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RAPID DEPHOSPHORYLATION OF A Mr 80000 PROTEIN, A SPECIFIC SUBSTRATE OF PROTEIN KINASE C UPON REMOVAL OF PHORBOL ESTERS, BOMBESIN AND VASOPRESSIN

Angeles Rodriguez-Pena, Ian Zachary, and Enrique Rozengurt

Imperial Cancer Research Fund, P O Box 123
Lincoln's Inn Fields, London WC2A 3PX, UK

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SUMMARY: The mitogens phorbol 12,13-dibutyrate, bombesin and vasopressin stimulate the phosphorylation of an acidic Mr 80000 cellular protein, a specific substrate of protein kinase C, in intact Swiss 3T3 cells. Phosphorylation of this substrate was rapidly reversed upon the removal of each of these agents. Dephosphorylation occurred with a similar half-life in each of the cases studied (2.2, 1.5 and 2 minutes for phorbol 12,13-dibutyrate, bombesin and vasopressin respectively) and agreed closely with the dissociation of the ligands from their specific high-affinity binding sites in Swiss 3T3 cells.

Protein kinase C which serves as a major receptor for tumour promoting phorbol esters is implicated in signalling a variety of both short and long-term cellular responses including cell growth (1-4). The enzyme is activated *in vivo* by membrane diacylglycerol transiently generated by the receptor-mediated breakdown of inositol phospholipids (1,3); diacylglycerol returns to basal levels within seconds or minutes after stimulation. However, transient activation of protein kinase C is not sufficient for the generation of a proliferative response (5). Recently, it has been proposed that the persistance of the effects of protein kinase C activation might be due to the stability of the phosphate group attached to the substrate protein (3). Since very little is known about the rate of dephosphorylation of substrates of protein kinase C in cells stimulated to proliferate, this hypothesis remains untested.

Abbreviations: DME, Dulbecco's modified Eagle's medium; PBt_2, Phorbol 12,13-dibutyrate; SP, substance P; Pmp^1-O-Me-Tyr^2-[Arg^8]vasopressin, [1-(β-Mercapto-β, β-cyclopentamethylene propionic acid), 2-(O-methyl) tyrosine]Arg^8-vasopressin; 80K, an acidic cellular protein of Mr 80000.

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Recently, an acidic cellular protein of Mr 80000 (termed 80K) has been identified as a major and specific substrate for protein kinase C in cultured cells (2,6-17). The phosphorylation of 80K is markedly and rapidly increased in response to phorbol 12,13-dibutyrate (PBt_2) (2,6) and to the mitogenic peptides bombesin (9,11) and vasopressin (13). The findings presented here demonstrate that this protein kinase C substrate is rapidly dephosphorylated in intact Swiss 3T3 cells in response to the removal of PBt_2, bombesin or vasopressin.

MATERIALS AND METHODS

Cell culture procedures (18,19), labelling of cells with $^{32}P_i$ (6) and two-dimensional polyacrylamide gel electrophoresis using isoelectric focusing in the first dimension and SDS-PAGE (8% polyacrylamide) in the second dimension were as previously described (10,11,20). The 80K and the Mr 60000 reference spot from the autoradiograms were scanned using a Joyce-Loebl double beam densitometer. The area of the peaks were measured with a Hewlett-Packard digitizer and the incorporation of $^{32}P_i$ into the 80K protein was determined as the ratio between the two peak areas.

Materials: PBt_2, Arg^8 vasopressin and bombesin were from Sigma Chemical Co., St Louis, Mo.; [1-(β-Mercapto-β, β-cyclopentamethylene propionic acid), 2-(O-Methyl) tyrosine] Arg^8-vasopressin (Pmp^1-O-Me-Tyr^2-[Arg^8] vasopressin), was from Peninsula Laboratories, Inc. (Belmont, CA); ($D\text{-}Arg^1$, $D\text{-}Pro^2$, $D\text{-}Trp^{7,9}$, Leu^{11}) substance P was obtained from Bachem Fine Chemicals (Saffron Walden, Essex, UK). $^{32}P_i$ and ^{125}I-gastrin releasing peptide were from the Radiochemical Centre (Amersham, UK) and [^3H]-PBt_2 and [^3H]-Arg^8 vasopressin were both supplied by New England Nuclear.

RESULTS AND DISCUSSION

The addition of PBt_2 (40 nM) to quiescent cultures of Swiss 3T3 cells prelabelled with $^{32}P_i$ markedly increased the phosphorylation of 80K (Fig. 1A, PBt_2 added). When the PBt_2-treated cultures were washed at 37°C with DME medium containing bovine serum albumin (5) and transferred for 10 min to the same medium with $^{32}P_i$ but lacking phorbol ester, the phosphorylation of 80K was reduced to control levels (Fig. 1A, PBt_2 removed). The decrease in the level of the 80K phosphoprotein was not due to the disappearance of either protein kinase C activity or of this protein kinase C substrate from the cells, since re-addition of PBt_2 to washed cultures caused the re-stimulation of 80K phosphorylation (Fig. 1A, PBt_2 re-added). The

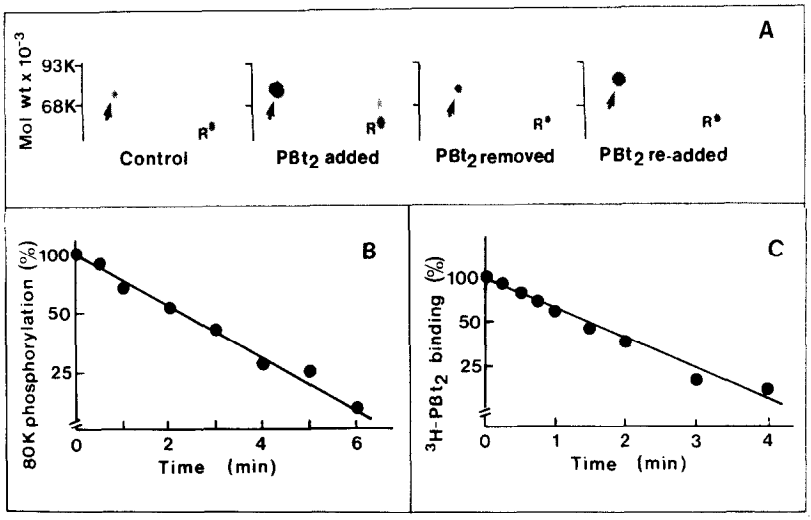

Figure 1. Decay of 80K phosphorylation in response to the removal of PBt_2. Panel A: Confluent and quiescent cultures of Swiss 3T3 cells were washed and labelled with $^{32}P_i$ (200 μCi/ml) for 5 hours at 37°C. Cells were then challenged with 40 nM PBt_2 (PBt_2 added) or an equivalent volume of solvent (Control) for 2 minutes. PBt_2-treated cultures were washed rapidly three times at 37°C with DME medium supplemented with 1 mg/ml bovine serum albumin (wash medium) and subsequently incubated for 10 minutes in 1 ml of wash medium containing 200 μCi/ml $^{32}P_i$ (PBt_2 removed). Other washed cultures were challenged for a further 1 minute with 40 nM PBt_2 (PBt_2 readded). All incubations were terminated as described in Materials and Methods. The arrow indicates the position of 80K. In this experiment, quantification of the stimulation of 80K phosphporylation showed relative increases of 2,95, 3,05 and 1, respectively for PBt_2 added, PBt_2 re-added, and PBt_2 removed. Panel B: Time-course of 80K dephosphorylation. Cultures of Swiss 3T3 cells were washed and labelled with $^{32}P_i$. They were then treated with 40 nM PBt_2 or an equivalent volume of solvent for 2 minutes. Cells were then rinsed three times with wash medium and incubated for various times in the same medium containing $^{32}P_i$. 80K phosphorylation was expressed as a percentage of the maximal value. The results shown represent the mean of two independent experiments. Panel C: Dissociation of [^{3}H]-PBt_2 from Swiss 3T3 cells. Cultures of confluent, quiescent Swiss 3T3 cells were washed twice with DME medium at 37°C and then incubated at 37°C with 1 ml of binding medium (0.14 M NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.0) supplemented with 1 mg/ml bovine serum albumin and [^{3}H]-PBt_2 (specific activity 10.8 Ci/mmol; 40 nM). After 2 minutes of incubation cultures were washed rapidly three times with the binding medium at 37°C and incubated for various times at 37°C in 1 ml of the same medium and then washed a further four times in phosphate-buffered saline (supplemented with 1 mg/ml bovine serum albumin) at 4°C. Cultures were extracted with 0.5 ml of 0.1 M NaOH containing 2% Na_2CO_3 and 1% sodium dodecyl sulphate, and total cell-associated radioactivity measured by scintillation counting in Pico-Flour. Non-specific binding defined as that observed in the presence of 2,000 fold excess unlabelled PBt_2 (80 μM) was approximately 14% of total binding. The results were expressed as the percentage of the control value (0.9 pmol/10^6 cells). All results are the mean of triplicate determinations.

level of 80K phosphorylation increased and decreased rapidly in response to three consecutive cycles of the addition and removal of PBt_2.

To examine the kinetics of 80K dephosphorylation, PBt_2-treated cultures were washed as described above for various times. At each time point the extracts were subjected to two dimensional gel electrophoresis and $^{32}P_i$ incorporation into the 80K phosphoprotein was measured relative to another reference spot of Mr 60000 (labelled R throughout) which remained unchanged. Fig. 1B shows that the decrease in the 80K phosphoprotein followed first order kinetics with a half-life of 2.2 min. This agrees with the dissociation of $[^3H]PBt_2$ from Swiss 3T3 which exhibited a half-life of 1.5 min (Fig. 1C). These findings suggested that the dephosphorylation of 80K was closely related to the deactivation of protein kinase C. This conclusion was further substantiated by experiments utilizing mitogenic hormones which activate protein kinase C in intact cells.

Bombesin and vasopressin bind to distinct receptors (21-23) and promote a rapid (within seconds) and marked increase in 80K phosphorylation (9,11,13). The availability of antagonists for these ligands which inhibit both binding and activation of protein kinase C (11,13,22,23) provided useful means for examining the dephosphorylation of the 80K phospoprotein when bombesin and vasopressin dissociate from their receptors. Fig. 2 shows that the stimulation of 80K phosphorylation by either bombesin (top-left) or vasopressin (top-right) was almost completely abolished when the cultures were washed and transferred to fresh medium lacking the hormones but containing the appropriate antagonist. The addition of PBt_2 for 1 min to the antagonist-treated cells stimulated 80K phosphorylation as effectively as in control cultures that did not receive the antagonists, demonstrating that the antagonists do not interfere with 80K phosphorylation in a non-specific manner. The time-course of 80K dephosphorylation upon removal of either bombesin or vasopressin (Fig. 2) was similar to that observed in response to the removal of PBt_2 (Fig. 1) with half-lives of 1.5 min and 2 min

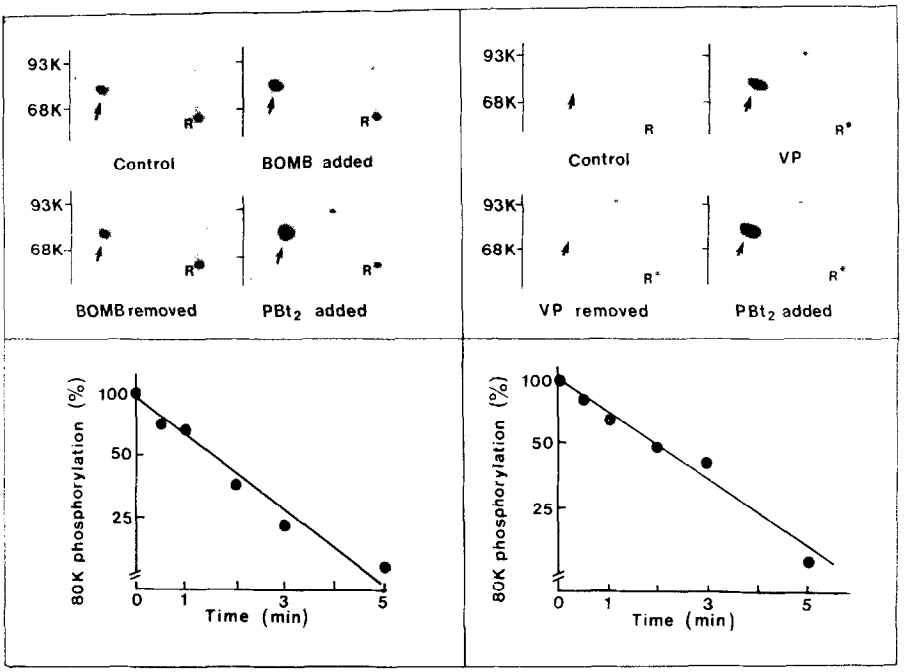

Figure 2. Decay of the 80K phosphoprotein produced in response to vasopressin and bombesin after removal of these ligands. Upper panel: Confluent and quiescent cultures of Swiss 3T3 cells were washed and labelled with $^{32}P_i$ (200 μCi/ml) for 5 h at 37°C. Cells were then challenged with either bombesin (top left) at 3nM for 1 min or vasopressin (top right) at 20 nM for 5 min. Other cultures were treated with an equivalent volume of solvent (top right, top left, control). Hormone-treated cultures were then washed rapidly three times at 37°C with wash medium and incubated a further 5 (bombesin) or 10 (vasopressin) min in 1 ml of DME medium containing $^{32}P_i$ and either 100 μM [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}] substance P or 1 μM Pmp^1-O-Me-Tyr^2-[Arg^8] vasopressin in the case of bombesin and vasopressin respectively (top-right, bombesin, vasopressin removed). Antagonist-treated cultures were subsequently challenged with 40 nM PBt_2 for 1 min (PBt_2 added, top left and right). 80K phosphorylation was increased 4.1 and 3.7 fold by addition of bombesin and vasopressin respectively. The arrow indicates the position of 80K. Lower panel: Time-course of 80K dephosphorylation after removal of bombesin and vasopressin. Cultures of Swiss 3T3 were washed and labelled with $^{32}P_i$. They were then exposed to bombesin or vasopressin before being washed and incubated for various times in the same medium containing $^{32}P_i$ and the correspondent antagonist. 80K phosphorylation was determined as described in Material and Methods and is expressed as a percentage of the value obtained for hormone-stimulated cultures above the value of the control cultures.

respectively. Like PBt_2, the decrease in bombesin and vasopressin-stimulated 80K phosphorylation caused by the antagonists occurred concomitantly with the dissociation of the ligands from their high-affinity sites in Swiss 3T3 cells. When cultures were preincubated for 1 minute at 37°C with the iodinated bombesin analogue ^{125}I-gastrin releasing peptide and transferred to medium without the

radiolabelled ligand but containing 100μM ($D\text{-}Arg^{1}$,$D\text{-}Pro^{2}$,$D\text{-}Trp^{7,9}$, Leu^{11}] substance P, cell-associated radioactivity decreased rapidly reaching 20% of the control value within 90 s. Similarly, only 19% of ^{3}H-vasopressin remained bound to cells 2 minutes after removal of the radioactive ligand and exposure to the antagonist ($Pmp^{1}\text{-}O\text{-}Met\text{-}Tyr^{2}\text{-}Arg^{8}$) vasopressin at a concentration of 1 μM.

The rapid reversibility of 80K phosphorylation shown here suggests that the level of the phospho-form of this substrate depends on the activities of both protein kinase C and a phosphoprotein phosphatase(s) in intact cells. Since the function of many key regulatory proteins is acutely modulated by rapid alterations in their state of phosphorylation, catalysed by cellular protein kinases and phosphatases (24), it is likely that 80K may play a role in the mechanism of action of protein kinase C.

Rapid reversal of protein kinase C mediated phosphorylation upon deactivation of the enzyme may not be confined to 80K. Diacylglycerol rapidly decreases the affinity of epidermal growth factor (EGF) for its receptor in 3T3 cells, an effect mediated by protein kinase C phosphorylation (9,25). The observation that this effect was rapidly reversed by removal of diacylglycerol (25) suggested that a similarly rapid reversal of EGF receptor phosphorylation might occur. Although the rates of phosphorylation and dephosphorylation of other protein kinase C substrates should be examined in mitogen-stimulated quiescent cells before a final conclusion can be drawn, the present findings are not consistent with the hypothesis that the long-term biological effects of protein kinase C activation, and in particular cellular proliferation, are due to the stability of the phosphate group attached to the substrate protein. Indeed, these results suggest the existence of an active phosphoprotein phosphatase(s) that reverses protein kinase C mediated phosphorylation in intact cells.

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