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14-3-3 proteins bind to histone and affect both histone phosphorylation and dephosphorylation

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Abstract

14-3-3 proteins appear to play a critical role in Ca^{2+}-stimulated secretion in permeabilized chromaffin cells. 14-3-3 proteins have been reported to be both stimulators and inhibitors of protein kinase C (PKC). We have found that 14-3-3 proteins, isolated on the basis of their ability to enhance secretory activity, stimulated histone phosphorylation by PKC, but they had no effect on myosin light chain phosphorylation by PKC. 14-3-3 proteins were also found to inhibit the rate of [^{32}P]histone dephosphorylation but not the rate of [^{32}P]myosin light chain dephosphorylation. Cross-linking experiments and affinity chromatography demonstrated that 14-3-3 proteins bind to histones. These results suggest that at least some of the reported effects of 14-3-3 proteins on PKC activity may result from 14-3-3 proteins binding to histone.

Key words: 14-3-3 protein; Protein phosphatase; Protein kinase C; Secretion

1. Introduction

Catecholamine secretion by digitonin-permeabilized chromaffin cells is both ATP- and Ca^{2+}-dependent. Incubation of these permeabilized cells in the absence of Ca^{2+} results in a progressive loss of proteins and secretory activity [1]. The addition of cytosolic proteins prevents this loss of Ca^{2+}-dependent secretion [1,2]. 14-3-3 proteins (or Exo1) appear to play a key role in this restoration [3,4], but their precise role in the secretory processes remains to be determined.

14-3-3 proteins are acidic, dimeric proteins with subunits of 27–30 kDa [5]. They are most abundant in brain but are present at lower levels in a variety of other tissues [6,7]. 14-3-3 proteins can activate both tyrosine and tryptophan hydroxylases [8]. Stimulation of these hydroxylases can not account for the effect of 14-3-3 proteins on Ca^{2+}-dependent secretion [3,4], and 14-3-3 proteins are in tissues which also do not contain hydroxylase [6,7]. It has been reported that a Ca^{2+}-sensitive phospholipase A_2 is a form of 14-3-3 protein [9]. However, 14-3-3 proteins, isolated on the basis of their ability to stimulate secretion, have no significant phospholipase A_2 activity ([10] and Chen and Wagner, unpublished results).

Toker et al. [11,12] have reported that 14-3-3 proteins inhibit protein kinase C (PKC) and have called them KCIPs, for kinase C inhibitor proteins. In contrast, Isobe et al. [13] have reported that 14-3-3 proteins stimulate PKC activity. Morgan and Burgoyne [14] found that 14-3-3 proteins, isolated on the basis of their ability to stimulate secretion (Exo1), have no effect on PKC activity.

Because an increase in the level of protein phosphorylation tends to increase Ca^{2+}-dependent secretion in permeabilized chromaffin cells [3,15], in the present investigation we examined the possibility that 14-3-3 proteins might inhibit protein phosphatases. We found that 14-3-3 proteins, isolated on the basis of their ability to stimulate secretion, inhibited the rate of histone dephosphorylation, but they had no effect on the rate of myosin light chain dephosphorylation. We also found that 14-3-3 proteins stimulated histone phosphorylation by PKC but not myosin light chain phosphorylation by PKC. Cross-linking experiments and affinity chromatography showed that 14-3-3 proteins bind to histones. These results suggest that at least some of the effects of 14-3-3 proteins on histone phosphorylation and dephosphorylation may result from 14-3-3 proteins binding to histone. Toker et al. [11,12] and Isobe et al. [13], but not Morgan and Burgoyne [14], used histone as the substrate for PKC.

2. Material and methods

PC12 cells were cultured as described previously [16]. 14-3-3 proteins were isolated from either bovine brain or adrenal medulla on the basis of their ability to enhance Ca^{2+}-stimulated secretion [4]. PKC was isolated from bovine brain [17]. The catalytic subunit of protein phosphatase 2A [18], smooth muscle myosin light chains [19], and myosin light chain kinase [20] were isolated from turkey gizzards.

PC12 cells were lysed by incubation in 5 mM EDTA and 5 mM Tris-HCl, pH 7.5 (10^5 cells/35 μl) for 5 min at room temperature [21]. The lysed cells were centrifuged for 5 min at 12,000 × *g*, and the soluble fraction used for protein phosphatase assays. Extracts from 7×10^4 cells were incubated in 100 μl of 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM dithiothreitol, 3 μM ^{32}P-phosphorylated histone (approximately 0.5 mol ^{32}P/mol histone) or 2 μM ^{32}P-phosphorylated myosin

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Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; KCIPs, kinase C inhibitor proteins; PKC, protein kinase C; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A.

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regulatory light chains (approximately 0.5 mol ^{32}P/mol light chain) at 30°C for 5 min [21,22], and the amounts of $^{32}PO_4$ released determined [23]. Less than 15% of the phosphorylated histones or myosin light chains were dephosphorylated, and the rates of dephosphorylation were linear. Histone (Sigma, type III-S) was phosphorylated by cAMP-dependent protein kinase (Sigma) [24] and myosin light chains by myosin light chain kinase [20]. Myosin light chains phosphorylated by myosin light chain kinase and histone phosphorylated by cAMP-dependent protein kinase are good substrates for both protein phosphatase 1 and protein phosphatase 2A [18,21,22,25], the two most abundant serine/threonine-specific protein phosphatases [26].

The assay buffer used for measuring PKC activity (60 μl total volume) contained 20 mM HEPES, pH 7.4, 1 mM dithiothreitol, 0.33 mM EDTA, 0.33 mM EGTA, 1.7 mM $CaCl_2$, 10 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP (2×10^4 cpm/nmol), 200 μg/ml phosphatidylserine, 1 μg/ml PKC [17] and either 0.25 mg/ml histone (Sigma; type III-S), 0.25 mg/ml myosin regulatory light chains, or 25 μg/ml of a synthetic peptide, VRKRTLRRL, that is a specific substrate for PKC [27]. After a 10 min incubation at 30°C, the reactions were terminated, and the amount of $^{32}PO_4$ incorporated into histone [13,17], myosin light chain [28], or synthetic peptide [29] determined.

Conditions used for cross-linking were 0.1 M triethanolamine, pH 8.5, 4 mM EDTA, 2 mg/ml dimethyl suberimidate, 0.1 mg/ml 14-3-3 proteins, and either 0.1 mg/ml histone H1 (Boehringer-Mannheim) or 0.1 mg/ml [^{32}P]histone H1. After 30 min at 37°C, the reactions were terminated by the addition of 50 mM Tris, pH 8.5. Cross-linked products were examined by SDS-PAGE and autoradiography. Cross-linking experiments were also performed with histone type III-S (Sigma). The results were identical to those obtained with histone H1 except the histones were more heterogeneous and, consequently, gave more bands when examined by SDS-PAGE.

Histone (3 mg; Sigma; type III-S) was coupled to 1 ml CNBr-activated Sepharose (Pharmacia) following the manufacturer's instructions.

3. Results and discussion

3.1. Effect of 14-3-3 proteins on protein phosphatase activity

The rate of [^{32}P]histone dephosphorylation by a soluble extract of PC12 cells was inhibited 35% by the addition of 100 μg/ml 14-3-3 proteins, but the rate of [^{32}P]myosin light chain dephosphorylation was not inhibited by 14-3-3 proteins (Table 1). The rate of [^{32}P]histone dephosphorylation by this cell extract was not inhibited by boiled 14-3-3 proteins or by similar concentrations of bovine serum albumin, calmodulin, or calpactin. As shown in Fig. 1, 50 μg/ml 14-3-3 protein gave maximal inhibition of histone dephosphorylation, and 12.5 μg/ml gave about half maximal inhibition. This concentration range for 14-3-3 proteins is similar to those reported for

Table 1
Effect of 14-3-3 proteins on the protein phosphatase activity in PC12 cell extracts

Substrate	pmol $^{32}PO_4$ released/5 min	
	Control	+ 100 μg/ml 14-3-3 proteins
[^{32}P]histone	13.0 ± 0.9	8.7 ± 0.6
[^{32}P]myosin light chain	13.3 ± 0.1	13.2 ± 1.0

Data are means ± S.D. for three trials. Assays were performed as described in section 2.

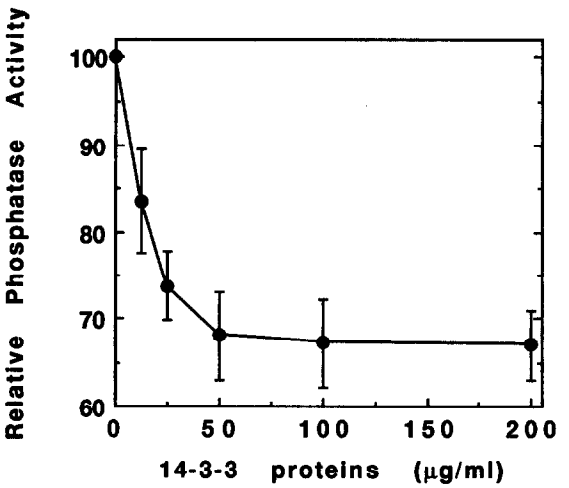

Fig. 1. Inhibition of protein phosphatase activity by 14-3-3 proteins. The protein phosphatase activity of a PC12 cell extract was determined as described in section 2 using 3 μM [^{32}P]histone as a substrate in the presence of the indicated concentrations of 14-3-3 proteins. Rates are expressed relative to that obtained in the absence of added 14-3-3 proteins. Data are means ± S.D. for three trials.

both the inhibition [11] and the stimulation [13] of histone phosphorylation by PKC and for the stimulation of catecholamine secretion [3,4]. As the phosphatase assays were performed in 2 mM EDTA, most of the phosphatase activity in the PC12 cell extract is presumably due to protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) [26]. Experiments with inhibitor-2 (a protein that inhibits PP1 but not PP2A) and okadaic acid (an inhibitor of protein phosphatases that can be used to distinguish between PP1 and PP2A) [26] indicated that the addition of 14-3-3 proteins to PC12 cell extracts partially inhibited the activities of both PP1 and PP2A towards [^{32}P]histone (data not shown). 14-3-3 proteins also gave 40% inhibition in the rate of histone dephosphorylation by the isolated catalytic subunit of PP2A; 7.3 ± 0.7 pmol $^{32}PO_4$ was released from [^{32}P]histone in the absence of 14-3-3 proteins, and 4.2 ± 0.1 pmol $^{32}PO_4$ was in the presence of 100 μg/ml 14-3-3 proteins. In contrast, the dephosphorylation of myosin light chains by purified PP2A was not affected by 14-3-3 proteins; 10.1 ± 0.1 pmol $^{32}PO_4$ was released from [^{32}P]light chains in the absence of 14-3-3 proteins, and 10.6 ± 0.8 pmol in the presence of 100 μg/ml 14-3-3 proteins.

The amount of inhibition of protein phosphatase activity by 14-3-3 proteins depended on histone concentration. When 2.5 μM [^{32}P]histone was used, 100 μg/ml 14-3-3 proteins (3.3 μM) gave about 50% inhibition of protein phosphatase activity in PC12 cell extracts, but when 10 μM [^{32}P]histone was used, 100 μg/ml 14-3-3 proteins gave only about a 20% inhibition of protein phosphatase activity.

The observations that (i) 14-3-3 proteins inhibited histone dephosphorylation but not myosin light chain

dephosphorylation, (ii) 14-3-3 proteins partially inhibited histone dephosphorylation by both PP1 and PP2A, and (iii) the extent of inhibition of histone dephosphorylation by 14-3-3 proteins depended on the histone concentration, all suggest that the inhibition of histone dephosphorylation by 14-3-3 proteins might result from an interaction of 14-3-3 proteins with histone rather than with the protein phosphatases.

3.2. *Effect of 14-3-3 proteins on PKC activity*

While Toker et al. [11,12] have reported that 14-3-3 proteins (KCIPs) inhibit PKC, Isobe et al. [13] have reported that 14-3-3 proteins stimulate PKC. Both groups used histone as a substrate for PKC. The reason for this difference in the effect of 14-3-3 proteins on histone phosphorylation by PKC is not clear, but it is possible that different isoforms of 14-3-3 proteins have different effects on histone phosphorylation by PKC. There are at least seven [30,31] and perhaps twelve [10] different isoforms of 14-3-3 proteins. The KCIPs preparation of Toker et al. [12] contains at least seven isoforms (α–η) of 14-3-3 proteins. Isobe et al. [13] used three different purified isoforms (ζ, β, and ε). As shown in Table 2, 14-3-3 proteins, isolated on the basis of their ability to stimulate secretion, stimulated histone phosphorylation by PKC almost two-fold, a result nearly identical to that obtained by Isobe et al. [13] with purified isoforms of 14-3-3 proteins. However, these 14-3-3 proteins had little or no effect on myosin light chain phosphorylation by PKC (Table 2). Morgan and Burgoyne [14] used a synthetic peptide substrate specific for PKC to examine the effect of 14-3-3 proteins (Exol) on PKC activity. They found that 14-3-3 proteins had no effect on the phosphorylation of this peptide by PKC. We also found that under the same assay conditions in which 14-3-3 proteins stimulated histone phosphorylation by PKC, 14-3-3 proteins had no effect on the phosphorylation of this peptide by PKC (Table 2). Like the KCIPs preparation of Toker et al. [11,12], our preparations of 14-3-3 proteins [4] and that of Morgan and Burgoyne [3,14] are heterogeneous and contain a variety of isoforms. At this time it is not known why KCIPs inhibit histone phosphorylation by PKC and our preparation of 14-3-3 proteins stimulate histone phosphorylation by PKC. However, it is possible that KCIPs contain an isoform of 14-3-3 proteins that is not present in our preparation of 14-3-3 proteins or that it is present in too low a concentration to inhibit histone phosphorylation by PKC.

In agreement with the results of Isobe et al. [13] and Morgan and Burgoyne [14], we observed no significant phosphorylation of 14-3-3 proteins by PKC (data not shown). Toker et al. [12], however, found that at least some of their 14-3-3 proteins are substrates for PKC.

The observation that 14-3-3 proteins affect PKC phosphorylation of histone but not PKC phosphorylation of either myosin light chain or a synthetic peptide suggests that 14-3-3 proteins might interact with histone and alter its rate of phosphorylation by PKC.

3.3. *14-3-3 proteins bind to histones*

The interaction of 14-3-3 proteins with histone was examined using the chemical cross-linker dimethyl suberimidate (Fig. 2). As expected from their dimeric structure, treatment of 14-3-3 proteins alone with this cross-linker generated a species with a molecular weight on SDS-PAGE of about 60 kDa (Fig. 2A, lane 2). Treatment of unlabeled histone (Fig. 2A, lane 4) or [^{32}P]histone (Fig. 2B, lane 2) alone with this cross-linker resulted in little, if any, cross-linking of histone to itself. Fig. 2B is an autoradiogram and therefore only [^{32}P]histone is detected. When a mixture of 14-3-3 proteins and histone were treated with the cross-linker, a species of about 100 kDa was formed (Fig. 2A, lane 3) and there was much less of the 60 kDa product than was obtained when 14-3-3 proteins were cross-linked by themselves. Fig. 2B shows that the 100 kDa species contained histone (lane 3) and that a large fraction of the [^{32}P]histone had been cross-linked. Thus, the 100 kDa product appears to be a dimer of 14-3-3 proteins cross-linked to histone(s). The precise amount of cross-linking of histones to 14-3-3 proteins was somewhat variable, ranging from a low of about 40% to almost complete cross-linking. The cross-linking experiments shown in Fig. 2 were performed in the presence of 2 mM EDTA. Similar results were ob-

Table 2
Effect of 14-3-3 proteins on protein kinase C activity

Substrate	nmol $^{32}PO_4$ incorporated/min/mg PKC	
	Control	+ 100 μg/ml 14-3-3 proteins
Histone in the presence of Ca/PL	650 ± 20	1,230 ± 50
Histone in the absence of Ca/PL	100 ± 10	110 ± 20
Myosin light chain in the presence of Ca/PL	570 ± 30	580 ± 50
Peptide VRKRTLRRL in the presence of Ca/PL	1,200 ± 50	1,140 ± 70

Data are means ± S.D. for three trials. Assays were performed as described in section 2 either in the presence or absence of 1.67 mM $CaCl_2$ and 200 μg/ml phosphatidylserine (Ca/PL).

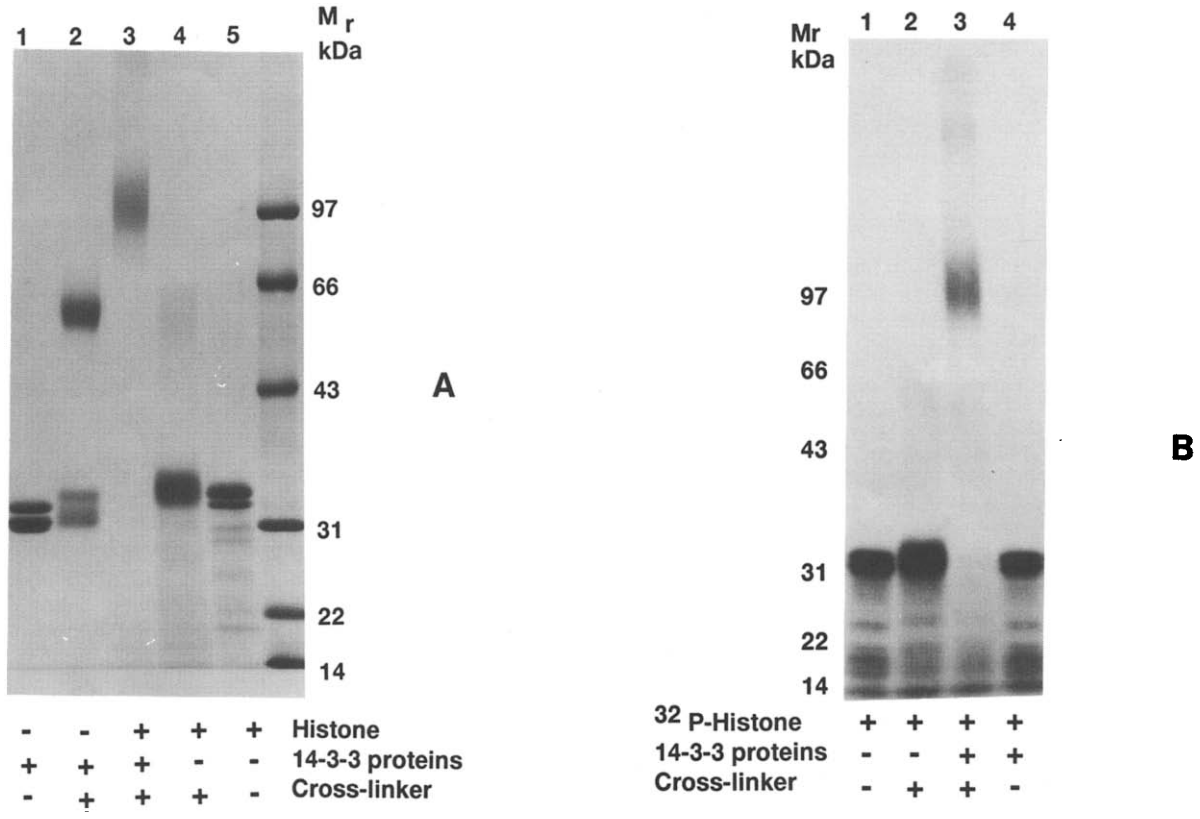

Fig. 2. Cross-linking of 14-3-3 proteins to histone H1. Cross-linking reactions were performed as described in section 2. (A) Samples separated by SDS-PAGE and stained with Coomassie brilliant blue: lanes (1) 14-3-3 proteins not treated with the cross-linker dimethyl suberimidate, (2) 14-3-3 proteins treated with dimethyl suberimidate, (3) 14-3-3 proteins and histone H1 treated with dimethyl suberimidate, (4) histone H1 treated with dimethyl suberimidate, and (5) histone H1 not treated with dimethyl suberimidate. (B) Samples separated by SDS-PAGE and [^{32}P]histone H1 detected by autoradiography: lanes (1) [^{32}P]histone H1 not treated with dimethyl suberimidate, (2) [^{32}P]histone H1 treated with dimethyl suberimidate, (3) [^{32}P]histone H1 and 14-3-3 proteins treated with dimethyl suberimidate, and (4) [^{32}P]histone H1 and 14-3-3 proteins not treated with dimethyl suberimidate.

tained in the presence of 1.7 mM $CaCl_2$ and 10 mM $MgCl_2$.

14-3-3 proteins were also reacted with dimethyl suberimidate in the presence of either 0.1 mg/ml ^{32}P-labeled myosin light chain or 3 μg/ml PKC autophosphorylated with [γ-^{32}P]ATP. The reaction products were separated by SDS-PAGE and examined by autoradiography. There was no detectable cross-linking of 14-3-3 proteins to either myosin light chains or PKC (data not shown).

Immobilized histone was also used to look for an interaction between 14-3-3 proteins and histones. 14-3-3 proteins were applied to either a histone–Sepharose column or a control column (CNBr-activated Sepharose reacted with ethanolamine) in a low salt buffer (Fig. 3). While most of the 14-3-3 proteins did not bind to the control column, more than 75% bound to the histone column. The 14-3-3 proteins which bound to the histone column were not eluted by 0.1 M NaCl but were eluted by 1 M NaCl (Fig. 3).

The results presented here show that 14-3-3 proteins can bind to histone. This is not unexpected as histones are basic proteins and 14-3-3 proteins are acidic. Other acidic proteins such as calmodulin can also bind to histones [32]. However, we found that, unlike 14-3-3 proteins, calmodulin had no effect on histone phosphorylation by PKC (580 nmol ^{32}P incorporated into histone/min/mg PKC in the presence and absence of 100 μg/ml calmodulin), and it did not inhibit histone dephosphorylation by PC12 cell extracts. Whether there is any physiological significance to the binding of 14-3-3 proteins to histones is not known, but 14-3-3 proteins in non-mammalian cells have been postulated to be involved in regulating gene expression (reviewed in [31]). The binding of 14-3-3 proteins to histone provides an explanation as to why 14-3-3 proteins affect both the phosphorylation and dephosphorylation of histones but not the phosphorylation and dephosphorylation of myosin light chains.

Because 14-3-3 proteins did not inhibit myosin light chain dephosphorylation or stimulate myosin light chain phosphorylation by PKC, it seems unlikely that 14-3-3 proteins act either as general inhibitors of protein phosphatases or as general stimulators of PKC. However, it is possible that 14-3-3 proteins affect secretion by

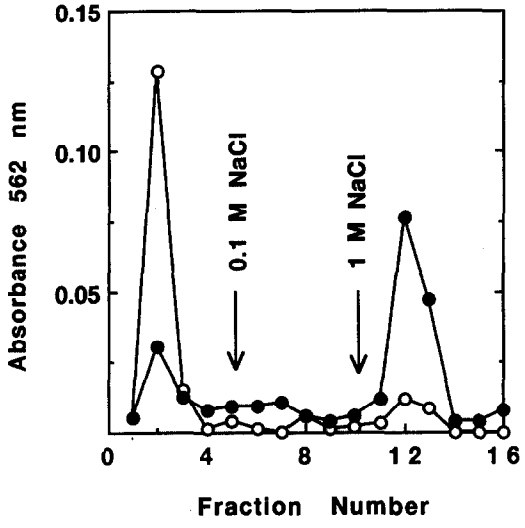

Fig. 3. Binding of 14-3-3 proteins to histone–Sepharose. 700 μl of 100 μg/ml 14-3-3 proteins were applied to a 1 ml column of either histone–Sepharose (●) or ethanolamine–Sepharose (○) equilibrated in 50 mM Tris, pH 7.5, 2 mM EDTA, and 0.5 mM dithiothreitol. The columns were washed successively with 5 ml of equilibration buffer, 5 ml of equilibration buffer plus 0.1 M NaCl, and 5 ml of equilibration buffer plus 1 M NaCl. 1 ml fractions were collected and protein detected using a bicinchoninic acid protein assays (Pierce) following the manufacturer's instructions.

binding to some unidentified protein in the cell and inhibiting its dephosphorylation or increasing its phosphorylation by PKC. Consistent with these possibilities is the result of Morgan and Burgoyne [3] that the enhancement of secretion by 14-3-3 proteins proteins is greater if the cells are also treated with phorbol ester to stimulate PKC.

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