Arachidonic acid-induced insulin secretion from rat islets of Langerhans

A. M. Band, P. M. Jones and S. L. Howell

Biomedical Sciences Division, King's College London, University of London, Campden Hill Road, Kensington, London W8 7AH

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ABSTRACT

There is growing evidence that arachidonic acid (AA) and/or its metabolites may be involved in the control of insulin secretion. We have now investigated the effect of AA on insulin secretion from rat islets, and the possible involvement of protein kinase C (PKC) in this process. Exogenous AA stimulated insulin secretion from intact islets at a substimulatory concentration of glucose (2 mM), but did not further enhance glucose-induced (20 mM) insulin secretion. AA-induced insulin secretion was temperature dependent. The secretory responses seen at 37°C were totally abolished by reducing the incubation temperature to ≤34°C. AA-induced insulin secretion was not dependent upon extracellular Ca²⁺ and was potentiated by omission of Ca²⁺ or bovine serum albumin from the media. PKC in rat islets can thus be stimulated by AA, but the stimulation of PKC is not required for AA-induced insulin secretion.

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INTRODUCTION

Several second messengers are thought to be involved in the regulation of insulin secretion from pancreatic islets, including Ca²⁺, cyclic AMP, inositol trisphosphate (IP₃) and diacylglycerol (DAG) (for reviews see Prentki & Matschinsky, 1987; Turk, Wolf & McDaniel, 1987). Evidence is also accumulating to suggest that arachidonic acid (AA) and its metabolites may play an important role in the control of insulin secretion (Turk et al. 1987; McDaniel, Wolf & Turk, 1988; Metz, 1988a). AA can be generated by the action of phospholipase A₂ on membrane phospholipids or by the action of DAG lipase on DAG (for reviews see Prentki & Matschinsky, 1987; Turk et al. 1987; Metz, 1988b). Glucose, the major physiological stimulator of insulin secretion, stimulates the release and metabolism of AA (Turk, Colca, Kogatagal & McDaniel, 1984; Wolf, Turk, Sherman & McDaniel, 1986; Wolf, Pasquale & Turk, 1991). However, the majority of released AA stays unmetabolized (Wolf et al. 1986) so it may be AA itself, rather than a metabolite, which has the second messenger function. For example, it has been shown that unmetabolized AA can release Ca²⁺ from intracellular stores of several tissues (Cheah, 1981; Beaumier, Faucher & Naccache, 1987; Chan & Turk, 1987; Alonso, Sanchez & Garcia-Sancho, 1990) including rat islets (Wolf et al. 1986; Metz, Draznin, Sussman & Leitner, 1987; Kindmark, Nilsson, Olsson et al. 1991). Furthermore, there is convincing evidence that unmetabolized AA can activate the Ca²⁺/phospholipid-dependent protein kinase C (PKC) (Sekiguchi, Tsukuda, Ogita et al. 1987; Shearman, Naor, Sekiguchi et al. 1989; Shearman, Shinomura, Oda & Nishizuka, 1991), an enzyme which is thought to play an important role in receptor-mediated insulin secretion (Yamatani, Chiba, Kadowaki et al. 1988; Persaud, Jones, Sugden & Howell, 1989a).

We have therefore investigated the effect of AA on insulin secretion from isolated rat islets, and the possible involvement of PKC in this process.

MATERIALS AND METHODS

Materials

Collagenase (type XI), bovine serum albumin (BSA, fraction V), ATP, AA, oleic acid and eicosatrienoic acid were purchased from Sigma Chemical Co. Ltd, Poole, Dorset, U.K. [γ-³²P]ATP (3000 Ci/mmol) was from Amersham International plc, Amersham, Bucks, U.K. Hank's buffered salt solution (HBSS) and RPMI 1640 were from Gibco Ltd, Uxbridge, Middx, U.K. All other reagents were of analytical grade and were obtained from commercial sources.
Islet isolation and incubations
Islets of Langerhans were isolated from male Wistar rats (200–250 g) by collagenase digestion of the pancreas (Bjaaland, Jones & Howell, 1988). Islets were preincubated with test substances or their solvents for 30 min at 37°C in bicarbonate buffer (Gey & Gey, 1936) which contained 2 mM d-glucose, 2 mM CaCl₂, 0.5 mg BSA/ml and 20 mM Hepes (pH 7.4). After preincubation, islets were stimulated by adding d-glucose to a final concentration of 20 mM and further incubated for 30 min at 37°C, unless otherwise described. The insulin content of the incubation media was measured by radioimmunoassay, as previously described (Jones, Salmon & Howell, 1988).

Culture of islets
HBSS was used to isolate islets for overnight culture. The buffer contained HBSS, 0.02% NaHCO₃, 50 U penicillin/ml, 5 µg streptomycin/ml and 1% fetal calf serum. The incubation was carried out in RPMI 1640 tissue culture medium in a humidified atmosphere of 5% CO₂ in air at 37°C in a Leec incubator. RPMI 1640 was supplemented with 2 mM glutamine, 0.02% NaHCO₃, 10% fetal calf serum, 100 U penicillin/ml, 10 µg streptomycin/ml and 200 µg 4β-phorbol myristate acetate (4β-PMA) or the inactive phorbol ester 4α-PMA. Islets were washed and incubated for 1 h at 37°C in bicarbonate buffer containing 2 mM glucose before use in experiments.

Assay of PKC
Islet PKC activity was estimated as Ca²⁺/phospholipid-dependent incorporation of ³²P into histone type IIIIs as described previously (Persaud, Jones, Sugden & Howell, 1989). Briefly, 800–1200 islets were sonicated and incubated for 30 min in ice-cold buffer A (20 mM Tris–HCl, 2 mM EDTA, 0.5 mM EGTA, 50 µg leupeptin/ml, 1 mM phenylmethylsulphonyl fluoride and 0.1% mercaptoethanol; pH 7.5) with 1% Nonidet P-40.

Islet sonicate was applied to a DEAE–Cellulose DE-52 column (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, U.K.) and washed with buffer A. PKC was eluted from the column by 120 mM NaCl in buffer A. Activity of PKC was assayed by measuring incorporation of ³²P from [γ-³²P]ATP into histone type IIIIs. Incubations were for 10 min at 30°C in the presence of 11.1 mM magnesium acetate, 1.3 mM CaCl₂, 1.11 mg histone/ml and 111 µM ATP (sp. act. 0.3 Ci/mmol). PKC activity is expressed as the increase on basal phosphorylation (fmol/islet per min) in the presence of 96 µg phos-
induced insulin secretion was further studied by increasing the glucose concentration from 2 to 20 mM in the presence of 200 μM AA, as shown in Fig. 2. AA stimulated insulin secretion to a similar level at all concentrations of glucose tested and this stimulation was not additional to that seen with 20 mM glucose. AA-induced insulin release at basal glucose concentration was temperature-dependent. AA was unable to induce insulin secretion at lower than 34°C (Table 1). At this temperature (34°C) glucose-induced insulin secretion was only partially inhibited.

AA-induced insulin secretion was not dependent on the presence of extracellular Ca²⁺. Indeed, removal of extracellular Ca²⁺ greatly potentiated the effect of AA, as shown in Fig. 3. Furthermore, the omission of either extracellular Ca²⁺ or BSA caused islets to respond to a concentration of AA (50 μM) which was ineffective as a stimulant of secretion in the presence of 2 mM extracellular Ca²⁺ and 0.05% BSA.

The requirement of extracellular Ca²⁺ influx was further studied by using the voltage-dependent Ca²⁺-channel blocker, verapamil. Verapamil (15 μM) inhibited glucose-induced insulin secretion by 80% but had no effect on AA-induced insulin release (Fig.4).

**AA and PKC activation in islets**

We studied the effect of AA on islet PKC activity by extracting enzyme suspension from freshly isolated islets. As shown in Table 2, islet PKC was activated by 200 μM AA and this activation was Ca²⁺-dependent. PKC was also partly activated by AA in the absence of PS, although not to the same extent as that seen in the presence of PS (Table 2).

The role of PKC on AA-induced insulin secretion was further investigated by down-regulation of the enzyme. Intact islets were incubated 20 h with the PKC activator, 4β-PMA, to cause down-regulation of PKC. The control islets were incubated with 4α-PMA which is an inactive phorbol ester. Down-regulation was confirmed by direct measurement of PKC activity extracted from 4α-PMA-treated and 4β-PMA-treated islets (Table 3). Prolonged ex-

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**TABLE 1. Temperature dependence of arachidonic acid (AA) induced-insulin secretion from rat islets.** The results are expressed as means±s.e.m. of the percentage of control incubations (2 mM glucose) at appropriate temperatures (n=6–9 observations)

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Insulin secretion (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>20 mM glucose 200 μM AA</td>
</tr>
<tr>
<td>34</td>
<td>484.8±39.7*                  446.7±58.8*</td>
</tr>
<tr>
<td>26</td>
<td>297.0±32.0*                  131.7±9.5</td>
</tr>
<tr>
<td>20</td>
<td>123.8±22.7                   76.3±9.8</td>
</tr>
<tr>
<td>4</td>
<td>102.4±15.5                   74.2±7.4</td>
</tr>
<tr>
<td></td>
<td>77.3±7.9                    81.6±14.8</td>
</tr>
</tbody>
</table>

*P<0.05 versus basal secretion (2 mM glucose) at appropriate temperatures (unpaired t-test).

**FIGURE 2.** Effect of arachidonic acid (AA) on glucose-induced insulin secretion. Rat islets were incubated at 37°C as described in Materials and Methods in the presence (hatched bars) or absence (open bars) of 200 μM AA and increasing concentrations of glucose. The values are means±s.e.m., n=7–9 observations. *P<0.01 versus 2 mM glucose (unpaired t-test).

**FIGURE 3.** Extracellular Ca²⁺-dependence of glucose and arachidonic acid (AA)-induced insulin secretion. Islets were incubated with 2 mM glucose and with increasing concentrations of AA in the presence of 2 mM CaCl₂ (open bars) or 1 mM EGTA (hatched bars) or with 2 mM CaCl₂ in the absence of bovine serum albumin (cross-hatched bars) in the media. Values are means±s.e.m., n=5–9 observations. *P≤0.06 and **P<0.01 versus basal secretion (unpaired t-test).
**Figure 4.** Effect of verapamil on glucose- and arachidonic acid (AA)-induced insulin secretion. Islets were incubated in the presence (hatched bars) and absence (open bars) of 15 μM verapamil in the presence of 2 mM or 20 mM glucose and AA as indicated. Values are means±s.E.M., n=7–9 observations. *P<0.01 versus absence of verapamil (unpaired t-test).

**Table 2.** Ca²⁺-dependence of stimulation of rat islet protein kinase C (PKC) activity. Data are expressed as means±s.E.M.; n=4–5 observations.

<table>
<thead>
<tr>
<th>PKC activity (fmol/islet per min)</th>
<th>+Ca²⁺ (1.3 mM)</th>
<th>−Ca²⁺ (1 mM EGTA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Additions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>149.2±12.3</td>
<td>0*</td>
</tr>
<tr>
<td>PS+Di</td>
<td>117.7±23.5</td>
<td>40.4±7.1*</td>
</tr>
<tr>
<td>AA</td>
<td>62.4±11.6</td>
<td>13.1±10.3*</td>
</tr>
<tr>
<td>AA+PS</td>
<td>133.2±10.5</td>
<td>0*</td>
</tr>
</tbody>
</table>

*P<0.01 versus the presence of 2 mM CaCl₂ (unpaired t-test).

Activity of PKC was estimated as Ca²⁺/phosphatidyserine (PS)-dependent incorporation of [³²P] into histone type IIIa. Activity without added Ca²⁺ was measured in the presence of 1 mM EGTA. 200 μM arachidonic acid (AA), 96 μg PS/ml and 6.4 μg diolein (Di)/ml and 200 nM PMA were used to stimulate PKC.

PKC-depleted islets no longer responded to 4β-PMA (500 nM), confirming that PKC down-regulation had occurred (4α-PMA-treated islets plus 500 nM PMA, 176±30% of control, P=0.06; PKC-depleted islets plus 500 nM PMA, 96±16% of control, P>0.2; mean±s.E.M., n=6–9 observations).

**Table 3.** Effect of prolonged exposure to phorbol myristate acetate (PMA) on rat islet protein kinase C (PKC) activity. Data are expressed as means±s.E.M.; n=4–5 observations.

<table>
<thead>
<tr>
<th>PKC activity (fmol/islet per min)</th>
<th>4α-PMA treatment</th>
<th>4β-PMA treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Additions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>112.8±17.9</td>
<td>2.9±0.5*</td>
</tr>
<tr>
<td>PS+Di</td>
<td>141.6±30.1</td>
<td>4.7±2.6*</td>
</tr>
<tr>
<td>PS+AA</td>
<td>93.9±4.5</td>
<td>18.6±4.6*</td>
</tr>
<tr>
<td>AA</td>
<td>64.8±7.3</td>
<td>2.4±2.3*</td>
</tr>
<tr>
<td>PS+PMA</td>
<td>158.3±4.5</td>
<td>13.7±3.7*</td>
</tr>
</tbody>
</table>

*P<0.01 versus 4α-PMA-treated (unpaired t-test).

Isolated islets were cultured (19 h) in RPMI 1640 tissue culture media in the presence of 200 nM 4β-PMA or the inactive phorbol ester 4α-PMA. Activity of PKC was estimated as previously described in Materials and Methods. 200 μM arachidonic acid (AA), 96 μg phosphatidyserine (PS)/ml, 6.4 μg diolein (Di)/ml and 200 nM PMA were used to stimulate PKC.

**Discussion**

Exogenous AA stimulated basal insulin secretion but did not increase glucose-induced insulin secretion from rat pancreatic islets. The stimulation of insulin secretion was concentration-dependent and sensitive to extracellular Ca²⁺ but it did not require an influx of extracellular Ca²⁺ since AA was effective in the absence of extracellular Ca²⁺, and since the effects of AA were not inhibited in the presence of verapamil. In Ca²⁺-free media there was a large potentiation of AA-induced insulin release in our experiments. A similar effect has been reported by Metz et al. (1987) who were able to demonstrate an AA-induced stimulation of insulin secretion only when extracellular Ca²⁺ was removed by EGTA. It is known that Ca²⁺ can bind to fatty acids and cause aggregation (Walters & Weiser, 1984; Watras, Messiino & Herbette, 1984) and it has been suggested that the free AA concentrations in buffers containing Ca²⁺ could be considerably lower than those in Ca²⁺-free buffers (Metz et al. 1987; Metz, 1988a). Since we wished to study interactions between AA and glucose, and since glucose-induced insulin release is dependent on extracellular Ca²⁺ (for review see Prentki & Matschinsky, 1987) it was necessary to include Ca²⁺ in the medium and thus to use the higher stimulatory concentrations of AA.
required under these conditions. Furthermore, the handling of islets necessitates the presence of some protein such as BSA in the medium, although it is known that fatty acids will bind to BSA (Chen, 1967; Chan & Turk, 1987). This was confirmed in our studies since when BSA was omitted from the medium insulin secretion was more sensitive to AA. Given the presence of Ca\(^{2+}\) and BSA in the incubation medium, the stimulatory concentrations of AA used in the present studies are not excessively high, particularly since it has been calculated that in glucose-stimulated islets intracellular concentrations of AA can reach 100–200 \(\mu\)M, or possibly even higher (Wolf et al. 1986; Metz, 1988c). However, it cannot be totally excluded that insulin release was due to a detergent effect of AA on the membrane. This explanation seems highly unlikely since (1) there was no increase in glucose-stimulated insulin release and (2) other unsaturated fatty acids, oleic acid and elcosatrienoic acid, did not cause insulin secretion in our studies. In other studies, where the effects of a variety of fatty acids on insulin secretion were tested, AA was the most potent polyunsaturated fatty acid (Metz et al. 1987; Metz, 1988a) and (3) AA-induced insulin release was temperature-dependent.

Activators of PKC have been shown to be powerful stimulators of insulin secretion (Virji, Steffes & Estensen, 1978; Jones, Stutchfield & Howell, 1985), so a likely explanation for the effect of AA on insulin secretion was through activation of PKC. The results of our studies demonstrate that rat islets contain a PKC activity that can be activated in vitro by AA in a Ca\(^{2+}\)-dependent manner. This is unlikely to be the AA-sensitive \(\gamma\) subform of PKC (Nishizuka, 1988), since islets are reported to contain \(\alpha\) and \(\beta\), but not \(\gamma\), isoforms of the enzyme (Ito, Saito, Taniguchi et al. 1989; Onoda, Hagiwara, Hachiya et al. 1990). However, recent reports from other tissues suggest that, in the presence of Ca\(^{2+}\), \(\alpha\) and \(\beta\) isoforms of PKC can be activated by the concentrations of AA used in our studies (Sekiguchi et al. 1987; Shearman et al. 1991).

Although we have demonstrated that AA can activate PKC in rat islets, this activation is not obligatory for the stimulation of insulin secretion by AA, since depletion of islet PKC activity by phorbol ester did not affect AA-induced insulin secretion. We have previously shown that prolonged exposure to PMA drastically reduces PMA- or diolein-stimulated Ca\(^{2+}\)/phospholipid-dependent protein kinase activity in rat islets (Persaud et al. 1989a). In the present study we have also shown that PMA pretreatment down-regulates the AA-activated PKC activity, without causing any concomitant reduction in AA-induced insulin secretion although PMA-induced secretion was, as expected, abolished. The abolition of PMA-induced secretion suggests a comprehensive down-regulation of PKC isoforms since the \(\alpha\), \(\beta\), \(\gamma\), \(\delta\) and \(\epsilon\) isoforms of PKC are known to be activated by this phorbol ester (Ryves, Evans, Olivier et al. 1991). This result appears to disagree with a previous report using digitonin-permeabilized islets in which AA-induced (110 \(\mu\)M) insulin secretion was totally blocked by depletion of PKC with phorbol ester (Metz, 1988a). However, in the same study, AA-induced insulin release from intact islets was only reduced by 40\(\pm\)6\%, suggesting that mechanisms other than PKC activation are involved in AA-induced insulin release, as implied by our results.

Other possible second messenger systems involving AA-induced insulin release have been suggested, including Ca\(^{2+}\) release from intracellular stores (Wolf et al. 1986; Metz et al. 1987), amplification of the voltage-sensitive Ca\(^{2+}\) channel opening (Wolf et al. 1991) and protein phosphorylation (Basudev, Jones, Band & Howell, 1991). Although AA-induced insulin release did not require an influx of extracellular Ca\(^{2+}\), it is possible that AA may increase cytosolic Ca\(^{2+}\) by liberating intracellular Ca\(^{2+}\), but it is uncertain whether this mechanism alone can explain the potent stimulation of insulin secretion by AA at substimulatory glucose concentrations. Thus, activators of phospholipase C, such as cholinergic agonists, can cause an IP\(_3\)-mediated release of intracellular Ca\(^{2+}\); but this is insufficient to initiate insulin secretion in the absence of a stimulatory concentration of glucose (Morgan, Rumford & Montague, 1985). Neither can the stimulation of insulin secretion by AA be fully explained by stimulation of opening of voltage dependent-Ca\(^{2+}\) channel (Wolf et al. 1991) since we were able to show AA-induced insulin secretion in the absence of extracellular Ca\(^{2+}\).

In conclusion, our results confirm that exogenous AA stimulates insulin secretion from intact islets both in the presence and absence of extracellular Ca\(^{2+}\). Although AA can activate islet PKC in vitro, this activation is not solely responsible for its effects on insulin secretion. Whether AA itself stimulates insulin secretion, or whether by the action of one of its lipoxygenase or cyclo-oxygenase metabolites remains to be clarified.

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