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Okadaic acid-sensitive protein phosphatases dephosphorylate MARCKS, a major protein kinase C substrate

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The myristoylated alanine-rich C kinase substrate (MARCKS) undergoes a rapid and, in certain circumstances, transient increase in phosphorylation in response to stimuli that activate protein kinase C. We have investigated the protein–serine/threonine phosphatase activity responsible for reversing the phosphorylation of MARCKS. In cell-free assays, protein phosphatases 1, 2A and 2C (PP1, PP2A and PP2C) all dephosphorylate recombinant MARCKS or a synthetic peptide based on its phosphorylation site domain. In intact Swiss 3T3 cells, okadaic acid, a specific inhibitor of PP1 and PP2A, had little effect on MARCKS phosphorylation on its own, but largely prevented the dephosphorylation of MARCKS that occured following activation of protein kinase C by bombesin with subsequent receptor blockade. These results indicate that although the dephosphorylation of MARCKS can be mediated by PP2C in vitro, this protein is dephosphorylated by okadaic acid-sensitive phosphatases in the intact cell.

MARCKS; Okadaic acid; Protein kinase C; Protein phosphatase

1. INTRODUCTION

A major and apparently specific intracellular substrate for protein kinase C is a 30 kDa protein known as the myristoylated alanine-rich C kinase substrate (MARCKS) ([1]; see [2,3] for review). MARCKS is phosphorylated rapidly when cells are treated with hormones, phorbol esters or diacylglycerols that activate protein kinase C. It does not appear to be phosphorylated to a biologically relevant extent by a number of other protein kinases [4,5], and its phosphorylation has frequently been used as a marker for protein kinase C activition in intact cells [2,3]. Although the cellular functions of MARCKS and its homologue, the MARCKS-related protein (MRP; also known as F52 and MacMARCKS), are not known, its phosphorylation state clearly alters some of its biochemical attributes. For example, phosphorylation markedly reduces its affinity for calmodulin [6], actin [7], and possibly cellular and synthetic membranes [8,9]. The hormone-stimulated phosphorylation of MARCKS is dynamic and transient in certain circumstances, indicating that phosphatase activity towards MARCKS is present in cells and could regulate the phosphorylation state of the protein [10]. However, the protein phosphatase(s) that acts on MARCKS has not been identified. MARCKS does not contain tyrosines in its primary sequence (nor does MRP); therefore tyrosine phosphatases are not relevant.

Studies on the activity of protein–serine/threonine phosphatases in intact cells and cellular extracts have been greatly facilitated by the discovery of highly specific inhibitors, such as okadaic acid [11–13]. This complex fatty acid polyketal is an inhibitor of the protein–serine/threonine phospatases termed protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), inhibiting PP2A much more potently in cell-free assays [14]. In combination with other criteria, okadaic acid can be used to distinguish the activities of PP1, PP2A and PP2C in cellular extracts [14,15]. It is also cell membrane permeable, allowing investigation of the roles of PP1 and PP2A in specific processes in intact cells [12,16]. Okadaic acid, like the phorbol esters which activate protein kinase C, is a potent tumour promoter [17]. Indeed, it has been suggested that its tumour promoting activity may be due, at least in part, to the inhibition of dephosphorylation of protein kinase C substrates [13].

In this study we have examined the activity of protein phosphatases towards the sites on the MARCKS protein phosphorylated by protein kinase C. We have used the intact protein and also a synthetic peptide containing the 3 or 4 sites phosphorylated by protein kinase C [18]. We find that both okadaic acid-sensitive and -insensitive phosphatases can dephosphorylate these phosphorylation sites in cell-free assays, but the dephosphorylation of MARCKS is largely prevented by okadaic acid in intact cells.

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2. MATERIALS AND METHODS

2.1. *Materials*

Recombinant MARCKS was expressed in *Escherichia coli* and purified as described in [19]. The synthesis and purification of a 25 amino acid peptide (KKKKKRFSFKKSFKLSGFSFKKNKK) corresponding to the phosphorylation site domain (PSD) of MARCKS [18] was as previously described [6]. The catalytic subunits of PP1 and PP2A were purified from rabbit skeletal muscle by D. Schelling, S. Dale and P. Dent, and inhibitor-2 by Drs. C. Holmes and M. Hubbard (at Dundee) [20]. Okadaic acid was a generous gift from Dr. Y. Tsukitani, Fujisawa Chemical Co., Japan or purchased from LC Services Corp., Woburn, MA, USA. Protein kinase C (a mixture of rat brain isozymes) was generously donated by Dr. P. Parker, ICRF, London. P81 phosphocellulose paper was purchased from Whatman and Sephadex G-25 and G-50 from Pharmacia, Milton Keynes, UK. Phosphatidylserine was from Lipid Products, Surrey, UK. Tetradecanoyl phorbol acetate (TPA, otherwise known as PMA) was from Sigma, Poole, UK. Bombesin was from Peninsula Labs., Belmont, OA, USA; (D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}) substance P (RPW-substance P) was from Calbiochem, LaJolla, CA, USA. Other chemicals were from BDH, Poole, UK.

2.2. *Preparation of phosphorylated MARCKS protein and PSD peptid.*

MARCKS protein (1–3 μM) or PSD peptide (20 μM) were phosphorylated at 30°C in incubations which contained 25 mM Na-HEPES (pH 7.5), 0.025% 2-mercaptoethanol, 2.5 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (2–5 $\times$ 10^5 cpm/nmol by Cerenkov counting), 1 mM $CaCl_2$, 1.25 mg/ml phosphatidylserine, 1.25 μg/ml TPA, 0.25% (v/v) Triton X-100. Phosphatidylserine, TPA and Triton X-100 were added as mixed micelles [21]. After 60 min, EDTA and NaF were added to 5 mM and 25 mM, respectively. Incorporation of phosphate was assessed by removing aliquots for precipitation by trichloroacetic acid (MARCKS protein) or spotting onto phosphocellulose paper (peptide) and was 2–3 mol phosphate/mol of protein or peptide. MARCKS protein or PSD peptide were separated from ATP by gel filtration on Sephadexes G-50 or G-25, respectively, previously equilibriated with 50 mM Na-HEPES (pH 7.2), 1 mM EGTA, 1 mM EDTA, 0.05% (v/v) 2-mercaptoethanol, 0.02% (w/v) Brij-35. The concentrations of phosphorylated MARCKS protein and peptide were determined using their specific radioactivities.

2.3. *Protein phosphatase assays*

MARCKS phosphatase assays were carried out in duplicate using the procedures established for other phosphatase substrates [20], stopping the reactions with trichloroacetic acid precipitation of the protein and measuring phosphatase activity by scintillation counting of the released radioactive phosphate. When the PSD peptide was used, reactions were stopped by the addition of sulphuric acid, followed by extraction of the phosphate molybdate complex in isobutanol/heptane (1:1) [20]. Except in the experiment shown in Fig. 1, the degree of dephosphorylation was below 30% and was linear with respect to time and phosphatase concentration. One unit of activity catalyses the release of 1 μmol of phosphate in 1 min.

2.4. *Dephosphorylation of MARCKS in intact cells*

These studies used a modification of the paradigm of Rodriguez-Pena et al. [10]. Briefly, confluent Swiss 3T3 cells were incubated for 16–18 h in serum-free medium to make them quiescent. They were then washed and incubated for 2 h in a Krebs–Ringer bicarbonate medium containing 0.2 mCi/ml $^{32}P_i$ and 1% (w/v) bovine serum albumin, as described previously [22]. After 1.5 h, okadaic acid, previously prepared as a stock solution at 1 mM in dimethyl sulfoxide (DMSO), was added to the medium in 1/1,000 vol. to give a final concentration of 1 μM. In controls, DMSO alone was added so that final DMSO concentrations in both cases were 0.1% (v/v). After an additional 0.5 h, the cells were treated with bombesin (3 nM); 1 or 2 min later, they were treated with the bombesin antagonist, RPW-substance P (100 μM).

The cells were washed and harvested at various times, and then used for the immunoprecipitation of [^{32}P]MARCKS, as described in [23]. In these studies, cellular homogenates were normalised for trichloroacetic acid precipitable radioactivity before being subjected to immunoprecipitation.

3. RESULTS

3.1. *Dephosphorylation of MARCKS by purified catalytic subunits of PP1 and PP2A*

MARCKS protein, phosphorylated to a high degree of stoichiometry using protein kinase C, could be almost completely dephosphorylated by the purified catalytic subunit of protein phosphatase 2A (PP2Ac). The purified catalytic subunit of protein phosphatase-1 (PP1c) also dephosphorylated MARCKS, although somewhat less efficiently (Fig. 1). Under conditions where the degree of dephosphorylation was proportional to the amount of phosphatase, the relative activities of purified PP1c and PP2Ac towards MARCKS were compared with activities towards a synthetic peptide based on the sequence of the phosphorylation site domain (PSD) of MARCKS, also phosphorylated using protein kinase C, and a well characterised phosphatase substrate, glycogen phosphorylase. The relative activities of purified PP1c and PP2Ac were similar towards glycogen phosphorylase and the PSD peptide. However, the intact MARCKS protein was a rather better substrate for PP2Ac than PP1c (Table I). Neither protein nor peptide appeared to be a significant substrate for PP2B, the Ca^{2+}/calmodulin-activated phosphatase, calcineurin (data not shown).

3.2. *Dephosphorylation of MARCKS and PSD peptide by native forms of PP1, PP2A and PP2C*

The relative activities of the native forms of PP1, PP2A and the Mg^{2+}-dependent protein phosphatase 2C (PP2C) towards MARCKS protein, the PSD peptide and phosphorylase were assessed using diluted post-mitochondrial extracts of rat liver as a source of phosphatases (Table II). PP1 activity was assessed as the proportion inhibited by 0.2 μM inhibitor-2 and PP2A as the proportion inhibited by 2 nM okadaic acid.

Table I

Relative activities of purified catalytic subunits of PP1 and PP2A towards glycogen phosphorylase, MARCKS and PSD peptide

Substrate	Concentration (μM)	PP1 activity (%)	PP2A activity (%)
Phosphorylase	10	100	100
MARCKS	3	86	199
PSD peptide	3	101	107

Activity values are the means of duplicate assays that agreed within ± 5% and are expressed relative to phosphorylase phosphatase activities which were 1.98 and 1.15 mU/ml for type 1 and 2A phosphatases, respectively.

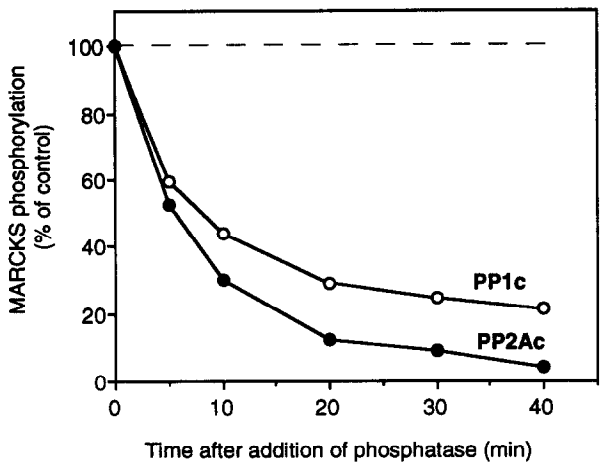

Fig. 1. Dephosphorylation of MARCKS by purified catalytic subunits of protein phosphatases 1 (PP1, closed circles) and 2A (PP2A, ●). Values are the means of duplicate experiments that agreed within ± 10%, and are expressed as a percentage of the stoichiometry of phosphorylation in control incubations lacking phosphatases in which no dephosphorylation was observed (indicated by the broken line). The initial stoichiometry of phosphorylation was 2 mol phosphate/mol protein. MARCKS was used at 3 μM, and PP1 and PP2A at 10 mU/ml assayed using phosphorylase as substrate.

Both PP1 and PP2A are inhibited completely by 1 μM okadaic acid, allowing PP2C to be assessed as the Mg^{2+}-dependent activity in the presence of 1 μM okadaic acid [14,15]. Total activity was taken as the sum of the activities of PP1, PP2A and PP2C. Similar to previous reports [24,25], just over half (58%) of the total phosphorylase phosphatase activity corresponded to PP1, and essentially all of the remaining phosphorylase phosphatase activity in the absence of divalent metal cations was accounted for by PP2A (37% of total). PP2C accounted for < 5% of the total phosphorylase phosphatase activity. Using the MARCKS protein as substrate, PP2A accounted for the greatest proportion of the total activity (52%), while PP1 was rather less active (21% of total). In contrast to phosphorylase phosphatase activity, a significant proportion of the total activity against MARCKS was accounted for by PP2C (27%). In assays performed using two different concentrations of the PSD peptide, PP1, PP2A and PP2C were all active, but PP1 accounted for a greater proportion of the total activity in the extracts (42% and 33% at 10 μM and 3 μM peptide, respectively) than when the MARCKS protein was used as substrate. This indicates that the PSD peptide is a better substrate than the intact protein for the native forms of PP1 in rat liver, consistent with results obtained using the purified phosphatase catalytic subunit (Table I). PP2C accounted for 27% of the total activity when assayed using 10 μM PSD peptide and 49% using 3 μM PSD peptide.

3.3. *Effect of okadaic acid on the phosphorylation and dephosphorylation of MARCKS in intact cells*

Rodriguez-Pena et al. [10] previously showed that, in Swiss 3T3 cells, bombesin-stimulated MARCKS phosphorylation rapidly decreased following the addition of a bombesin receptor antagonist. Using a minor modification of the same experimental paradigm, we found that pretreatment of cells with okadaic acid (1 μM for 30 min) resulted in only a minor increase in MARCKS phosphorylation (Fig. 2a,c). Similarly, there was little difference between okadaic acid-treated and untreated cells 1 min after bombesin stimulation. However, while the cells not treated with okadaic acid exhibited rapid MARCKS dephosphorylation after the addition of the bombesin receptor antagonist ($t_{1/2}$ about 4 min), the protein was minimally dephosphorylated during this time in the cells pretreated with okadaic acid (Fig. 2a,c). The incorporation of $^{32}P_i$ into proteins in the total cellular homogenates in this experiment is shown in Fig. 2b. Even in the absence of bombesin stimulation (0 time point), a large increase in the phosphorylation of many proteins was seen with okadaic acid treatment.

Since the difference in MARCKS phosphorylation appeared to be greatest in the later time points in this experiment, we repeated the experiment and evaluated four plates each of okadaic acid-treated and untreated cells 13 min after RPW-substance P treatment. The autoradiograph shown in Fig. 3a clearly demonstrates markedly increased phosphorylation of MARCKS in the okadaic acid-treated cells. Densitometric evaluation of these data (Fig. 3b) revealed about a 3.4-fold increase in MARCKS phosphorylation in the okadaic acid-treated cells, a difference that was highly statistically significant ($P < 0.0005$ using Student's t-test). A comparison of the magnitude of this change with the initial stimulation of phosphorylation by bombesin (Fig. 2a)

Table II

Effect of inhibitor-2, okadaic acid and magnesium ions on phosphorylase phosphatase (Phos-P), MARCKS phosphatase (MARCKS-P) and PSD peptide phosphatase (PSDP-P) activities in post-mitochondrial extracts of rat liver

Additions	Phos-P activity (%)	MARCKS-P activity (%)	PSDP-P activity (%)	PSDP-P activity (%)
1. None	100	100	100	100
2. 0.2 μM inhibitor-2	45	71	54	59
3. 2 nM okadaic acid	65	27	76	78
4. 1 μM okadaic acid	3	0	1	4
5. 1 μM okadaic acid plus 10 mM Mg^{2+}	5	38	29	60
Substrate concentration (μM)	10	3	10	3

Assays were carried out at 1:150 dilution of extract. Activity values are the means of duplicate assays that agreed within ± 10%, and are given relative to those measured in the standard assay (EGTA without Mg^{2+}) which were: 32.8 mU/ml (phosphorylase phosphatase), 4.8 mU/ml (MARCKS phosphatase), 41.7 mU/ml (10 μM PSD peptide phosphatase) and 12.6 mU/ml (3 μM PSD peptide phosphatase). One unit of activity catalyses the release of 1 μmol of phosphate in 1 min.

suggests that much, if not all, of the cellular MARCKS phosphatase activity in these cells was inhibited by okadaic acid.

4. DISCUSSION

The present studies have demonstrated that phosphorylated recombinant MARCKS and its synthetic PSD peptide are excellent substrates for the catalytic subunuts of both of the okadaic acid-sensitive phosphatases, PP1 and PP2A. In fact, the intact protein appears to be superior to glycogen phosphorylase as a substrate for PP2A, while the PSD peptide is similar to phosphorylase. In cell-free extracts, the native forms of PP1 and PP2A account for all of the phosphatase activity towards MARCKS in the absence of divalent metal cations. The difference between the PSD peptide and the protein persisted in the experiments using extracts; the activities of the native forms of PP1 and PP2A towards the PSD peptide and glycogen phosphorylase were very similar, whereas MARCKS was a relatively better substrate for PP2A and a somewhat worse one for PP1. These differences are noteworthy given the near-identity of the kinetic responses of the protein and peptide as substrates for protein kinase C [4,19]. They suggest that there are determinants on MARCKS outside of the immediate primary structure at the phosphorylation sites that affect its rate of dephosphorylation by these phosphatases. Neither protein nor peptide appeared to be a significant substrate for PP2B, the Ca^{2+}/calmodulin-activated phosphatase, calcineurin. However, both were substrates for the okadaic acid-resistant and Mg^{2+}-dependent phosphatase, PP2C. In this case, the PSD peptide appeared to be superior to the protein as a substrate.

These findings raised the interesting question of whether PP2C might represent a significant component of the MARCKS dephosphorylating activity in intact cells. For this reason, we performed experiments similar to those of Rodriguez-Pena et al. [10], examining the dephosphorylation of MARCKS in Swiss 3T3 cells. The results of these studies show clearly that the rapid MARCKS dephosphorylation that occurs with bombesin activation of protein kinase C followed by a bombesin receptor antagonist can be almost completely prevented by pretreatment of the cells with 1 μM okadaic acid. In other words, at least in Swiss 3T3 cells, MARCKS dephosphorylation is likely to be mediated by PP1 and/or PP2A. Distinguishing between these two activities in intact cells is more difficult than in cell

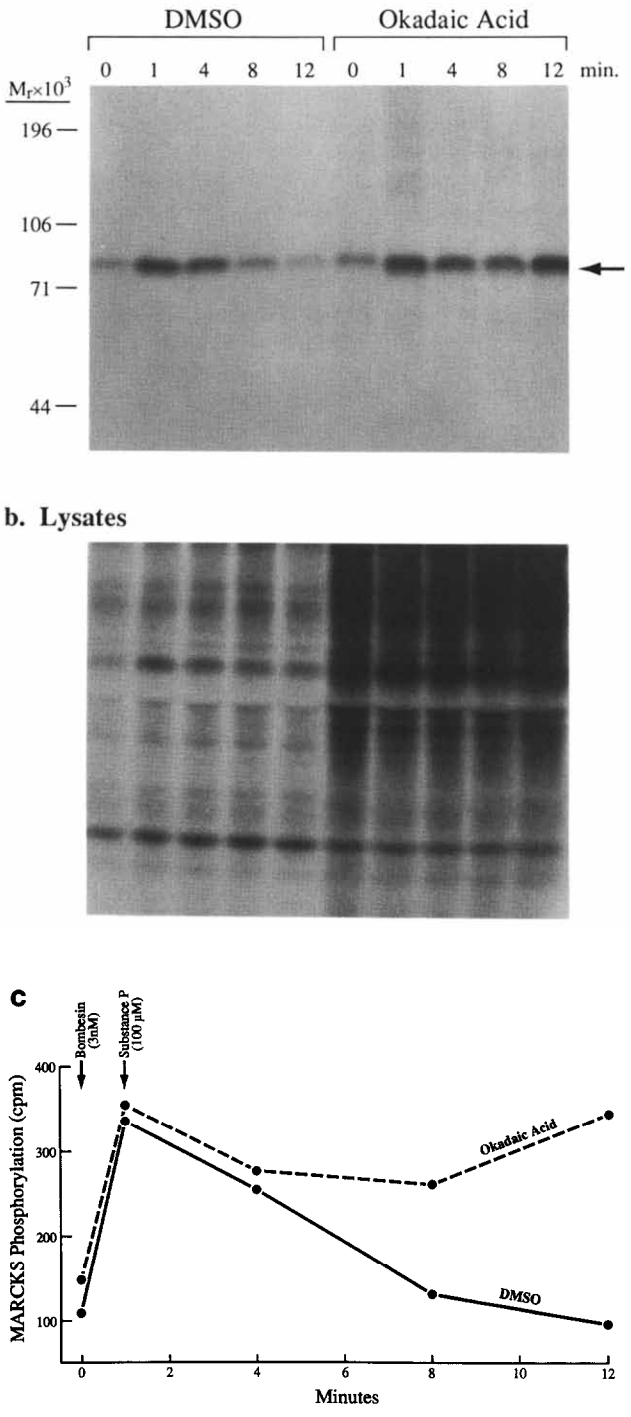

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Fig. 2. Effects of okadaic acid on MARCKS dephosphorylation in Swiss 3T3 cells. Confluent, serum-deprived Swiss 3T3 cells were labelled with $^{32}P_i$ and then pre-treated for 30 min with either okadaic acid (1 μM in 0.1% (v/v) DMSO) or 0.1% (v/v) DMSO as a control. At time 0, both sets of cells were treated with 100 μM RPW-substance P. Individual plates of cells were harvested at the indicated times, and whole cell lysates were normalised for trichloroacetic acid-precipitable radioactivity. These matched extracts were then used for the immunoprecipitation of ^{32}P-labelled MARCKS, indicated by the arrow in a. Although MARCKS has a calculated molecular mass of 30 kDa, it migrates anomalously at about 80 kDa on denaturing gels. An autoradiograph of a gel in which equal volumes of the cell lysate were subjected to electrophoresis is shown in b, and illustrates the effect of okadaic acid. In c is shown a quantitative analysis of the ^{32}P-labelled MARCKS protein in a, determined by excising the bands containing MARCKS from the gel and subjecting them to scintillation counting. 'Substance P' denotes RPW-substance P.

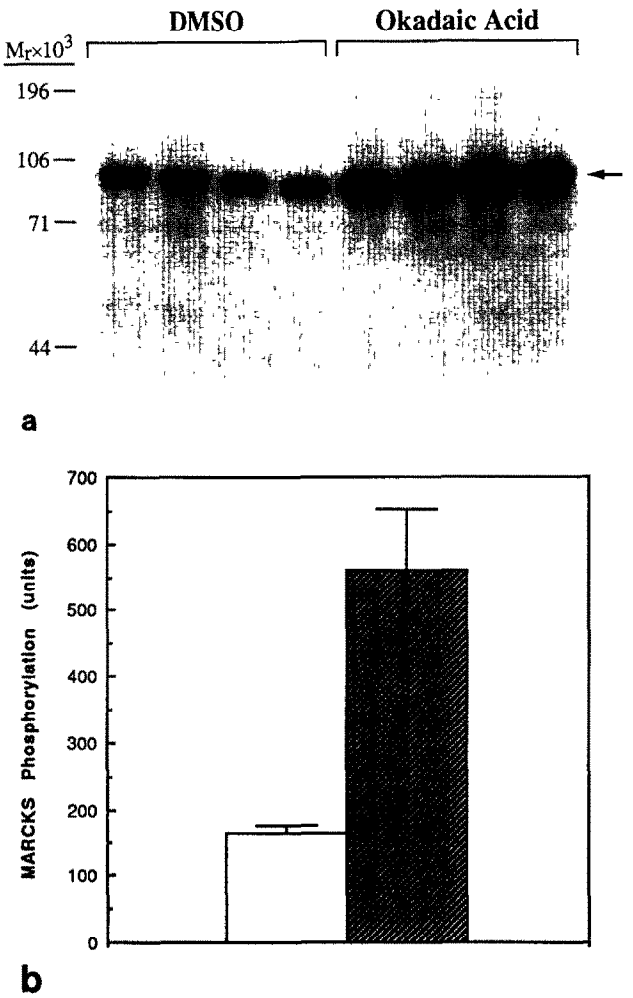

Fig. 3. Okadaic acid causes a significant inhibition of MARCKS dephosphorylation in Swiss 3T3 cells. Serum-deprived, confluent Swiss 3T3 cells were treated as in the legend to Fig. 2, except that bombesin treatment (3 nM) was for 2 min before the addition of RPW-substance P (100 μM) for an additional 13 min. The cells were then harvested, and four plates of cells for each treatment (okadaic acid and 0.1% (v/v) DMSO control) were matched for trichloroacetic acid-precipitable radioactivity and subjected to immunoprecipitation with an anti-MARCKS antibody. In a is shown an autoradiograph of these data; ^{32}P-labelled MARCKS is indicated by the arrow. In b is shown a densitometric evaluation of the extent of MARCKS phosphorylation; the hatched bar indicates the mean ± S.D. from the four plates of cells treated with okadaic acid; the open bar indicates the mean ± S.D. from the four control plates of cells treated with 0.1% (v/v) DMSO alone.

extracts, but could conceivably be accomplished by the use of careful okadaic acid dose–response analyses, combined with the use of tautomycin, a toxin more potent for PP1 than PP2A [26].

Several previous studies have evaluated changes in MARCKS phosphorylation state with okadaic acid treatment of cells, and the results have been somewhat conflicting. For example, Mahadevan et al. [27] found that okadaic acid did not significantly enhance phorbol ester-stimulated MARCKS phosphorylation in a mouse fibroblast cell line. On the other hand, Bhat [28] showed that okadaic acid enhanced phorbol ester-stimulated MARCKS phosphorylation in oligodenroglial progenitor cells, while Thelen et al. [8] demonstrated that okadaic acid inhibited MARCKS dephosphorylation in fMet-Leu-Phe-stimulated human neutrophils. More recently, Amess et al. [29] found that okadaic acid appeared to inhibit MARCKS dephosphorylation from one or two (but not three) of its serine phosphorylation sites in mouse fibroblasts. Thus, most studies in intact cells to date agree that okadaic acid-sensitive phosphatases are involved in dephosphorylation of at least some phosphorylated serines in MARCKS in intact cells. Such phosphatases seem to be important in controlling the phosphorylation state of MARCKS during the recovery period after the transient activation of protein kinase C. However, our results also suggest that under basal conditions, at least in Swiss 3T3 cells, protein kinase C activity is very low and the phosphorylation state of MARCKS is not greatly influenced by the activity of okadaic acid-inhibited phosphatases. Furthermore, they indicate that okadaic acid does not activate protein kinase C.

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