Identification of 14-3-3 proteins in human platelets: effects of synthetic peptides on protein kinase C activation

Caroline P. D. WHEELER-JONES*, Michele P. LEARMONTH†, Harry MARTIN† and Alastair AITKEN†‡

*Vascular Biology Research Centre, Biomedical Sciences Division, King's College London, Campden Hill Road, Kensington, London W8 7AH, U.K., and

†Laboratory of Protein Structure, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

The 14-3-3 proteins inhibit protein kinase C (PKC) activity *in vitro* and contain conserved sequences that resemble the pseudo-substrate domain of PKC and the C-terminus of the annexins. In the present study we have identified the isoforms of 14-3-3 in human platelets and used synthetic peptides derived from the regions with similarity to PKC and annexins to examine the potential role of 14-3-3 in regulating platelet PKC activity. Immunoblotting studies with isoform-specific antisera raised against the acetylated peptides corresponding to the N-termini of 14-3-3 showed that these cells express high levels of the β , γ and ζ 14-3-3 isoforms. In addition, low levels of the ϵ and η 14-3-3 isoforms were detected. In washed, saponin-permeabilized platelets incubated with [γ - 32 P]ATP, thrombin- and phorbol 12-myristate 13-acetate (PMA)-induced phosphorylation of several

proteins (66, 45, and 20 kDa) was inhibited by preincubation with AS peptide (KNVVGARRSSWRVISSIEQK) based on the pseudosubstrate-like region of the 14-3-3 family. A control peptide of similar size had no effect on PKC-mediated phosphorylation. PMA caused a rapid translocation of PKC activity from the cytosol to the particulate fraction of saponin-permeabilized platelets that was unaffected by either the AS peptide or a peptide derived from the annexin-like 14-3-3 domain (MKGDYYRYLAEVATGDD). These results suggest that isoforms of the 14-3-3 family may play an important physiological role as inhibitors of PKC activity in human platelets but are unlikely to be involved in controlling association of PKC with the membrane.

INTRODUCTION

The 14-3-3 proteins are a highly conserved, widely distributed eukaryotic protein family that consists of several isoforms with apparent molecular masses ranging from 29 to 33 kDa. The varied functions documented for members of the 14-3-3 family suggest that these proteins may be particularly important as regulators of signal transduction processes.

Members of this family of proteins are found in a broad range of eukaryotes, including plants and invertebrates, and have been implicated in several signalling mechanisms in a variety of cell types (reviewed in [1]). A recent near-exponential increase in publications has suggested many additional biological roles for the 14-3-3 family [2]. So far seven distinct products of genes coding for 14-3-3 isoforms have been purified and sequenced and are known to be differentially expressed among mammalian tissues. Two of the abundant mammalian and avian brain isoforms (β and ζ) have recently been shown to associate with and possibly activate Raf protein kinase in yeast and in the cytosol and at the membrane of mammalian cells [2–4]. These two isoforms occur in brain as phosphorylated forms *in vivo* (α and δ) [5]. Expression of 14-3-3 in *Xenopus* oocytes also leads to activation of Raf and promotes Raf-dependent maturation of oocytes [6]. These findings provide strong evidence that 14-3-3 proteins are involved in the regulation of cell growth and proliferation through interactions with Raf and other key proteins that are central to the mitogen-activated protein kinase cascade. Raf-1 is a substrate for protein kinase C (PKC) *in vitro*. The latter kinase is activated by a wide variety of mitogenic stimuli, including thrombin [7].

The recent suggestion that mitogen-activated protein kinase might regulate exocytosis in non-proliferative cells (e.g. platelets [8]) raises the possibility that 14-3-3 is involved in the control of

platelet secretion, in addition to the role recently ascribed to 14-3-3 in secretion/exocytosis from adrenal medulla [9].

A sheep platelet phospholipase A_2 (PLA $_2$) with high (submicromolar) Ca^{2+} sensitivity was identified on the basis of peptide sequences as the 14-3-3 ζ isoform [10]; when expressed in *Escherichia coli* it formed a stable arachidonyl-enzyme intermediate and the recombinant protein was shown to bind Ca^{2+} . The authors proposed that the recombinant protein 'mediated phospholipolysis' and some recent publications describe ζ 14-3-3 as 'human PLA $_2$ '. Among these is a paper [11] that describes an association with platelet glycoprotein GPIb-IX. The authors suggest this as a possible mechanism for thrombin-induced activation although they did not confirm that thrombin elevated PLA $_2$ activity. It has in fact been shown by several laboratories that 14-3-3 isoforms do not have PLA $_2$ activity. Furthermore, mammalian brain and recombinant 14-3-3 proteins (including ζ) do not activate or inhibit PLA $_2$ [12]. The crystal structure of 14-3-3 that we have recently determined does not support the existence of a lipase or similar active site [13].

In platelets, excitatory agonists promote the generation of Ins(1,4,5) P_3 and diacylglycerol, which respectively result in an elevation of intracellular Ca^{2+} and activation of PKC, the major receptor for tumour-promoting phorbol esters [14]. There is now substantial evidence to support the involvement of PKC in both the initiation and termination of cellular signals elicited by receptor-operating platelet agonists [15], and at least three isoforms of PKC have been identified in human platelets [16]. Although there is some evidence that the different isoforms of PKC are differentially regulated during platelet activation [17,18], there is no information available about the potential physiological role of endogenous PKC inhibitors in these cells.

In the present study we have identified isoforms of 14-3-3 present in human platelets by using polyclonal antisera raised

against peptides corresponding to the N-termini of sheep brain 14-3-3 isoforms [19,20]. In addition, we have introduced peptide sequences derived from two regions of 14-3-3 into saponin-permeabilized platelets to examine the possible role of the 14-3-3 family in controlling platelet PKC activity.

MATERIALS AND METHODS

[γ - 32 P]ATP (4500 Ci/mmol) was obtained from ICN Flow (High Wycombe, Bucks., U.K.). Human α -thrombin, phorbol 12-myristate 13-acetate (PMA) and indomethacin were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Chemicals for gel electrophoresis were from Bio-Rad (Hemel Hempstead, Herts., U.K.) and National Diagnostics (Hessle, Hull, North Humberside, U.K.). All other materials were of analytical reagent grade and were purchased from either Sigma or BDH (Poole, Dorset, U.K.). N-terminally acetylated peptides for the production of anti-14-3-3 antibodies and other peptides were synthesized by using standard *N*-9-fluorenylmethoxycarbonyl chemistry on an Applied Biosystems 430A peptide synthesizer with reagents supplied by the manufacturer. Peptides were purified by reverse-phase HPLC. Their structures were verified by mass spectrometry and peptide concentrations were verified by amino acid analysis.

Purification of kinase C inhibitor protein (KCIP) 1 and fractionation of its isoforms by reverse-phase HPLC

14-3-3 protein was isolated and purified from sheep brain and from human platelets by a combination of anion-exchange and hydrophobic-interaction chromatographies as described previously [21]. Purified preparations of 14-3-3 protein (500 mg) were applied to a reverse-phase HPLC column (Aquapore RP300, 100 mm $\times$ 2.1 mm, 7 μ m, Brownlee) and proteins were eluted at a flow rate of 0.25 ml/min with a three-step gradient of 0.1 % aqueous trifluoroacetic acid in acetonitrile (containing 0.082 % trifluoroacetic acid by vol.). Peaks (A_{215}) were collected manually. Samples were concentrated, analysed by SDS/PAGE (12.5 % gel) and either revealed with Coomassie Blue or silver staining or Western blotted as described [19,20].

Production of anti-14-3-3 antibodies

Antibodies against the acetylated N-terminal sequences of mammalian brain 14-3-3 isoforms, a T-cell isoform (τ) and an epithelial-cell-specific isoform (σ) were raised in rabbits as described [19,20]. Some cross-reaction has previously been observed between the β isoform and antiserum against the ζ 14-3-3 isoform [19]. This is not unexpected because the N-terminal sequences of these isoforms are almost identical. An additional antiserum was therefore raised against a peptide with the N-terminal sequence Ac-Thr-Met-Asp-Lys-Ser instead of Ac-Met-Asp-Lys-Ser. This sequence derives from the 40 % of the β isoform that uses an alternative initiator methionine codon six nucleotides upstream of the other initiation site. This initiator Met is processed, resulting in species commencing with the N-acetylated threonine as shown. Results were identical, regardless of which antisera were used, except that the latter is totally specific for the β (and α) isoform [20]. These antisera do not cause immunoprecipitation of the native proteins, as this region is involved in dimerization [13,22]; because this additional residue at the N-terminus is masked in the native protein, it is very unlikely that this would affect the localization of the two forms of β 14-3-3. An antiserum specific for the C-terminal region of the ϵ isoform [22] was also used where stated.

Preparation of washed human platelets

Human blood was obtained by forearm venepuncture of healthy volunteers who had denied taking medication for at least 10 days. Blood was drawn into 3.2 % (w/v) trisodium citrate (1 vol.) and platelet-rich plasma was obtained by centrifugation at 200 *g* for 15 min at room temperature as previously described [23]. The platelet-rich plasma was acidified to pH 6.5 with 0.1 M citric acid and subsequently centrifuged at 1500 *g* for 10 min in the presence of prostacyclin (60 nM). The resulting platelet pellet was resuspended in washing buffer consisting of 36 mM citric acid, 103 mM NaCl, 5 mM KCl, 5 mM D-glucose and 60 nM prostacyclin (pH 6.5). Washed platelets were finally resuspended in a Hepes buffer (10 mM Hepes, 145 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 10 mM indomethacin, pH 7.4) and aliquots of platelet suspension were used for HPLC separation of 14-3-3 isoforms or for the immunoblotting studies, or were prepared for fractionation as described below. Platelets to be used in the phosphorylation studies were resuspended at a final count of 6×10^8 /ml in a permeabilization buffer composed of 10 mM Hepes, 0.42 mM NaH₂PO₄, 140 mM KCl, 1 mM MgCl₂, 6 mM NaHCO₃, 5 mM D-glucose and 10 mM indomethacin, pH 7.35. For determination of PKC activity in saponin-permeabilized platelets, washed platelets were resuspended to a count of $(2-3) \times 10^9$ /ml in a modified permeabilization buffer consisting of 15 mM Hepes, 120 mM KCl, 4 mM MgCl₂, 25 mM NaCl, 1 mM NaH₂PO₄, 1 mM EGTA, 10 mM indomethacin and CaCl₂ to give a predicted free Ca²⁺ concentration of 50 nM at pH 7.4 [24].

Preparation of platelet membrane and cytosolic fractions

Aliquots of washed platelet suspension (10^9 cells/ml) in Hepes buffer containing 1 mM EGTA were incubated with vehicle or α -thrombin (1.0 unit/ml) and the reactions were terminated after 5 min by rapid microcentrifugation. The supernatants were removed and the platelet pellets were each resuspended in 200 μ l of sonication buffer (140 mM sodium glutamate, 15 mM Hepes, 2 mM MgSO₄, 1 mM EGTA, 1 mM 4-(2-aminoethyl)-benzenesulphonylfluoride, 0.01 mM 7-amino-1-chloro-3-L-tosylamidoheptan-2-one, 50 mg/ml leupeptin, pH 7.0) and sonicated on ice (5×10 s, 10 μ m) with an MSE Soniprobe. Platelet homogenates were centrifuged at 80 000 *g* (30 min, 4 °C) on a Beckman Optima TLX Ultracentrifuge, and the supernatant (cytosolic) fractions were dispensed into separate tubes. The membrane pellets were resuspended in 200 μ l of buffer and dispersed by brief sonication on ice (3×5 s). Proteins in aliquots of particulate and cytosolic fractions were separated by SDS/PAGE and transferred to a poly(vinylidene difluoride) membrane, which was then probed with anti-14-3-3 antisera as described below.

Saponin concentrations

Permeabilization of washed platelets with saponin was carried out essentially as described previously [23]. Standard aggregometry was used to measure the extent of light transmission which was monitored in 300 μ l aliquots of platelet suspension. Saponin concentrations were chosen that caused maximal permeabilization of the plasma membrane at the appropriate cell count but did not cause less than a 15 % increase in light transmission when monitored over 6–8 min. Thus 12–14 μ g/ml saponin was sufficient to permeabilize platelets at a count of 6×10^8 /ml but greater concentrations (20 μ g/ml) were required to achieve permeabilization at the higher cell count $[(2-3) \times 10^9$ /ml].

Measurement of protein phosphorylation in intact and saponin-permeabilized platelets

Incubations were performed, with stirring, with 70 μ l aliquots of washed platelet suspension and were started by the addition of a further 10 μ l of permeabilization buffer containing saponin and the peptide of interest. Concentrations were calculated to give 12–14 μ g/ml saponin and 10 or 50 μ M peptide in a final incubation volume of 100 μ l. [γ - 32 P]ATP (10 μ l, 25 μ M final concentration) and 10 μ l of agonist (0.2 unit/ml thrombin or 100 nM PMA) were added simultaneously to the cuvette 5 min after the addition of saponin. Reactions were terminated 2.5 min later by the direct addition of 100 μ l of twice-concentrated sample buffer. All samples were heated at 90 °C for 5 min, centrifuged (5 min, 10000 *g*) to remove insoluble material and stored at –20 °C until analysis.

Separation of phosphoproteins by SDS/PAGE was performed as previously reported [23] on 7–20 % gradient gels with 50–100 μ l of sample per lane. Coomassie Blue-stained gels were dried under vacuum and the extent of protein phosphorylation was assessed by autoradiography (overnight at –70 °C, Fuji XR X-ray film).

Assay of PKC activity in saponin-permeabilized platelets

Aliquots (300 μ l) of stirred platelet suspension (3×10^9 /ml) were pre-incubated with saponin (20 μ g/ml) for 30 s followed by a 5 min exposure to either vehicle or the peptide of interest, at a final concentration of 50 μ M. PMA (160 nM) was then added to the cuvette, the suspension stirred for 3 s and the remainder of the 1 min challenge performed without stirring. Saponin at 20 μ g/ml caused a 10–15 % increase in light transmission when monitored over a 6 min incubation period at a platelet count of 3×10^9 /ml (results not shown). Incubations were terminated by rapid centrifugation (1500 *g* for 5 min at 4 °C), the supernatant was discarded and the platelet pellet was resuspended in 100 μ l of a buffer consisting of 20 mM Tris/HCl, 2 mM EDTA, 0.5 mM EGTA, 50 μ g/ml leupeptin, 1 mM PMSF and 1 % (v/v) 2-mercaptoethanol. Particulate and cytosolic platelet fractions were prepared and PKC was partly purified by ion-exchange chromatography as described [25]. Column eluates were assayed for PKC activity by measuring the incorporation of 32 P from [γ - 32 P]ATP into histone H1 in the absence and presence of added phospholipids as previously reported [25].

Western immunoblotting

Total platelet homogenates or crude platelet membrane and cytosolic fractions were transferred from polyacrylamide gels onto poly(vinylidene difluoride) by using an LKB Novablot semi-dry blotting apparatus as described previously [19,20]. Primary and secondary antibodies were used at dilutions of 1:3000 and immunoreactive bands were revealed with the enhanced chemiluminescence detection system (Amersham, U.K.) with X-OMAT-AR film.

RESULTS

Identification of 14-3-3 isoforms

Figure 1 (upper panel) shows the elution profiles obtained after reverse-phase HPLC separation of human platelet 14-3-3 and sheep brain 14-3-3 (inset). The profiles indicate the existence of at least three major isoforms, which correspond to the β , γ and ζ isoforms. Low levels of the ϵ and η isoforms seemed to be present (the former was a shoulder on the peak of γ 14-3-3). Isoforms of 14-3-3 were also identified by Western blotting with antibodies raised against the synthetic peptides described in the

Figure 1 Identification of isoforms of 14-3-3 in human platelets

Upper panel: reverse-phase HPLC of KCIP-1 from human platelets and sheep brain. Purified preparations of KCIP-1 protein prepared from human platelets and sheep brain (inset) were applied to a reverse-phase column (Brownlee, RP300, 100 mm $\times$ 2.1 mm). Proteins were eluted by the application of a three-step gradient of 0.1 % aqueous trifluoroacetic acid at a flow rate of 0.25 ml/min. Peaks (A_{215}) are labelled according to the nomenclature in [19,21]. Lower panel: immunoblots probed with antibodies raised against the N-termini of all known isoforms of mammalian 14-3-3. Proteins were revealed by using enhanced chemiluminescence [19].

Materials and methods section. When proteins from human platelet homogenates were separated by SDS/PAGE, Western blotted, and probed with each of the anti-14-3-3 antisera (Figure 1, lower panel), the presence of high levels of the β , γ and ζ 14-3-3 isoforms and smaller amounts of the ϵ and η isoforms was confirmed. No significant levels of either a T-cell isoform (τ) or an epithelial-cell-specific isoform (σ) were expressed.

A series of experiments was performed to examine the cellular distribution of the 14-3-3 proteins and to determine whether platelet activation leads to changes in the localization of the various isoforms. Particulate and cytosolic fractions were prepared from resting platelets and from platelets stimulated with 1.0 unit/ml α -thrombin for 5 min. Subsequent analysis of the fractions by SDS/PAGE and immunoblotting (Figure 2) showed that under resting conditions the γ , ϵ and ζ 14-3-3 isoforms were present at a higher level within the membrane compartment compared with the cytosol and that an apparent change to a more equal cellular distribution of these proteins occurred in thrombin-stimulated platelets.

In contrast, there were lower levels of the β and η 14-3-3 isoforms in the particulate fraction and little change in distribution of these proteins occurred in thrombin-stimulated platelets.

Figure 2 14-3-3 isoforms in membrane and cytosolic fractions of human platelets

Washed platelets were incubated with either vehicle or thrombin (1.0 unit/ml, 5 min) and particulate (P) and cytosolic (S) fractions were prepared as described in Materials and Methods. Proteins in each of the fractions were analysed by SDS/PAGE (12.5% gel) followed by Western blotting. Immunoreactive proteins were revealed by using enhanced chemiluminescence. Blots were probed with antibodies against the acetylated N-terminal sequences [19,20] of the 14-3-3 isoforms shown and with an antibody raised against the C-terminus of the ϵ 14-3-3 isoform [22]. This latter antibody has a higher titre than the antibody that recognizes the N-terminus of this isoform (results not shown). The enhanced chemiluminescence reaction was therefore stronger than the low levels of ϵ isoform indicated in Figure 1, although the results showing the relative levels of the ϵ isoform in each fraction are valid. Thrombin-treated and control samples are indicated. Equal amounts of protein were loaded on each lane. The figure is representative of two separate experiments with similar results. The fifth lane (labelled b) is a control containing mammalian brain protein extract.

Effects of a peptide derived from the pseudosubstrate-like region on protein phosphorylation

Stimulation of platelets with phorbol esters or with receptor-operating agonists that lead to PKC activation results in the characteristic phosphorylation of several protein substrates, most notably pleckstrin (approx. 45 kDa) and myosin light chain (20 kDa) [26]. To examine whether 14-3-3 modulates platelet PKC activity *in situ* and to determine the potential involvement of the PKC pseudosubstrate-like domain in this modulation, a peptide (AS) corresponding to residues 50–69 of the 14-3-3 family (KNVVGARRSSWRVISSIEQK) was introduced into permeabilized platelets and its effects on protein phosphorylation were monitored. Figure 3 (upper panel) shows the effects of the AS peptide and a control peptide from another region of 14-3-3 sequence (ELVQKAKLAQEARY) on PMA-induced protein phosphorylation in saponin-permeabilized cells. PMA enhanced the incorporation of 32 P into several proteins, the major bands being those with approximate molecular masses of 20, 36, 45 and 66 kDa (lane 3). Preincubation with the control peptide (lane 4) had little effect on PMA-induced protein phosphorylation. However, addition of the AS peptide with saponin reduced the phosphorylation seen in the presence of PMA; 10 μ M peptide marginally reduced phosphorylation of the 66 kDa protein (lane 5) whereas total inhibition of PMA-induced phosphorylation was observed at a final peptide concentration of 50 μ M (lane 6).

Figure 3 (lower panel) shows the results from a similar experiment in which the effects of the peptides on thrombin-induced phosphorylation were examined. Exposure to thrombin (0.2 unit/ml) markedly enhanced 32 P incorporation into several proteins (lane 6). Thrombin-induced phosphorylation was unaffected by the control peptide (lane 7) but was dose-dependently inhibited in the presence of the AS peptide (lanes 8 and 9).

A dense autoradiographic band coinciding with the gel front

Figure 3 Effects of the pseudosubstrate-like peptide of 14-3-3 on PMA- and thrombin-induced phosphorylation in permeabilized platelets

Aliquots of human platelet suspension were preincubated with saponin (12 μ g/ml) and vehicle, a control peptide or the AS peptide. Platelets were subsequently challenged with either PMA (100 nM, upper panel) or thrombin (0.2 unit/ml, lower panel) in the continued presence of saponin. Reactions were terminated and the phosphoproteins were analysed by SDS/PAGE and autoradiography as described in the Materials and methods section. The arrows indicate approximate molecular masses, and the location of phosphorylated AS peptide (PAS). Results are from two separate experiments and are representative of three with PMA and two with thrombin. Upper panel: lane 1, no additions; lane 2, 50 μ M AS peptide; lane 3, PMA (100 nM); lanes 4 and 7, PMA (100 nM) plus control peptide (50 μ M); lane 5, PMA (100 nM) plus 10 μ M AS peptide; lane 6, PMA (100 nM) plus 50 μ M AS peptide. Lower panel: lane 1, no additions; lane 2, 50 μ M AS peptide, no saponin; lane 3, saponin (12 μ g/ml) only. Lanes 4–9 contained the following additions plus saponin (12 μ g/ml): lane 4, AS peptide (50 μ M), lane 5, control peptide (50 μ M); lane 6, thrombin, (0.2 unit/ml); lane 7, thrombin plus control peptide (50 μ M); lane 8, thrombin plus AS peptide (10 μ M); lane 9, thrombin plus AS peptide (50 μ M).

was observed in those lanes in which permeabilized cells were incubated with the AS peptide. Microsequencing studies have subsequently shown that this represents the AS peptide in its phosphorylated state (results not shown). Phosphorylation of the AS peptide was not observed in intact platelets incubated with [γ - 32 P]ATP [Figure 3 (lower panel), lane 2], indicating that permeabilization is a prerequisite for this phosphorylation event.

Effects of peptides on PMA-induced PKC translocation in saponin-permeabilized platelets

To investigate the potential role of 14-3-3 in controlling the cellular distribution of PKC, PMA-induced PKC translocation

Table 1 Effects of a peptide derived from the annexin-like region of 14-3-3 on PMA-induced PKC translocation in saponin-permeabilized platelets

Indomethacin-treated platelet suspensions were incubated (37 °C) with saponin (20 µg/ml) for 30 s followed by the addition of either control peptide (Con) or MK peptide (annexin-like) at final concentrations of 50 µM in the continued presence of saponin. Platelets were challenged 5 min later with either vehicle or PMA (160 nM) for 1 min. Reactions were terminated and the PKC activity associated with cytosolic (C) and particulate (P) fractions of each sample was determined as described in the Materials and methods section. Results from three individual experiments are shown. The columns headed Total represent the total activity recovered in stimulated platelets expressed as a percentage of that recovered in platelets treated with saponin alone. The values given in parentheses are the PKC activities obtained in each fraction expressed as a percentage of the total (particulate plus cytosol) activity in each incubation.

Incubation conditions	PKC activity (fmol of ³² P incorporated/min per mg of protein)								
	Experiment 1*			Experiment 2			Experiment 3		
	P	C	Total (%)	P	C	Total (%)	P	S	Total (%)
Saponin	16.7 (31)	37.0 (69)	100	22.1 (34)	42.8 (66)	100	12.8	32.9	100
Saponin + PMA	24.8 (72)	9.6 (28)	64	38.7 (67)	19.1 (33)	89	32.7	14.0	102
Con + saponin + PMA	36.3 (69)	16.3 (31)	98	34.1 (59)	23.7 (41)	89	35.4	11.8	103
MK + saponin + PMA	39.2 (76)	12.4 (24)	96	40.7 (64)	22.9 (36)	98	29.3	17.9	103

* Permeabilization buffer did not contain EGTA.

was measured in saponin-permeabilized platelets in the absence and presence of a 17-residue synthetic peptide (MKGDYYRYLAEVATGDD) based on the conserved annexin-like domain of 14-3-3 [27]. The results from three individual experiments are presented in Table 1. As previously reported [25], approx. 70 % of the PKC activity was associated with the cytosolic compartment under resting conditions, i.e. in the presence of saponin alone. Exposure of unstirred saponin-permeabilized platelets to PMA caused a marked reduction in cytosolic PKC activity that was accompanied by a parallel increase in the enzyme activity associated with the membrane fraction. In all three experiments the PMA-induced redistribution of enzyme activity was unaffected by pre-exposure to either a control peptide or the annexin-like peptide. The AS peptide was also ineffective (results not shown), although this was examined in platelets from only one donor. In addition, in experiments 2 and 3, the total PKC activity recovered (membrane plus cytosol) was similar under all conditions examined. In contrast, in experiment 1, in which an EGTA-free permeabilization buffer was employed, PMA caused an apparent reduction in total activity, which seemed to reverse in the presence of peptide.

DISCUSSION

Endogenous inhibitors of PKC isolated from sheep brain and termed KCIP-1 were subsequently shown to be members of the 14-3-3 family [21]. These proteins potently inhibit PKC *in vitro* by a mechanism whose effects were overcome by the addition of diacylglycerol or phorbol ester. In our dispersion assay the IC₅₀ is similar for a protein or peptide substrate and for different isoforms this varies between 0.32 and 1.1 µM [12]. Some isoforms of 14-3-3 have been shown to bind phospholipid [28], and approx. 40 % of certain brain isoforms bind the plasma membrane of synaptic vesicles [29] via a domain in the N-terminal 12 kDa region [22]. 14-3-3 forms homodimers and heterodimers *in vivo* [30] and could therefore interact with PKC and other signalling proteins *in vivo* to function as a linker in a complex or to mediate interaction with membrane proteins.

In the present study we have shown that human platelets contain five of the 14-3-3 proteins known to be expressed in mammalian tissues [19]. Reverse-phase HPLC and immunoblotting studies with isoform-specific antisera raised against the acetylated N-terminal peptides of mammalian 14-3-3 showed

that platelets express high levels of the β, γ and ζ 14-3-3 isoforms and to a smaller extent the ε and η 14-3-3 isoforms. Very low levels of the α and δ isoforms (the phosphorylated forms) were present, compared with brain tissue. A considerable proportion of each form was distributed between cytosolic and membrane compartments and there was some evidence for a change in cellular distribution of at least some isoforms (γ, ε and ζ) after exposure to thrombin. The presence of these proteins in short-lived cells that are essentially devoid of protein synthesis raises the possibility that platelet 14-3-3 may have a physiological role in rapid signalling events. We also have evidence that individual 14-3-3 isoforms regulate distinct PKC isoenzymes [31]. Because the phosphorylated form *in vivo* of ζ, the δ isoform [5], interacts more strongly with PKC than does ζ 14-3-3 [31], this phosphorylation could also be a mechanism for regulating the interaction with Raf and other proteins. We are currently examining the relative amounts of the phospho- and dephosphoforms of these 14-3-3 isoforms in platelets and whether this is altered in response to physiological stimuli.

Platelets represent a convenient cell type in which to examine PKC activation because they exhibit a well-defined, characteristic series of phosphorylation events in response to phorbol ester or receptor-mediated PKC activation [26]. Most notable is the multisite phosphorylation of pleckstrin (approx. 45 kDa), a cytosolic protein whose phosphorylation is believed to be involved in the control of platelet aggregation and secretion [26]. Inhibition of PKC and PKC-mediated events by pseudosubstrate peptides has been demonstrated in several permeabilized cell preparations, including platelets [32–34]. In the latter case, introduction of the pseudosubstrate peptide into saponin-permeabilized cells completely inhibited ³²P incorporation into the 45 kDa substrate in response to thrombin and additionally blocked thrombin-induced secretion [32]. Few studies so far have addressed the potential functional role of the 14-3-3 proteins in controlling PKC activity *in situ*, despite growing evidence from studies *in vitro* for their involvement in modulating the activity of this enzyme [12,21,31]. Comparison of the sequences of members of the 14-3-3 family has shown that they share several conserved domains. In the present study we have exploited the fact that members of the 14-3-3 family contain domains that resemble the pseudosubstrate domain of PKC [27]. Pseudosubstrate or auto-inhibitory sequences have been proposed to function as endogenous regulators of several protein kinases, and peptides spanning this region of PKC are potent inhibitors of the enzyme

[35]. A second region within the 14-3-3 proteins resembles the conserved C-terminus of the annexin family [27]. It has recently been shown that members of the annexin family can act as cytoskeletal receptors for activated PKC and that a synthetic peptide based on the annexin C-terminus, and therefore on the annexin-like region of 14-3-3, inhibits the binding of PKC to the cytoskeletal receptors [36]. Those authors therefore proposed that binding to the receptors might be a physiologically important component of activation-induced PKC translocation. The existence of an 'annexin-like' structural domain is confirmed by crystallography studies and is at a location that has a high probability of being involved in 14-3-3 interactions with signalling proteins [13].

Peptides derived from either the annexin-like or the pseudosubstrate-like regions of 14-3-3 did not significantly modulate PMA-induced redistribution of PKC activity in saponin-permeabilized platelets. It may be worthy of note that the presence of PMA or diacylglycerol can overcome the inhibition of PKC by KCIP-1 to a maximum of 60% [12]. These results do not provide support for the proposal by Mochly-Rosen et al. [36] that the annexin-like 14-3-3 domain might be important in the control of PKC translocation. This does not rule out the possibility that peptides derived from this domain or from the pseudosubstrate-like region affect translocation of a phorbol ester-insensitive isoform of PKC, or that they might modify the transient PKC translocation observed after agonist stimulation [25]. Recent studies, however, indicate that all the PKC isoforms expressed in human platelets are capable of translocating to the particulate fraction after challenge with PMA or physiological agonists [16]. In marked contrast, and under similar experimental conditions, the pseudosubstrate-like peptide completely attenuated phosphorylation of the 45 kDa protein induced either by direct activation of PKC by PMA, or by receptor-mediated stimulation. The fact that total inhibition of phosphorylation occurred with maximal concentrations of peptide supports the observation that pleckstrin is a substrate for α , β and δ PKC isoenzymes and that its phosphorylation is not due to selective activation of a particular PKC isoform [17]. Taken together, our findings suggest that the pseudosubstrate-like peptide does not interfere with the relocation of PKC to the membrane compartment but can modulate the subsequent activity of the enzyme once translocated. This may be due to binding of the peptide either to PKC itself or to substrates/binding protein(s) at or near the site of membrane attachment; in the latter case binding would result in decreased phosphorylation. In this regard, Liao and Jaken [37] have recently identified several cytoskeletal proteins that may represent membrane association sites for PKC and have speculated that interaction of PKC with these proteins is mediated in part through the pseudosubstrate domain. These results, together with those from the present study, therefore suggest that 14-3-3 could bind to the same proteins as PKC resulting in an indirect inhibition of the enzyme by blockade of substrate interaction rather than through direct association with PKC.

Recent work on permeabilized chromaffin cells has indicated that the annexin-like domain of the 14-3-3 proteins might be important for the regulation of calcium-dependent secretion [9,38]. It would seem to play a stimulatory role because a 16-residue synthetic peptide based on the C-terminus of annexin II partly inhibited secretion; this effect was not due to a direct interaction with PKC nor to an effect on PKC-regulated events [38]. In our hands a similar peptide did not modify the distribution of PKC between membrane and cytosol, indicating that the annexin-like region is probably not essential for translocation. It may be significant, however, that the inhibitory effect of purified

14-3-3 on PKC can be alleviated in the presence of this peptide (A. Aitken and Y. Soneji, unpublished work), suggesting that the inhibitory mechanism, at least *in vitro*, may involve binding through this site.

Microsequencing of the dense band migrating just above the gel front showed that this represents the peptide phosphorylated on Ser-64 (C. P. D. Wheeler-Jones, M. P. Learmonth, H. Martin and A. Aitken, unpublished work). These findings indicate that the pseudosubstrate-like peptide can be phosphorylated *in situ* and support previous studies *in vitro* showing PKC-mediated phosphorylation of this residue in specific isoforms of 14-3-3 at low stoichiometry [39]. We have recently shown that mutant forms of 14-3-3 can be phosphorylated at high stoichiometry *in vitro* [22]. This may occur *in vivo* under conditions where the conformation of the normally dimeric 14-3-3 is altered in a complex.

In summary, these studies in permeabilized platelets using synthetic peptides suggest that the isoforms of the 14-3-3 family that we have detected in platelets may be endogenous regulators of rapid signalling events in these cells through their inhibitory effect on the enzymic activity of PKC.

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