity to a similar level (4.4 ± 0.2 -fold) the dose-effect curve was markedly displaced to the left (Figure 2). This, we believe, is the first demonstration of a substance that can augment calmodulin's action as a type-I PDE activator; with the presence of poly(L-lysine) causing an approx. 7-fold decrease in the EC_{50} value for activation of PDE ($0.3 \pm 0.1 \text{ nM}$; $n = 5$ separate experiments). Thus poly(L-lysine) serves not only to allow the insulin receptor to interact with and thus tyrosyl phosphorylate calmodulin (Sacks et al., 1989; Saville and Houslay, 1993), but it also increases the affinity of interaction of Ca^{2+} /calmodulin with type-I PDE. It did not, however, lead to any change in (basal) PDE activity assessed in the absence of added calmodulin ($< 5\%$), showing that its action was calmodulin-dependent. As for the ability of the insulin receptor to effect the phosphorylation of calmodulin (Saville and Houslay, 1993), similar results were obtained using poly(D-lysine) (molecular mass 65 kDa; results not shown), indicating that such effects were probably due to local charge effects.

As poly(L-lysine) clearly affected the dose-dependent ability of calmodulin to stimulate type-I PDE activity it was essential to remove this polybasic substance from phosphorylation mixtures with calmodulin in order to be able to examine the action of P-Tyr⁹⁹-calmodulin on PDE activity. To do this we developed a rapid and simple method based upon dissociating the poly(L-lysine)/calmodulin complex with NaCl (Fujita-Yamaguchi et al., 1989b; Saville and Houslay, 1993) and then selectively removing poly(L-lysine) on the strongly cationic Dowex-50 resin (see the Materials and methods section). This method, using both native and Tyr⁹⁹-calmodulin, allowed for the recovery of $> 99\%$ of calmodulin in the supernatant fraction and the removal of all ($> 99\%$) of the poly(L-lysine) as a complex with the Dowex-50 resin. As evidence of the utility of this procedure we were able to isolate calmodulin from the poly(L-lysine)-containing 'phosphorylation mixture' (1:5; poly(L-lysine)/calmodulin) and demonstrate that it yielded an identical ($< 5\%$ difference in EC_{50} value) dose-dependence for the activation of type-I PDE activity, in contrast with the approx. 7-fold difference noted with the mixture of poly(L-lysine) with calmodulin (see above).

Using various calmodulin preparations, which had been Tyr⁹⁹-phosphorylated to between 0.8–1.0 mol of phosphate/mol of calmodulin (range, $n = 5$ different preparations), we identified no difference, compared with native calmodulin, in either the maximal stimulation of PDE activity achieved (4.4 ± 0.1 -fold) or the dose-dependency of calmodulin action (EC_{50} of $2.1 \pm 0.2 \text{ nM}$). Furthermore, if calmodulin was incubated in the 'phosphorylation mixture', but without the insulin receptor, then the isolated calmodulin behaved in the same way as the native material with regard to activation, dose-dependence and mobility on SDS/PAGE ($< 5\%$ difference).

As with native calmodulin, poly(L-lysine) (1:5; poly(L-lysine)/P-Tyr⁹⁹-calmodulin) caused a marked left-shift in the dose-dependent ability of P-Tyr⁹⁹-calmodulin to stimulate type-

Figure 3 Both calmodulin and P-Tyr⁹⁹-calmodulin are activated in a similar dose-dependent fashion by Ca²⁺

Assays were done in the presence of 1.8 nM concentrations of either native calmodulin (■) or P-Tyr⁹⁹-calmodulin (○). Free Ca²⁺ concentrations were calculated as described in the Materials and methods section for assays done in HEDTA buffers. PDE activity is expressed as a percentage of the maximum stimulation obtained using either native calmodulin or P-Tyr⁹⁹-calmodulin. A typical experiment is shown of one done five times with triplicate assays of PDE activity. Here, P-Tyr⁹⁹-calmodulin was phosphorylated to a stoichiometry of 0.85 mol of phosphate/mol of calmodulin.

I PDE activity (EC_{50} of 0.3 ± 0.2 nM), while not affecting the degree of activation observed (4.4 ± 0.2 -fold; $n = 5$ separate experiments).

Activation kinetics of type-I PDE by calmodulin were subjected to Hill-plot analysis (results not shown). This indicated that normal saturation kinetics were obeyed for the activation of this enzyme by both native and Tyr⁹⁹-calmodulin, in the absence of poly(L-lysine), yielding similar Hill coefficients of near unity, 1.2 ± 0.2 and 1.2 ± 0.1 respectively. Interestingly, however, the presence of poly(L-lysine) [1:5; poly(L-lysine)/calmodulin] engendered activation kinetics indicative of apparent positive co-operativity, with Hill coefficients being 2.0 ± 0.3 and 1.7 ± 0.1 ($n = 3$ separate experiments) for native and P-Tyr⁹⁹ calmodulin respectively. This may have functional consequences if it should occur in the cell, in that polybasic substances may serve to increase the sensitivity of PDE-I to calmodulin.

The dose-dependent activation of type-I PDE by Ca²⁺ was similar using both native calmodulin and P-Tyr⁹⁹-calmodulin, yielding EC_{50} values for free [Ca²⁺] of 0.8 ± 0.1 and 0.9 ± 0.1 μ M respectively ($n = 5$ separate experiments). Using both forms of calmodulin, Ca²⁺ stimulated PDE activity with kinetics of apparent positive co-operativity, exhibiting Hill coefficients of 2.3 ± 0.3 with native calmodulin and 2.1 ± 0.3 with P-Tyr⁹⁹-calmodulin.

There are classes of calmodulin antagonists which bind to calmodulin and alter its conformation (Wang et al., 1990), thus attenuating the stimulatory effect of calmodulin on target proteins. These antagonists do not act competitively with respect to calmodulin and thus bind to a different domain of calmodulin than does the target protein (Wang et al., 1990). As such they can be used to explore the effect of the phosphorylation of Tyr⁹⁹ upon a distinct functional domain of calmodulin. Here then we investigated the effect of two chemically distinct antagonists (Wang et al., 1990) on calmodulin's stimulation of type-I PDE

activity, namely the phenothiazine, trifluoroperazine (TFP), and the naphthalenesulphonamide, W7. Intriguingly, using both of these antagonists we found a marked decrease of approx. 3–4-fold in their efficacy as inhibitors when P-Tyr⁹⁹-calmodulin, rather than native calmodulin, was used to stimulate PDE activity (Figure 3). Thus, TFP inhibited the stimulatory effect of calmodulin with an IC_{50} of 3.7 ± 0.2 μ M and that of P-Tyr⁹⁹-calmodulin with an IC_{50} of 8.7 ± 0.2 μ M. Similarly, the calmodulin antagonist W7 exhibited IC_{50} values of 12 ± 3 μ M and 51 ± 4 μ M for calmodulin and P-Tyr⁹⁹-calmodulin respectively ($n = 4$ separate experiments). Hill plots showed that the use of P-Tyr⁹⁹-calmodulin led to a noticeable increase in the value of the Hill coefficients seen for inhibition by the calmodulin antagonists. With TFP we observed Hill coefficient values of 0.39 ± 0.05 and 0.71 ± 0.05 and with W7 we observed values of 0.90 ± 0.09 and 1.60 ± 0.11 for native and P-Tyr⁹⁹-calmodulin respectively ($n = 3$ separate experiments). Such results indicate that these two chemically dissimilar compounds, while both acting by binding calmodulin, exert inhibition in rather different ways. This is shown by the very different Hill coefficients observed and also the fact that, unlike with TFP, low concentrations of W7 elicited a small stimulation of PDE activity, which was more apparent using P-Tyr⁹⁹-calmodulin than native calmodulin (Figure 4). However, under the experimental conditions used, they both exerted similar maximal inhibitory effects with either native ($48 \pm 3\%$ TFP; $50 \pm 5\%$ W7) or P-Tyr⁹⁹-calmodulin ($50 \pm 4\%$ TFP; $55 \pm 3\%$ W7). Thus the effect of tyrosyl phosphorylation appears to be restricted to one of affecting the affinity and co-operativity of calmodulin for interaction with these antagonists rather than altering their maximal inhibitory action.

The phosphorylation of Tyr⁹⁹, occurring within the third high-affinity Ca²⁺-binding domain of calmodulin, appears to elicit a distinct conformational change, which is evident from the profound reaction in the ability of Ca²⁺ to alter the mobility of phosphorylated calmodulin upon SDS/PAGE. However, it is intriguing to find that functional aspects of this effect are highly localized in that while the ability of P-Tyr⁹⁹-calmodulin to activate a physiological effector, type-I PDE, is unaltered, the action of calmodulin antagonists is clearly affected by Tyr⁹⁹ phosphorylation, resulting in a reduction in their affinity for calmodulin and altered co-operativity of inhibition. Such a localization of the conformational change may reflect the highly ordered structure of this peptide, especially when it is in its functionally active state associated with Ca²⁺ (Babu et al., 1988; Klee, 1988). Whether, among other calmodulin-stimulated systems, there are similar selective responses to the conformational change induced by Tyr⁹⁹ phosphorylation remains to be seen. This might be expected if some systems couple to linear epitopes exposed as a result of the binding of Ca²⁺ to calmodulin, whereas others demand the formation of domains formed from non-contiguous sequences. Certainly the possibility that some, but not other, effector proteins may be influenced by the tyrosyl phosphorylation of calmodulin may mean that selective signalling actions could result. Indeed, the fact that forms of calmodulin from a large number of non-mammalian species do not have tyrosine at position 99 (Klee, 1988) might indicate that its occurrence in calmodulin species from mammals is of some importance. The requirement for calmodulin to form a complex with polybasic substances in order for it to be phosphorylated (Saville and Houslay, 1993) may compartmentalize any such action. One possible location is the nucleus, as not only are polybasic substances found there but calmodulin exerts actions at this site (Bossier et al., 1993) and occupied insulin receptors have been shown to be translocated there (Renston et al., 1980). Thus, while there is still controversy over whether functionally

Figure 4 Tyr⁹⁹ phosphorylation of calmodulin shifts the dose-dependent inhibition achieved by TFP and W7

In (a) is shown data using TFP and in (b) data using W7. Assays were performed in the presence of approx. half-maximally activating concentrations of calmodulin (3.5 nM). This was done using either native calmodulin (■) or P-Tyr⁹⁹-calmodulin (○). Total Ca²⁺ concentrations were 100 μM. PDE activity is expressed as a percentage of the maximum stimulation obtained using either native calmodulin or P-Tyr⁹⁹-calmodulin. A typical experiment is shown of one done three times with triplicate assays of PDE activity. Here, P-Tyr⁹⁹-calmodulin was phosphorylated to a stoichiometry of 0.9 mol of phosphate/mol of calmodulin. Over the range of concentrations used then neither TFP or W7 affected type-I PDE activity (< 4%) measured in the absence of added calmodulin.

significant levels of tyrosyl phosphorylation of calmodulin occur in cells, our study addresses, for the first time, the question as to whether such a modification can alter the functioning of this key regulatory peptide. Such data thus provides a novel insight concerning structure-function relationships of calmodulin as regards the lack of any clear functional effect on the domain which serves to regulate a particular effector system, namely type-I cyclic AMP phosphodiesterase, an alteration in the functioning of the domain at which antagonists bind and a profound effect on the Ca²⁺-induced conformational change.

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