

# Arachidonic acid induces phosphorylation of an 18 kDa protein in electrically permeabilised rat islets of Langerhans

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Arachidonic acid (AA) was shown to induce concentration-dependent, calcium-independent, *in situ* phosphorylation of a protein of approximate molecular weight 18 kDa in electrically permeabilised rat islets of Langerhans. This protein did not appear to be a substrate for protein kinase C (PKC) since stimulation of PKC by  $4\beta$  phorbol myristate acetate ( $4\beta$  PMA) did not result in  $^{32}\text{P}$  incorporation into an 18 kDa protein, and since AA-induced phosphorylation was observed in islets in which PKC had been down-regulated by prolonged exposure of islets to  $4\beta$  PMA. These results suggest that AA stimulates protein phosphorylation by a mechanism other than PKC activation.

Islets of Langerhans; Protein phosphorylation; Arachidonic acid; Protein kinase C

## 1. INTRODUCTION

Protein phosphorylation is thought to play an important role in insulin secretion, and intracellular messengers such as  $\text{Ca}^{2+}$ , cyclic AMP and diacylglycerol, which may be involved in the regulation of insulin secretion, can activate specific protein kinases which have been identified in islets of Langerhans and insulin-secreting cell lines [1]. Evidence is accumulating to suggest that arachidonic acid (AA) is involved in the control of B cell function [2]. Glucose and other insulin secretagogues cause rapid elevations in the concentrations of AA in islets [3], and exogenous AA can stimulate insulin secretion from intact [4–6] and from permeabilised [2] islets. AA has been reported to affect kinase activity or protein phosphorylation in several tissues [7,8] and AA is known to activate  $\alpha$ ,  $\beta$  and  $\gamma$  subtypes of protein kinase C (PKC) *in vitro* [9,10]. Pancreatic B cells are reported to contain the  $\alpha$  and  $\beta$  isoforms of PKC [11,12], and PKC extracted from rat islets can be activated by exogenous AA [5,13]. However, studies using PKC-depleted islets suggest that the effects of AA in insulin secretion cannot be entirely accounted for by PKC activation [2,5]. We have therefore measured the effect of AA on the phosphorylation of endogenous substrates in electrically permeabilised rat islets, and determined whether the effects of AA on islet protein phosphorylation are mediated solely by activation of PKC.

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## 2. MATERIALS AND METHODS

### 2.1. Materials

Collagenase (type XI), bovine serum albumin (BSA, fraction V),  $4\alpha$  and  $4\beta$  phorbol myristate acetate (PMA) and adenosine 5'-triphosphate (ATP) were obtained from Sigma (Dorset, UK). Tissue culture medium was from Gibco (Middx., UK).  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (3000 Ci/mmol) was from Amersham International (Bucks., UK). Acrylamide and methylenebisacrylamide were from Uniscience (Oxon., UK). All other reagents were of analytical grade from BDH (Dorset, UK).

### 2.2. Islet isolation and permeabilisation

Islets of Langerhans were isolated from rat pancreata by collagenase digestion [14] and incubated for 30 min at  $37^\circ\text{C}$  in a bicarbonate-buffered physiological salt solution [15] supplemented with 2 mM  $\text{CaCl}_2$ , 2 mM glucose and 0.5 mg/ml BSA. In order to down-regulate PKC, islets were cultured for 20–24 h in the presence of the PKC activator  $4\beta$  PMA (200 nM), as previously described [16]. Control islets were cultured with the inactive phorbol ester,  $4\alpha$  PMA (200 nM). Freshly isolated islets or cultured islets were washed 5 times ( $4^\circ\text{C}$ ) in a buffer ('permeation buffer') containing 1 mM EGTA, 2 mM  $\text{MgSO}_4$ , 15 mM HEPES and 140 mM potassium glutamate, pH 6.6, with  $\text{CaCl}_2$  added to produce 50 nM  $\text{Ca}^{2+}$ , and permeabilised by 5 exposures (200  $\mu\text{s}$ ) to an electric field of 3.4 kV/cm [17]. For secretion studies, groups of 5 permeabilised islets were pre-incubated for 5 min at  $4^\circ\text{C}$  in permeation buffer supplemented with 5 mM ATP, 7 mM  $\text{MgSO}_4$ , 5.6 mM glucose, 0.5 mg/ml BSA and other test substances to allow for equilibration to occur and then incubated for 30 min at  $37^\circ\text{C}$ . Islets were pelleted by centrifugation ( $9000 \times g$ , 15 s) and insulin secretion was measured by radioimmunoassay [18].

### 2.3. Protein phosphorylation in electrically permeabilised islets

Electrically permeabilised islets were incubated in 80  $\mu\text{l}$  of permeation buffer containing 10  $\mu\text{Ci}$  of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (spec. radioactivity 12.5 mCi/mmol) and the test substances of interest for 15 min at  $4^\circ\text{C}$  to permit equilibration of the radiolabelled ATP across the permeabilised plasma membrane of the islet cells. Phosphorylation was initiated by incubating the islets for 1 min at  $37^\circ\text{C}$  and the reaction was halted by separation of islets from medium by centrifugation ( $9000 \times g$ , 15 s). The supernatant was removed, 20  $\mu\text{l}$  of a solution containing 2% (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.1% (w/v) Bromophenol blue in 6.06 M Tris/HCl buffer, pH 6.8 ('sample buffer')

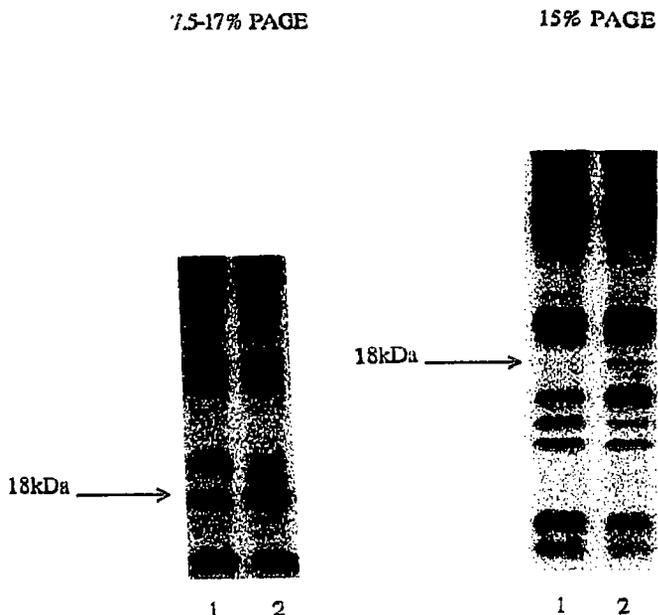


Fig. 1. Autoradiograph of <sup>32</sup>P incorporation into proteins in electrically permeabilised islets separated by 7.5-17% gradient or 15% slab PAGE. The islets were incubated for 1 min at 37°C in 50 nM Ca<sup>2+</sup> (lane 1) or 50 nM Ca<sup>2+</sup> supplemented with 100 μM AA (lane 2). Arrow shows position of AA-induced <sup>32</sup>P incorporation into islet protein of approximate molecular weight 18 kDa calculated from a calibration curve by using migration positions of proteins of known molecular mass.

were added to the islet pellets, and samples were incubated for 5 min in a boiling water bath. SDS-polyacrylamide gel electrophoresis [19] was performed using either 7.5-17% gradient or 15% slab gels and 5% stacking gels. Ten calibration proteins of known subunit mass over the range of 14.1-205 kDa were separated on each gel. Electrophoresis

was carried out at 25 mA/gel unit the dye front reached the separating gel, and at 35 mA/gel thereafter. Proteins were detected by Coomassie blue staining after which gels were dried by heating under vacuum and <sup>32</sup>P incorporation was detected by autoradiography using Kodak X-Omatic regular intensifying screen and X-Omat S film. In some experiments <sup>32</sup>P incorporation was quantified by measuring the intensity of autoradiogram bands with a LKB Ultrascan laser densitometer and 2190 Gelscan interface and software.

2.4. Protein kinase C assay

PKC was partially purified from islet homogenates using DEAE-cellulose DE52 columns and PKC activity in eluate samples was assayed by measuring incorporation of <sup>32</sup>P from [ $\gamma$ -<sup>32</sup>P]ATP into histone type III, as previously described [20].

3. RESULTS

Preliminary experiments indicated that AA consistently stimulated <sup>32</sup>P incorporation from [ $\gamma$ -<sup>32</sup>P]ATP into an islet protein of molecular weight approximately 18 kDa. Fig. 1 shows AA-induced phosphorylation of this protein detected after polyacrylamide gel electrophoresis (PAGE) separation of radiolabeled islet proteins on either gradient (7.5-17%) gels or on slab (15%) gels. In 14 such experiments the molecular weight of the substrate was found to be 18.3 ± 0.2 kDa (mean ± SEM). The AA-induced phosphorylation was rapid, being detectable after incubation for 1 min at 37°C. In some of these experiments (5/14) AA also stimulated phosphorylation of a protein of approximate molecular weight 21 kDa (see Fig. 1 gradient gel). Varying the concentration of AA from 12.5-100 μM resulted in a concentration-dependent increase in <sup>32</sup>P incorporation into the 18 kDa protein, with effects of AA being observed initially at 50 μM and maximally with 100 μM AA.

AA stimulated <sup>32</sup>P incorporation into the 18 kDa

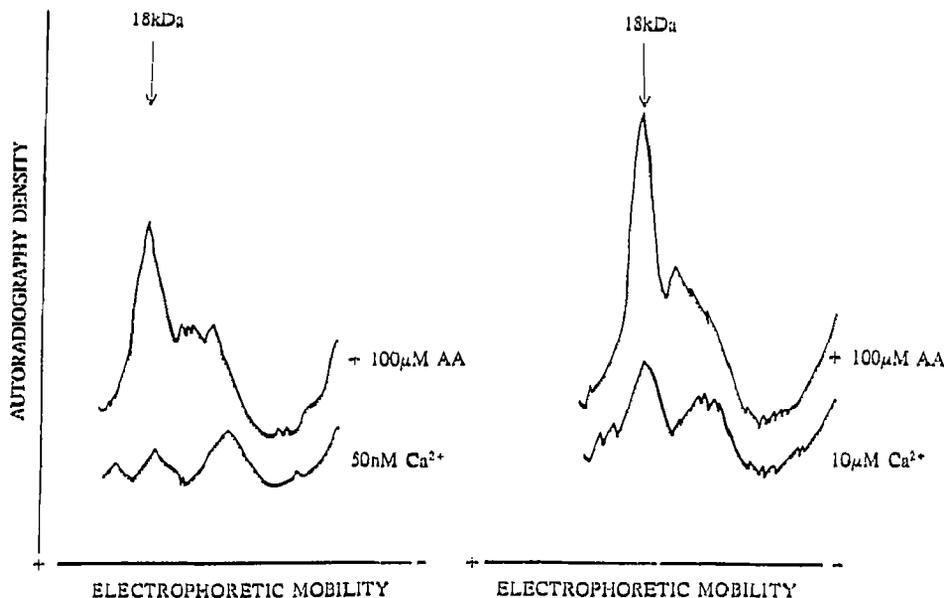


Fig. 2. Scanning-densitometer traces from autoradiographs of radiolabelled proteins from electrically permeabilised islets after a 1 min incubation at 37°C in the presence of 50 nM Ca<sup>2+</sup> supplemented with 100 μM AA (left panel) or 10 μM Ca<sup>2+</sup> supplemented with 100 μM AA (right panel). Arrow shows position of <sup>32</sup>P incorporation into islet protein of approximate molecular weight 18 kDa.

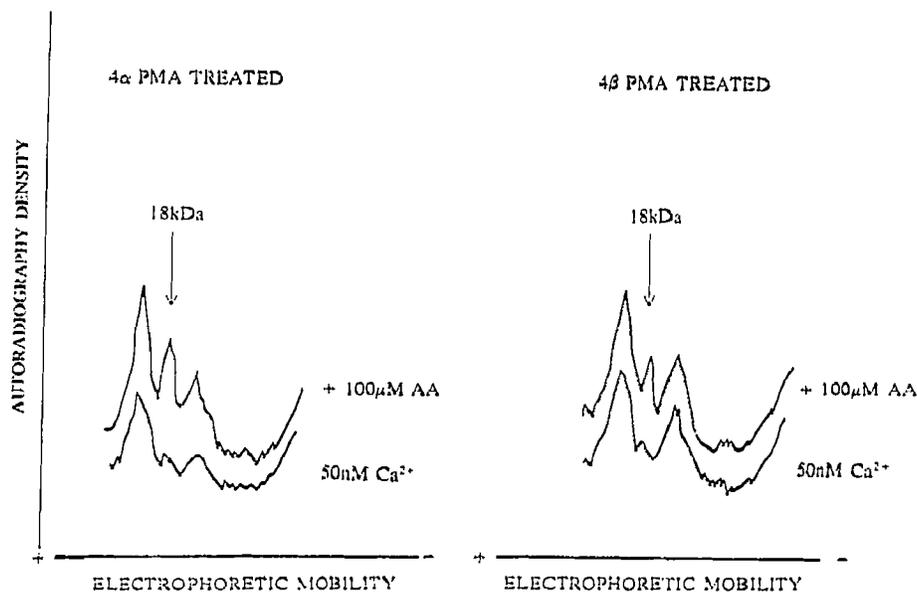


Fig. 3. Scanning-densitometer traces from autoradiographs of  $^{32}\text{P}$  incorporation into proteins from electrically permeabilised islets cultured for 20–24 h in the presence of either 200 nM  $4\alpha$  PMA (left panel) or 200 nM  $4\beta$  PMA (right panel). Control in each case represents phosphorylation in the presence of 50 nM  $\text{Ca}^{2+}$  alone. Arrow shows AA-induced (100  $\mu\text{M}$ ) phosphorylation of 18 kDa protein.

protein in the presence of either a substimulatory (50 nM) or a maximum stimulatory (10  $\mu\text{M}$ )  $\text{Ca}^{2+}$  concentration. Fig. 2 shows scanning densitometer traces of an autoradiograph of  $^{32}\text{P}$  incorporation into islet proteins after incubation (1 min,  $37^\circ\text{C}$ ) of permeabilised islets in the presence of 100  $\mu\text{M}$  AA. AA clearly stimulated the phosphorylation of the 18 kDa protein irrespective of the  $\text{Ca}^{2+}$  concentration although there was a slight increase in radiolabel incorporation into the 18 kDa protein in the presence of 10  $\mu\text{M}$   $\text{Ca}^{2+}$  alone.

The 18 kDa protein did not appear to be a substrate for PKC since stimulation of PKC by  $4\beta$  PMA (500 nM) did not result in  $^{32}\text{P}$  incorporation into the 18 kDa protein. However, other substrates for PKC-dependent phosphorylation were detected, including a protein of approximate molecular weight 21 kDa which could be a substrate for AA-induced phosphorylation. To further investigate whether the AA-induced phosphorylation of the 18 kDa protein was mediated via PKC activation we measured the effects of AA on protein phosphorylation in islets in which PKC had been down-regulated. Direct measurements of PKC activity indicated that prolonged exposure of islets to  $4\beta$  PMA resulted in the down-regulation of PKC ( $4\alpha$  PMA-treated  $43 \pm 10$  fmol/islet/min;  $4\beta$  PMA-treated  $< 5 \pm 1$ ,  $n=5$ ,  $P < 0.01$ ). This was further confirmed by the results of secretion experiments in which 500 nM  $4\beta$  PMA had no effect on insulin secretion from PKC-depleted islets ( $98 \pm 10\%$  of 50 nM  $\text{Ca}^{2+}$  controls, mean  $\pm$  SEM,  $n=9$ ,  $P < 0.2$ ), but significantly stimulated insulin secretion from control ( $4\alpha$  PMA)-treated islets

( $187 \pm 10\%$   $P < 0.01$ ). AA (100  $\mu\text{M}$ ) caused a marked increase in radiolabel incorporation into the 18 kDa substrate in PKC-depleted islets as shown by the scanning densitometer traces in Fig. 3. In the experiment shown in Fig. 3 there was a slight reduction in  $^{32}\text{P}$  incorporation into the 18 kDa protein in PKC-depleted islets when compared to control islets. However, the magnitude of the AA-induced phosphorylations varied between experiments and there was no consistent decrease in AA-induced  $^{32}\text{P}$  incorporation in PKC-depleted islets. Thus, in 5 separate experiments of the type shown in Fig. 3, AA (100  $\mu\text{M}$ ) caused a  $253 \pm 52\%$  increase in  $^{32}\text{P}$  incorporation over 50 nM  $\text{Ca}^{2+}$  alone in control ( $4\alpha$  PMA-treated) islets, whereas in PKC-depleted islets AA caused a  $353 \pm 106\%$  increase in radiolabelling of the 18 kDa substrate.

#### 4. DISCUSSION

AA can be generated within B cells directly from membrane phospholipids by the action of phospholipase  $\text{A}_2$  [21] or more indirectly from products of phospholipase C- or phospholipase D hydrolysis of membrane phospholipids [21–23]. The actions of AA in the B cell are complex because of the many possible mechanisms through which AA could influence insulin secretion. Thus, AA may mobilise intracellular  $\text{Ca}^{2+}$  [3,24], enhance  $\text{Ca}^{2+}$  influx [6] or activate PKC [5,13]. The situation is further complicated by the metabolism of AA into a number of biologically active products which may affect B cell function [21].

In the present study we have shown that AA stimulates phosphorylation of an islet protein of 18 kDa molecular weight. The concentrations of AA required to produce this effect are comparable with those measured in rat islets after exposure to stimulatory concentrations of glucose [6]. Although our results suggest that the 18 kDa protein may be a minor substrate for  $\text{Ca}^{2+}$ -dependent kinases, the effects of AA are unlikely to be due to an indirect activation of  $\text{Ca}^{2+}$ -dependent kinases as a consequence of AA-induced increases in intracellular  $\text{Ca}^{2+}$ , since the effects were observed in permeabilised islets in which intracellular  $\text{Ca}^{2+}$  was clamped at either sub- or suprastimulatory concentrations by  $\text{Ca}^{2+}$ /EGTA buffers.

AA can activate PKC [9,10], an enzyme which is thought to play an important role in receptor-mediated insulin secretion [25]. PKC activity and endogenous substrates for the enzyme have been detected in insulin secreting tissues [1,12,18], and PKC extracted from islets can be activated by AA in vitro [5,13]. In the present study AA occasionally stimulated phosphorylation of a 21 kDa protein which also appeared to be a substrate for PKC. However, the AA-induced phosphorylation of the 18 kDa protein detected in the present studies cannot be attributed solely to activation of PKC since this effect of AA was not mimicked by 4 $\beta$  PMA which is known to be a powerful activator of PKC. Furthermore, down-regulation of PKC activity, with the subsequent loss of secretory response to PMA, did not abolish AA-induced phosphorylation.

In conclusion, our results suggest that AA can promote protein phosphorylation in rat islets independently of the activation of  $\text{Ca}^{2+}$ -dependent kinases or of PKC, perhaps reflecting activation of a novel AA-dependent protein kinase or inactivation of a phosphatase. Further studies are required to determine whether the phosphorylation observed in these studies was stimulated by AA itself or by a product of its metabolism.

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