Amylin evokes phosphorylation of P20 in rat skeletal muscle

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Abstract To investigate the signal transduction events underlying amylin's actions, the amylin-evoked protein phosphorylation cascade was analysed using two-dimensional gel electrophoresis. We found that phosphorylation of three isoelectric variants of P20 (termed ARPP1, ARPP2 and ARPP3) was associated with amylin's actions in rat skeletal muscle. Amylin decreased phosphorylation of ARPP1 and increased phosphorylation of ARPP2 and ARPP3 in a dose-dependent manner. Insulin inhibited amylin-evoked phosphorylation of ARPP2 and ARPP3. The amylin-selective antagonist rat amylin-(8–37) completely reversed amylin's action on ARPP3 and partially decreased phosphorylation of ARPP2. By contrast, the CGRP-selective antagonist, human CGRP-(8–37) blocked phosphorylation of ARPP2 but had little effect on ARPP3. These results suggest that amylin modifies phosphorylation of P20 via two independent mechanisms, and that P20 might be a molecule mediating amylin's biological functions.

Key words: Amylin; P20; Phosphorylation; Signal transduction

1. Introduction

Amylin, a 37-amino acid polypeptide, is the major protein component of the islet amyloid typically found in the pancreatic islets of non-insulin-dependent diabetes mellitus patients [1]. Amylin is cosecreted from the pancreatic islet β-cells along with insulin [2]. Many studies have demonstrated that amylin is involved in controlling glucose homeostasis [3]. In skeletal muscle, amylin promotes net loss of glycogen and increases lactate output, probably by inactivating glycogen synthase, inhibiting glucose uptake and activating glycogen phosphorylase [4–6]. It has also been reported to antagonise insulin's metabolic functions in vitro and in vivo [7,8]. Although earlier results suggested that amylin's biological effects on fuel metabolism were only of pharmacological interest [3], more recent in vivo studies with an amylin-selective antagonist have shown that they are likely to be of physiological relevance [8]. More recently, amylin has been proposed as a brain-gut peptide participating in the rapid endocrine response during digestion to maintain euglycaemia [9]. It can inhibit acid gastric secretion, gastric emptying and food uptake [10]. Possible roles for amylin in causing insulin resistance have been suggested. Agonists and antagonists of amylin are currently under development for clinical trials in diabetic patients [11].

Despite considerable investigation, little is known about the receptor and post-receptor events linking amylin to its final biological activities. Amylin has about 50% sequence identity with calcitonin gene-related peptide (CGRP) [3]. Some of amylin's effects such as vasodilation and inhibition of muscle contraction appear to be mediated by a CGRP receptor [12–14]. On the other hand, there is evidence arguing for the existence of an amylin-specific receptor [15]. Studies are also conflicting as to whether or not amylin elicits its functions through a cAMP-mediated pathway [5,16].

We show here that amylin-evoked signalling cascades converge on P20 in rat hindlimb skeletal muscle. P20 is a small heat shock-related protein whose function has recently been linked to muscle contractile activity [17]. Amylin, by acting through at least two independent mechanisms, modulates phosphorylation of P20 to produce three phosphorylated isoforms. This effect can be differentially blocked by amylin antagonists rat amylin-(8–37), human CGRP-(8–37) and insulin.

2. Materials and methods

2.1. Muscle dissection and metabolic labelling

Dissection and isolation of rat extensor digitorum longus (EDL) muscle strips from 18-h fasted male Wistar rats (~250 g) were carried out under anaesthesia with pentobarbital. The muscle strips were preincubated in a shaking incubator at 30°C for 1 h in 5 ml of Dulbecco's modified Eagle's medium without sodium phosphate. Subsequently the muscle strips were transferred to similar flasks containing identical medium plus 0.25 mCi/ml [^{32}P]orthophosphate (ICN) and incubated for a further 4 h at 30°C to equilibrate the internal ATP pool. All incubation media were gassed with a mixture of 95% O_2 and 5% CO_2. Rat amylin, rat amylin-(8–37), human CGRP-(8–37) (Bachem, Torrance, CA) or human insulin (Actrapid, NovoNordisk) were added to the incubation medium to stated final concentrations. Reactions were terminated by freezing muscle strips in liquid nitrogen immediately after incubation.

2.2. Preparation of muscle extracts and two-dimensional gel electrophoresis (2-DE)

^{32}P-labelled muscle strips were homogenised in 2-DE lysis buffer (9 M urea, 4% w/v CHAPS, 2% v/v Pharmalyte pH 3–10, 200 mM DTT, 8 mM PMSF). The lysates were briefly sonicated, incubated on ice for 20 min and microcentrifuged at 12 000 rpm for 10 min to remove debris. Protein concentrations were determined by the Bradford method and radioactivity was measured by liquid scintillation counting. ^{32}P-labelled lysates with equivalent amounts of radioactivity were isoelectrically focused on IPG Drystrip pH 4–7 Linear gels (Pharmacia) using a multiphor RII electrophoresis system according to the manufacturer's instructions. Second-dimensional electrophoresis was carried out on the ExcelGel precast 12–14% acrylamide gradient gels (Pharmacia). After electrophoresis the gels were fixed and the proteins visualised by Coomassie brilliant blue R250 staining, autoradiography or phosphorimaging. In all figures, the gels are displayed with the acid end of the isoelectric focusing dimension to the right and the direction of SDS-PAGE from top to bottom.

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Abbreviations: ARPP, amylin-responsive phosphoprotein; CGRP, calcitonin gene-related peptide; 2-DE, two-dimensional gel electrophoresis; HSP, heat shock protein; EDL, extensor digitorum longus

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2.3. In-gel trypsin digestion, RP-HPLC and amino acid sequencing

Protein spots of interest were excised and pooled from Coomassie brilliant blue-stained preparative gels. Gel pieces were subjected to in-gel trypsin digestion as described by Rosenfeld et al. [18]. The extracted peptide mixture was fractionated by reverse phase HPLC (RP-HPLC) on a Jupiter 5 μ C18 column (Phenomenex) and collected manually. Amino acid sequencing was performed using the Edman degradation method with a Perkin Elmer (Procise, Model 492) protein sequencer.

2.4. Immunoprecipitation

^{32}P-labelled EDL muscle strips were solubilised by homogenising in lysis buffer (1% Triton X-100, 50 mM HEPES pH 7.5, 100 mM NaCl, 10% glycerol, 5 mM EDTA, 2 mM PMSF, 10 U/ml apyrase) and incubated on ice for 30 min with shaking. The debris was removed by microcentrifugation and immune complexes formed by shaking the clarified muscle lysate with antisera against P20 (provided by Dr. Kanefusa Kato) [19] overnight at 4°C, then recovered following addition of protein A Sepharose for 1 h. ^{32}P-labelled immunoprecipitated proteins were eluted from the beads by incubating with 2-DE lysis buffer at 60°C for 30 min and separated by 2-DE as above.

2.5. Data analysis

Autoradiography films were scanned and digitised using a Sharp JX-325 scanner, and protein spots detected, quantitated and analysed using Imagemaster 2D software (Pharmacia). Radioactivity of protein spots was also simultaneously quantitated by phosphorimager (BAS 2000). All the results presented are based on at least three independent experiments.

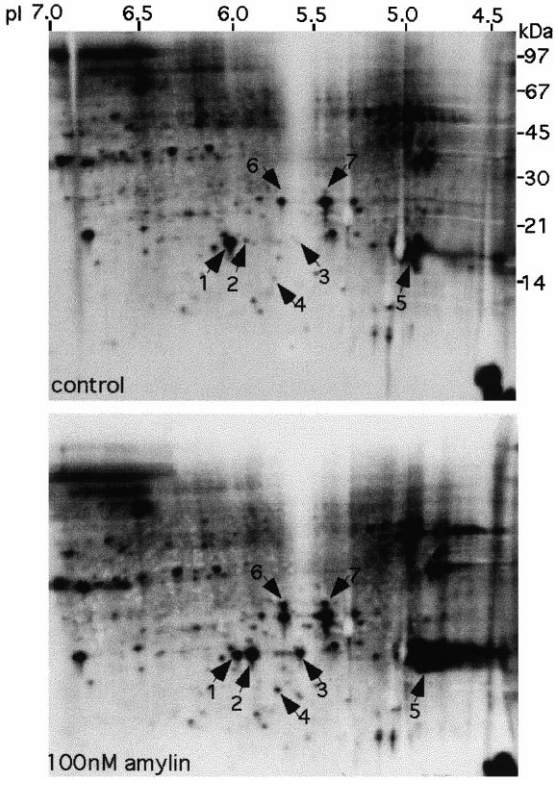

Fig. 1. Changes of protein phosphorylation in rat EDL muscle after amylin stimulation. ^{32}P-prelabelled (4 h) EDL muscle strips were treated without or with 100 nM rat amylin for 1 h. Samples with equivalent amounts of radioactivity were separated by 2-DE and visualised by autoradiography. The arrows and numbers indicate the spots affected by amylin as described in the text.

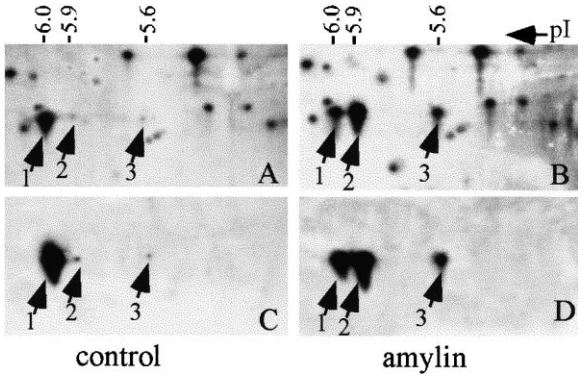

Fig. 2. Multiple phosphorylated isoforms of P20 exist in rat EDL. ^{32}P-prelabelled muscle extracts treated with or without amylin were immunoprecipitated using anti-P20 antibody. The muscle lysates (A,B) or the immunoprecipitated complexes (C,D) were separated by 2-DE and visualised by autoradiography. The three phosphorylated isoelectric variants of p20 are numbered 1, 2 and 3.

3. Results

3.1. Rat amylin alters the pattern of protein phosphorylation in rat hindlimb skeletal muscle

To detect phosphoproteins that are responsive to amylin's action, EDL muscle strips were prelabelled with ^{32}P for 4 h and subsequently treated with or without 100 nM amylin for 1 h. Alterations of phosphorylation/dephosphorylation in several groups of proteins were consistently observed (all these proteins are here termed ARPP (amylin-responsive phosphoprotein) for convenience) (Fig. 1). Phosphorylation of ARPP1 (p*I* 6.0, MW 20 kDa) was decreased 2.1-fold. Phosphorylation of four proteins was significantly increased after stimulation, by on average 3–8-fold individually (ARPP2 (p*I* 5.9, MW 20 kDa), ARPP4 (p*I* 5.7, MW 14 kDa), ARPP5 (p*I* 4.8, MW 17 kDa) and ARPP7 (p*I* 5.4, MW 28 kDa)). ^{32}P labelling signals of two proteins (ARPP3 (p*I* 5.6, MW 20 kDa) and ARPP6 (p*I* 5.7, MW 28 kDa)), which were only just detectable in control muscle strips, became very prominent in amylin-treated samples.

3.2. ARPP1, ARPP2 and ARPP3 are different phosphorylated isoforms of P20

Alterations in phosphorylation of ARPP1, ARPP2 and ARPP3 occurred as early as 3 min after commencement of amylin stimulation (data not shown), suggesting that they might be early components in amylin-evoked signal transduction. In addition, the three proteins are abundant enough to be visible on silver-stained gels (data not shown). Thus we decided to further investigate these three ARPPs. Both ARPP1 and ARPP2 were identified as P20 by amino acid sequencing of the RP-HPLC-separated tryptic peptides from these two proteins. P20 is a small heat shock-related protein that has been reported to exist as three phosphorylated isoforms with similar molecular weights and different p*I* in smooth muscle [17]. Immunoprecipitation of ^{32}P-labelled muscle lysates using antibody against P20 further confirmed that ARPP1, ARPP2 and ARPP3 are three phosphorylated isoforms of P20 in rat skeletal muscle. The pattern of increased phosphorylation of ARPP2, ARPP3 and decreased phosphorylation of ARPP1 mirrors that detected in the above phosphoprotein maps (Fig. 2). The identities of these three

proteins were also verified by Western blotting (data not shown).

3.3. Amylin-evoked phosphorylation of P20 is concentration-dependent

The responsiveness of three isoforms of P20 to amylin's action differs with varying amylin concentrations (Fig. 3). Phosphorylation of ARPP3 is significantly increased by 1 nM amylin, reaching a maximum by 10 nM. Conversely, there is only a slight increase in phosphorylation of ARPP2 and a decrease in phosphorylation of ARPP1 at 1 nM amylin; maximum effects on these two isoforms were only achieved at 100 nM amylin.

3.4. Effects of amylin antagonists on amylin-evoked phosphorylation of P20

Rat amylin-(8–37) and human CGRP-(8–37) have been shown to partially or completely block amylin's functions [8,12,15]. Therefore, their actions on the amylin-associated phosphorylation of P20 were also evaluated in this study. When EDL muscle strips were co-stimulated by rat amylin and rat amylin-(8–37) at a molar ratio of 1:500, amylin-stimulated phosphorylation of ARPP3 was completely blocked (Fig. 4C), whereas the phosphorylation of ARPP2 decreased by only about 20% compared with that from samples stimulated by amylin alone (Fig. 4B). By increasing the molar ratio to 1:5000, phosphorylation of ARPP2 was further decreased (by 81%), but was still not totally suppressed (Fig. 4D). By contrast, a 500-fold molar excess of CGRP-(8–37) over amylin slightly inhibited phosphorylation of ARPP2 but had no effect on ARPP3 (Fig. 4E). A 5000:1 ratio of CGRP-(8–37) over amylin blocked phosphorylation of ARPP2 by 86% compared to that in samples treated with an equivalent concentration of amylin alone, whereas phosphorylation of ARPP3 was still little affected (Fig. 4F). Insulin inhibited amylin-evoked phosphorylation of ARPP2 and ARPP3 by 72% and 76% respectively (Fig. 4G).

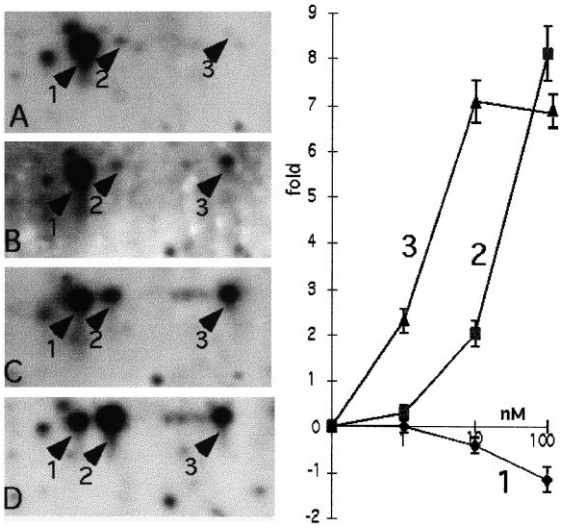

Fig. 3. Distinctive phosphorylation patterns of P20 in response to varying amylin concentrations. ^{32}P-prelabelled rat EDL muscle strips were treated for 1 h without amylin (A), or with 1 nM (B), 10 nM (C), or 100 nM amylin (D), then analysed as described. The graph in the right panel represents fold increases or decreases in radioactivity of each phosphorylated form of P20 in relation to that in the untreated muscle strips.

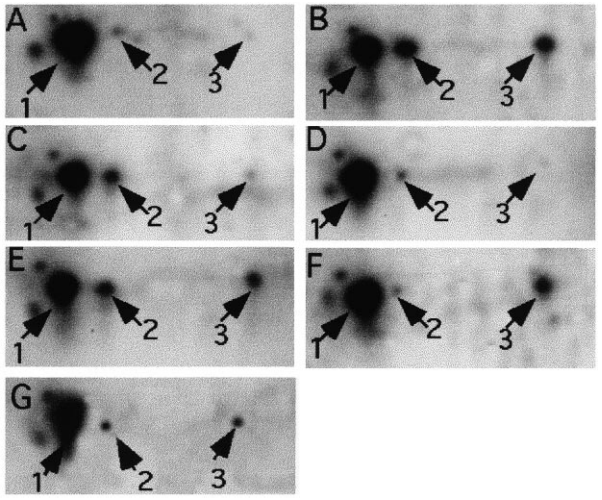

Fig. 4. Amylin antagonists influence amylin-stimulated phosphorylation of P20. ^{32}P-prelabelled rat EDL muscle strips were incubated for 1 h without (A) or with (B) 10 nM rat amylin, 10 nM rat amylin plus 5 μM rat amylin-(8–37) (C), 10 nM rat amylin plus 50 μM rat amylin-(8–37) (D), 10 nM rat amylin plus 5 μM human CGRP-(8–37) (E), 10 nM amylin plus 50 μM human CGRP-(8–37) (F) or 10 nM amylin plus 71 nM insulin and analysed by 2-DE (G).

4. Discussion

P20 is a small heat shock-related protein that was recently isolated from rat and human skeletal muscle as a by-product of the purification of HSP28/27 [19]. While it is ubiquitously distributed, the expression level is much higher in skeletal, smooth and heart muscle [20]. Rat P20 is composed of 162 amino acid residues and has significant sequence similarities with αB crystallin (47%) and HSP27 (35%). However, unlike these two proteins, P20 is not inducible by heat shock stress in rat skeletal muscle [19]. The physiological function of P20 is still uncertain.

In the current study, we found that amylin modulated phosphorylation of P20 to produce three isoelectric isoforms (ARPP1 with p*I* 6.0; ARPP2 with p*I* 5.9 and ARPP3 with p*I* 5.6) in rat skeletal muscle. Our data support the notion that amylin stimulates phosphorylation of ARPP2 and ARPP3 through two independent mechanisms. (I) The responsiveness of these two isoforms to different amylin concentrations is distinct. Phosphorylation of ARPP3 is significantly increased by 1 nM and saturated by 10 nM amylin, whereas ARPP2 is maximally stimulated only by 100 nM amylin (Fig. 3). (II) The amylin-selective antagonist rat amylin-(8–37) has separate efficacy on blocking amylin's action on these two isoforms (Fig. 4). A 500-fold molar excess of rat amylin-(8–37) over amylin completely reversed amylin-evoked phosphorylation of ARPP3, whereas a 5000-fold ratio still could not completely block amylin's action on ARPP2.

It remains to be determined whether amylin acts through a specific receptor, or only a CGRP receptor. Previous studies, which were based on either binding activity [21–23] or sensitivity to antagonists' blocking effects on similar post-receptor effects [15,24], could not provide an unequivocal answer. Our

study showed that amylin antagonists have separate effects on amylin-evoked phosphorylation of different P20 isoforms (Fig. 4). We thus infer that both an amylin-specific receptor and a CGRP receptor are likely to be involved in amylin-evoked phosphorylation of P20 in skeletal muscle. Low concentrations of amylin (<10 nM) evoke phosphorylation of ARPP3, perhaps via its specific receptor with high affinity and low abundance, which can be completely blocked by the specific antagonist amylin-(8–37). In contrast, amylin-stimulated phosphorylation of ARPP2 is likely to be mediated by non-specific binding of amylin to a G_s-coupled CGRP receptor with higher capacity and lower affinity, through the cAMP pathway [25], which requires higher concentrations of amylin and is more sensitive to antagonism by CGRP-(8–37).

Several recent studies have linked the function of P20 with muscle contractile activity [20,26,27]. Phosphorylation of P20 has been linked to vasodilation [17] and contraction of smooth muscle [28]. P20 might regulate vasorelaxation through a direct interaction with contractile regulatory proteins such as actin and myosin [29]. Coincidentally, many of amylin's biological functions are associated with muscle contractile activity. For example, amylin has been shown to induce vasorelaxation [12,13], inhibit electrically stimulated muscle contraction [14,30,31] and decrease gastric motility [9]. Moreover, the contractile status of muscle is also an important factor in regulating fuel metabolism [32,33]. Therefore, P20 could be a critical signalling molecule which mediates amylin's physiological functions. It is interesting to note that insulin inhibits amylin's action on phosphorylation of P20. Thus, P20 could serve as a marker to dissect the mechanisms of the interplay between amylin and insulin.

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