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Activation of Bovine Rod Outer Segment Phosphatidylinositol-4,5-bisphosphate Phospholipase C by Calmodulin Antagonists Does Not Depend on Calmodulin†

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ABSTRACT: Calmodulin antagonists stimulated phosphatidylinositol-4,5-bisphosphate phospholipase C in soluble and particulate fractions of bovine rod outer segments. Antagonists tested include trifluoperazine, melittin, calmidazolium, compound 48/80, W-13 [*N*-(4-aminobutyl)-5-chloro-1-naphthalenesulfonamide], and W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide]. All were effective, but W-7 was chosen for further characterization of the effect, which was most pronounced in the soluble fraction. Phospholipase C activity in the soluble fraction did not increase linearly with the quality of enzyme assayed, suggesting the presence of an endogenous inhibitor or an inhibitory self-association of the enzyme. W-7 appeared to counteract this inhibition, resulting in a linear activity–quantity relationship. Stimulation by W-7 was therefore largest when large amounts of crude enzyme were assayed and small or nil when small amounts were assayed. The effect of W-7 was also dependent on [Ca^{2+}], with half-maximal stimulation occurring between 0.1 and 1 μM. W-7 and W-13 were much more effective than their nonchlorinated analogues W-5 and W-12 at increasing phospholipase C activity. While this pattern of effectiveness is typical of calmodulin-mediated processes, the absence of any effect by added calmodulin and the retention of W-7 sensitivity by purified CaM-free enzyme argue against regulation by CaM. Octyl glucoside, a nonionic detergent, mimicked some of the effects of CaM antagonists, suggesting that the antagonists act by interfering with protein–protein interactions. It appears likely that CaM antagonists prevent an inhibitory multimerization or aggregation of at least one form of ROS phospholipase C.

We have previously reported (Gehm & McConnell, 1990) that bovine retinal rod outer segments (ROS)[1] contain both soluble and particulate PIP_2 phospholipase C activities which are strongly affected by Ca^{2+} concentration. Ca^{2+} is absolutely required, and half-maximal activity is obtained at concentrations of ~0.1 μM for the soluble fraction and ~3 μM for the particulate. These results prompted us to examine the possibility that phospholipase C activity in ROS is regulated by calmodulin (CaM), a widely occurring Ca^{2+}-dependent regulatory protein (Means & Dedman, 1980).

CaM is present in ROS in both soluble and membrane-bound forms (Kohnken et al., 1981), but its function in ROS is unknown. Since ROS contain endogenous CaM, our initial experiments employed CaM antagonists to evaluate a potential role for CaM in phospholipase C regulation. Unexpectedly, we found that CaM antagonists stimulate PIP_2 phospholipase C in both soluble and particulate ROS fractions, raising the possibility that ROS phospholipase C is inhibited by CaM. Such inhibition would be extremely significant, as phospho-

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[1] Abbreviations: BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; EC_{50}, 50% effective concentration; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; calmidazolium, 1-[bis(4-chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]ethyl]-1H-imidazolium chloride; CaM, calmodulin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IC_{50}, 50% inhibitory concentration; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; ROS, retinal rod outer segments; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; W-7, *N*-(6-(aminohexyl)-5-chloro-1-naphthalenesulfonamide; W-12, *N*-(4-aminobutyl)-1-naphthalenesulfonamide; W-13, *N*-(4-aminobutyl)-5-chloro-1-naphthalenesulfonamide.

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Table I: Effect of Calmodulin Antagonists on PIP_2 Phospholipase C[a]

addition	[^{3}H]PIP_2 hydrolyzed (dpm)[b] particulate	soluble
none (control)	3213 ± 26	4535 ± 439
100 μM trifluoperazine	4094 ± 42	14461 ± 206
100 μM W-7	4813 ± 52	14497 ± 861
200 μM HCl (control)[c]	2742 ± 35	3577 ± 139
200 μM HCl + 100 μM haloperidol	4235 ± 84	5771 ± 452
1% ethanol (control)[c]	4854 ± 168	4765 ± 287
1% ethanol + 10 μM calmidazolium	7265 ± 129	19958 ± 590

[a] Particulate (600 μg of protein/assay) and soluble (5 μg of protein/assay) fractions were assayed as described under Materials and Methods with the indicated additions. [b] Means ± standard errors for triplicate assays. [c] HCl and ethanol were used as solvents for haloperidol and calmidazolium, respectively.

lipase C has not previously been shown to be CaM-regulated, and CaM activates, not inhibits, those enzymes it is known to regulate. However, as Hartshorne (1985) has observed, "there is no *a priori* reason why calmodulin interaction must activate enzymatic activity, and it is conceivable that calmodulin-dependent inhibition may occur." Furthermore, Rhee et al. (1989) have presented reasons for expecting phosphoinositide-specific phospholipase C activity to be inhibited by an as yet undiscovered negative regulatory protein. We therefore carefully examined the possibility that ROS phospholipase C is down-regulated by CaM. Our results indicate that at least one form of ROS phospholipase C is inhibited by protein–protein interactions that can be blocked by CaM antagonists, but the enzyme is not regulated by CaM per se.

Materials and Methods

Materials. Tritiated phosphoinositides were purchased from NEN. Unlabeled phosphoinositides, calmodulin antagonists, *n*-octyl glucoside, and BSA were purchased from Sigma, calmodulin (bovine brain) and Pansorbin were from Calbiochem, and BAPTA was from Fluka. ROS soluble and particulate fractions were prepared as previously described (Gehm & McConnell, 1990). Sheep anti-calmodulin was prepared and purified as described by Chafouleas et al. (1979, 1983).

Phospholipase C Assays. Phospholipase C activity was measured as described previously (Gehm & McConnell, 1990). Briefly, aliquots of ROS fractions were incubated for 30 min at 30 °C in 200 mM potassium Hepes, pH 7.5, 50 mM KCl, 30 μM $CaCl_2$, and 10 μM [^{3}H]PIP_2 (2.5 μCi/μmol) or other phosphoinositides as noted. The total assay volume was 500 μL. Reactions were terminated by the addition of 500 μL of trichloroacetic acid and 100 μL of 5% BSA (Inoue et al., 1985). After centrifugation and neutralization, acid-soluble ^{3}H was quantified by liquid scintillation counting.

CaM Assays and Immunoprecipitation. CaM was quantified by Western blot and by radioimmunoassay using the method of Chafouleas et al. (1979). CaM immunoprecipitation with specific sheep anti-CaM was performed as previously reported (Chafouleas et al., 1981, 1983).

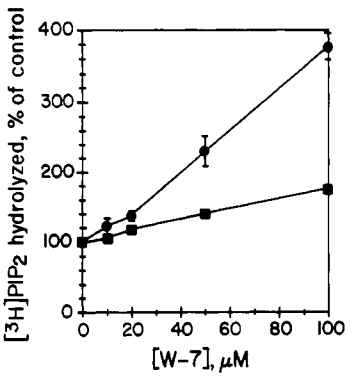

FIGURE 1: Effect of W-7 on soluble and particulate phospholipase C. Soluble (●, 32 μg of protein/assay) and particulate (■, 260 μg of protein/assay) fractions of ROS were assayed for PIP_2 phospholipase C in the presence of the indicated concentrations of W-7. Activities are expressed as percent of no-W-7 control, mean ± SE for duplicate determinations. Error bars for the particulate assays are smaller than the symbols.

Protein Determinations. Protein concentrations were estimated by the method of Lowry et al. (1951).

Results

Calmodulin Antagonists Stimulate PIP_2 Phospholipase C. As shown in Table I, the CaM antagonists trifluoperazine, W-7, haloperidol, and calmidazolium all increased PIP_2 hydrolysis in both soluble and particulate fractions of ROS. Chlorpromazine, mellitin, and compound 48/80 also stimulated PIP_2 hydrolysis (data not shown). The effects of the antagonists were concentration dependent; Figure 1 shows enzyme activity as a function of W-7 concentration.

Since more pronounced stimulation of phospholipase C activity was observed in the soluble fraction than in the particulate, further experiments concentrated primarily on the soluble fraction. W-7 was routinely used as a representative CaM antagonist.

Effects on Hydrolysis of Other Phosphoinositides. W-7 affected hydrolysis of PI, PIP, and PIP_2 in different ways. As shown in Table II, it appeared to alter phospholipase C substrate specificity in favor of polyphosphoinositides at the expense of PI. In contrast to PIP and PIP_2, PI hydrolysis decreased in the soluble fraction and increased only slightly in the particulate fraction when W-7 was added. Similar results were obtained with trifluoperazine (data not shown). Inhibition of PI phospholipase C by CaM antagonists has been reported previously (Wightman et al., 1981; Craven & DeRubertis, 1983; Benedikter et al., 1985; Schwertz et al., 1987, Bronner et al., 1987), but these studies did not attempt to determine the role of CaM, if any, in this inhibition.

Comparison of Chlorinated and Nonchlorinated Naphthalenesulfonamides. Hidaka and Tanaka (1983) have prepared a series of *N*-aminoalkylnaphthalenesulfonamides

Table II: Effect of W-7 on Phosphoinositide Hydrolysis[a]

substrate	spec act[b] (μCi/μmol)	acid-soluble label released (dpm)[c] particulate −W-7	particulate +W-7	soluble −W-7	soluble +W-7
[^{3}H]PI	9.1	4235 ± 75	4808 ± 53	1022 ± 62	348 ± 40
[^{3}H]PIP	5.0	6102 ± 388	11322 ± 390	4652 ± 475	5548 ± 65
[^{3}H]PIP_2	2.5	5348 ± 277	7868 ± 902	1558 ± 118	2898 ± 30

[a] Particulate and soluble fractions (400 and 36 μg of protein/assay, respectively) were assayed for phospholipase C activity as described under Materials and Methods, with or without the addition of 100 μM W-7. Similar results were obtained using trifluoperazine instead of W-7. [b] Values for specific activities of substrates do not reflect dilution of the label by endogenous lipids in the enzyme preparations, which could be significant for the particulate fraction. [c] Means ± standard errors for triplicate (particulate) or duplicate (soluble) assays.

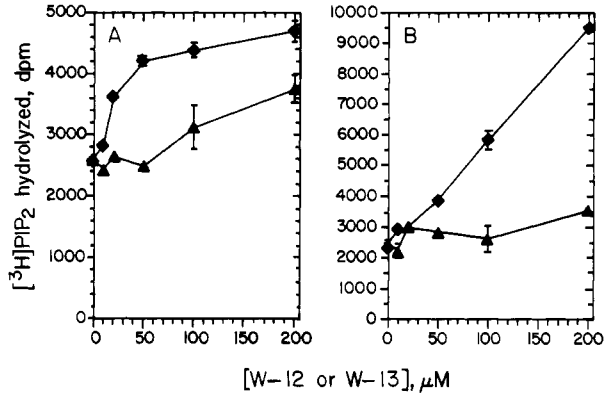

FIGURE 2: Effects of W-12 and W-13 on soluble and particulate phospholipase C. Particulate (A, 260 μg of protein/assay) and soluble (B, 32 μg of protein/assay) fractions were assayed for PIP_2 phospholipase C in the presence of the indicated concentrations of W-12 (▲) and W-13 (◆). Activities are shown as means ± SE for duplicate assays.

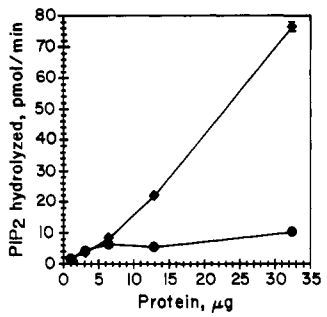

FIGURE 3: Effect of enzyme concentration on W-7 stimulation of phospholipase C. Aliquots of soluble fraction (0.13 mg of protein/mL) containing the indicated amounts of total protein were assayed for PIP_2 phospholipase C activity in the presence (◆) or absence (●) of 100 μM W-7. Results are presented as means ± SE of duplicate assays. Where not shown, error bars are smaller than symbols.

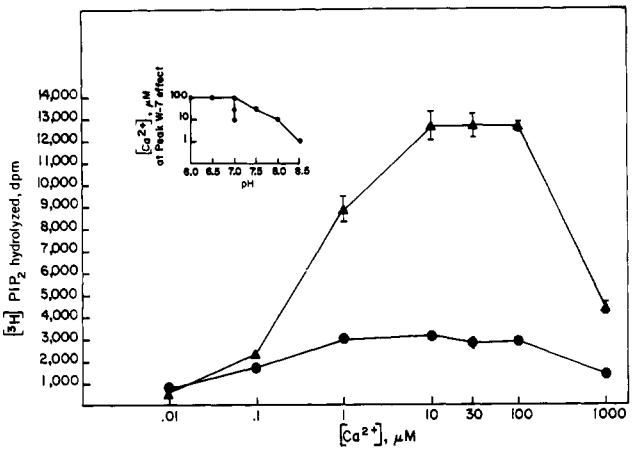

FIGURE 4: Ca^{2+} dependence of W-7 stimulation of phospholipase C. Soluble fraction (30 μg of protein/assay) was assayed for PIP_2 phospholipase C activity at pH 7.0 in the presence (▲) or absence (●) of 100 μM W-7. Ca^{2+} concentrations were set with a Ca-BAPTA buffer as described previously (Gehm & McConnell, 1990). Results are shown as means ± SD for duplicate assays. Where not shown, SD < 100 dpm. (Inset) Effect of pH on Ca^{2+} concentration required for maximal activation by W-7. Ca^{2+} concentration curves like that shown in the main figure were obtained in Hepes buffer at the indicated pH. The Ca^{2+} concentration producing the largest stimulation by 100 μM W-7 at each pH is indicated.

Table III: Effect of W-7 on Kinetics of Soluble Phospholipase C[a]

	-W-7	+W-7
K_m	5.8 ± 0.9	6.5 ± 0.7
V_{max}	11.8 ± 0.6	28.2 ± 1.1

[a] Soluble phospholipase C activity was assayed as described, but with various concentrations of [^{3}H]PIP_2 and in the presence or absence of 100 μM W-7. Duplicate assays were performed at each of five concentrations of [^{3}H]PIP_2 over the range 2–30 μM. Plots of $1/v$ vs $1/S$ were linear for both treatments. The results were analyzed by the method of Wilkinson (1961).

that are widely used as probes of CaM regulation. Among the most widely used are W-7 and W-13 and their non-chlorinated analogues, W-5 and W-12. W-7 and W-13 are much more potent CaM antagonists than W-5 and W-12 but are similar to them in hydrophobicity. Comparison of W-13 with W-12 and of W-7 with W-5 has been proposed as a way of distinguishing CaM-mediated from non-CaM-mediated effects of CaM antagonists (Hidaka et al., 1981; Chafouleas et al., 1982). Figure 2 shows the effects of W-13 and W-12 on phospholipase C activity in the particulate (A) and soluble (B) fractions. In both fractions, W-13 was much more effective than W-12 at stimulating the enzyme. Comparison of W-7 and W-5 gave similar results, except that in the particulate fraction the effect of W-7 was maximal at 100 μM and declined at 200 μM (data not shown).

Dependence of CaM Antagonist Effects on Quantity of Enzyme. As previously reported (Gehm & McConnell, 1990), phospholipase C activity in ROS soluble and particulate fractions was not linear with respect to quantity of material assayed. The apparent specific activity declined with increasing amounts of crude enzyme. This is unsurprising in the particulate fraction, which contains endogenous unlabeled phosphoinositides, but was unexpected in the soluble fraction. Such a decline in specific activity is frequently due to the presence of an endogenous inhibitor in the enzyme preparation. As Figure 3 illustrates, W-7 prevented this decline, producing an approximately linear relationship between phospholipase C activity and quantity of soluble fraction assayed. Consequently, the magnitude of the W-7 effect can be made larger or smaller by altering the quantity of crude enzyme used in the assay. The minimum quantity needed to observe W-7 stimulation varied from one preparation to the next, but qualitatively similar results were obtained with several preparations of soluble fraction.

Effects of Ca^{2+} and pH on W-7 Stimulation. The effect of W-7 on PIP_2 phospholipase C activity was calcium dependent, as shown in Figure 4. For the soluble activity, half-maximal stimulation was obtained between 0.1 and 1 μM Ca^{2+}, with a broad peak of maximum effect from 10 to 100 μM. These results were obtained at pH 7. At higher pH lower Ca^{2+} concentrations were needed for maximal stimulation, while at lower pH 100 μM Ca^{2+} was required (inset). Below pH 6.5 little or no stimulation was produced by W-7, trifluoperazine, melittin, or compound 48/80, in the presence of 30 μM Ca^{2+} (data not shown).

Effect of W-7 on PIP_2 Phospholipase C Kinetics. As shown in Table III, W-7 increased the V_{max} of phospholipase C in the soluble fraction without appreciably altering the apparent K_m for PIP_2 (~6 μM). This suggests that the effects of the antagonists are not due to interaction with substrate, although CaM antagonists have been reported to interact with phospholipids.

No Effect of Anti-CaM Antibodies. We attempted to remove endogenous CaM from ROS soluble fraction by immunoprecipitation with a specific sheep anti-CaM antibody and Pansorbin (Chafouleas et al., 1979, 1983). Use of ^{125}I-labeled CaM as a tracer demonstrated that quantitative immunoprecipitation of CaM could not be achieved with practical amounts of anti-CaM unless the quantity of soluble fraction

Table IV: Added CaM Does Not Affect Phospholipase C Activity[a]

CaM added (μg)	[^{3}H]PIP_2 hydrolyzed (dpm)[b]	
	−W-7	+W-7
0	2875 ± 310	2525 ± 56
0.2	2741 ± 95	2477 ± 98
2.0	2498 ± 150	2701 ± 60

[a] ROS soluble fraction was incubated overnight at 4 °C in the presence of 10 μM $CaCl_2$ and varying amounts of added CaM. Aliquots containing 1.2 μg of soluble fraction protein and the indicated quantity of CaM were assayed for PIP_2 PLC activity as described under Materials and Methods. A total of 0.005 μg of endogenous CaM (as determined by radioimmunoassay) was also present in each assay. [b] Means ± standard errors for triplicate determinations.

used was extremely low (i.e., less than 1 μg of protein; data not shown). At such low concentrations of soluble fraction protein, no endogenous inhibition was observed and CaM antagonists had no effect (cf. Figure 3). Anti-CaM, with or without Pansorbin, had little effect on soluble fraction phospholipase C activity (data not shown).

No Effect of Added CaM. Stimulation of phospholipase C activity by CaM antagonists suggested that addition of CaM should produce inhibition. In order to avoid masking the effect of added CaM, we used a small amount (1.2 μg) of soluble fraction protein in these assays, so that no endogenous inhibition was observed and W-7 was without effect (cf. Figure 3). As shown in Table IV, addition of excess CaM had no effect on phospholipase C activity under these conditions. In experiments using large amounts of soluble fraction, added CaM (up to 250 μg/mL) affected neither basal nor W-7-stimulated phospholipase C activity (data not shown). Moreover, the activity of a purified form of ROS phospholipase C free of endogenous CaM (described below) was unaffected by added CaM (data not shown).

Inhibition by BSA. In various experiments involving the addition of antibodies or other proteins to phospholipase C assays, bovine serum albumin (BSA) was used as a control. Unexpectedly, BSA inhibited PIP_2 hydrolysis (IC_{50} = ~50 μg/mL in assays of ROS soluble fraction). W-7, but not W-5, was able to prevent this inhibition. No inhibition was produced by 50 μg/mL chicken egg ovalbumin, carp parvalbumin, preimmune sheep IgG (all purchased from Sigma), or bacteriological grade gelatin (Difco). Purified BSA depleted of fatty acids and thyroxine (Sigma A7030) was as inhibitory as standard fraction V (Sigma A4503).

Other Assay Systems. To examine the possibility that the effects of CaM antagonists might be an artifact of our assay procedure, soluble fraction PIP_2 phospholipase C activity in the presence and absence of 100 μM W-7 was measured according to the methods of Rebecchi and Rosen (1987), Ryu et al. (1987), and Katan and Parker (1987). Stimulation by W-7 was apparent in the first two, albeit more modest than in our usual assay, possibly due to the lower pHs (6.8 and 7.0, respectively). No stimulation by W-7 could be detected in the assay of Katan and Parker; we attribute this to the pH (6.0) and Ca^{2+} concentration (0.1 μM), which were much lower than in our assay (pH 7.5, 30 μM Ca^{2+}). As shown in Figure 4, the effect of W-7 was very slight at 0.1 μM Ca^{2+}.

Effect of Detergent. Octyl glucoside, a nonionic detergent, increased [^{3}H]PIP_2 hydrolysis by the soluble fraction. Like the effect of W-7 (Figure 3), this effect was dependent on the amount of soluble fraction protein present in the assay (data not shown). Octyl glucoside concentrations of ca. 0.3% (10 mM) produced maximal (typically about 3-fold) stimulation, and no additional stimulation was produced by 100 μM trifluoperazine. The effect of octyl glucoside on ROS particulate fraction was variable, increasing PIP_2 phospholipase C activity in some cases and decreasing it in others.

Effect of W-7 on Purified Phospholipase C. ROS contain multiple forms of phospholipase C (Gehm & McConnell, 1990). One form obtained from the soluble fraction was purified to homogeneity by ion-exchange chromatography followed by HPLC hydrophobic interaction chromatagraphy (data not shown, to be published). A single A_{214} peak obtained by HPLC had high enzyme activity and yielded a single silver-stained band when applied to a nondenaturing polyacrylamide electrophoresis gel. The addition of 2.5% β-mercaptoethanol, which abolishes enzyme activity, to the applied samples produced two HPLC peaks and three bands on the electrophoresis gel, none of which were attributable to β-mercaptoethanol alone and none of which comigrated with CaM. Addition of 1 mM W-7 to the enzyme before HPLC caused the enzyme to comigrate with W-7 (one peak observed). The purified enzyme contained no CaM detectable by Western blotting or radioimmunoassay. Although full characterization of the purified enzyme must await development of a method for large scale purification, we have determined that the purified CaM-free enzyme is activated by CaM antagonists. Triplicate phospholipase C assays of 1-μg samples yielded 15 160 ± 310 cpm in the presence of 100 μM W-7 and 6216 ± 106 cpm in its absence (assay volume 100 μL).

Discussion

Many of the results obtained with CaM antagonists were initially suggestive of regulation of phospholipase C by CaM. All CaM antagonists tested (W-7, W-13, trifluoperazine, chlorpromazine, haloperidol, melittin, calmidazolium, and compound 48/80) produced significant increases in phospholipase C activity. The effects of the antagonists were dose dependent at concentrations comparable to those reported for inhibition of CaM-dependent enzymes (Nelson et al., 1983; Gietzen, 1986), although higher concentrations were sometimes required for maximum effect. Some antagonists produced biphasic responses, i.e., stimulation at low concentrations, inhibition at higher ones. The relative effectiveness of W-13 and W-7 compared to W-12 and W-5 was consistent with CaM regulation, as was the Ca^{2+} dependence of W-7 stimulation. The ability of W-7 to prevent the decrease in phospholipase C specific activity observed when increasing amounts of ROS soluble fraction were assayed (Figure 3) suggested the presence of an endogenous inhibitor which the drug antagonizes.

However, the inability of added CaM to inhibit the activity either of crude enzyme (Table IV) or of a purified form of ROS phospholipase C argues strongly against CaM being an endogenous inhibitor, as does the retention of W-7 activation by purified CaM-free enzyme. The latter result further implies that W-7, and presumably other CaM antagonists, interacts directly with at least one form of ROS phospholipase C.

How this interaction produces activation remains unclear. The dependence on enzyme concentration suggests that the phospholipase C may undergo some form of self-association, e.g., aggregation or multimerization, that inhibits the enzyme and that W-7 prevents. Although our present work is in vitro, it is possible that activation by CaM antagonists mimics a physiological regulatory process involving interaction of the enzyme with its membranous or cytosolic environment in the ROS.

Stimulation of soluble fraction phospholipase C activity by the nonionic detergent octyl glucoside was similar to, and not additive with, stimulation by CaM antagonists. This may seem to suggest that the CaM antagonists exert only nonspecific

hydrophobic effects, but comparison of the effective concentrations casts doubt on this interpretation: octyl glucoside was maximally stimulatory at ~10 mM, trifluoperazine and W-7 at ~100 μM, calmidazolium at ~10 μM, and melittin at <1 μM (data not shown). Thus, CaM antagonists are 100–10000-fold more potent than octyl glucoside at activating ROS phospholipase C. The ability of high concentrations of detergent to mimic the effects of CaM antagonists supports the idea that CaM antagonists activate ROS phospholipase C by preventing or disrupting inhibitory protein associations.

The observed Ca^{2+} dependence of stimulation by W-7 may mean that ROS phospholipase C binds CaM antagonists Ca^{2+} dependently. There are a number of proteins beside CaM that bind CaM antagonists in a Ca^{2+}-dependent fashion; this property has been exploited in their isolation via affinity chromatography. Moore and Dedman (1982) isolated several proteins that bind Ca^{2+}-dependently to phenothiazine and W-7 affinity columns; they later coined the name "calcimedins" to describe these proteins, which differ considerably from CaM in size. The physiological function of most of these proteins is unknown. When we applied ROS soluble fraction to a W-7 affinity column, phospholipase C activity was retained on the column but could not be eluted by EGTA. Partial recovery of the activity was achieved by eluting with octyl glucoside (data not shown).

The inhibitory effect of BSA raises the question of whether it could be an endogenous inhibitor (presumably present as a contaminant derived from blood during dissection of the cattle eyes). This appears very unlikely. Inhibition by exogenous BSA required quantities approximating the total protein content of the ROS soluble fraction used in the assay. This exceeds by at least 2 orders of magnitude the possible amount of BSA present in ROS soluble fraction, as judged by polyacrylamide gel electrophoresis. The ability of W-7 to prevent inhibition by BSA suggests that BSA may inhibit phospholipase C via an adventitious hydrophobic interaction which W-7 can prevent, just as it may prevent inhibitory self-association of the enzyme.

In sum, we find that CaM antagonists activate at least one form of ROS PIP_2 phospholipase C by a CaM-independent mechanism. In addition to its significance to the study of phospholipase C in photoreceptors and other cells, this finding has important implications for the use of CaM antagonists as probes of CaM function.

We have also observed that CaM antagonists activate crude PIP_2 phospholipase C from bovine brain.[2] Several of these antagonists (e.g., haloperidol, trifluoperazine, chlorpromazine) are used clinically as antipsychotics and antidepressants. The activation of PIP_2 phospholipase C by these drugs may be of pharmacological interest.

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[2] F. E. Wilkinson, J. G. Chafouleas, B. D. Gehm, and D. G. McConnell, unpublished observations.

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