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Isolation and Characterisation of Two Amylin Responsive Proteins from Rat Skeletal Muscle

Shao Chin Lee

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biological Sciences, The University of Auckland, 1997
To My Family
ABSTRACT

Two amylin responsive proteins, here designated ARPI and ARP2, were discovered from rat skeletal muscle through two dimensional gel electrophoresis analysis. ARPI was detected only in amylin-stimulated muscles where the insulin-stimulated glucose incorporation into glycogen was inhibited. This protein incorporated $^{32}\text{Pi}$ but not $[^{35}\text{S}]-\text{methionine}$ in the metabolic labelling experiments. Subsequent molecular characterisation revealed that ARPI was a novel monomeric form (designated form 1) of protein p20, and two other monomeric forms (designated forms 2 and 3 respectively) of protein p20 were also characterised. The production of ARPI was not affected by the presence of insulin, but calcitonin gene-related peptide (CGRP) was found to evoke the production of ARPI in the presence or absence of insulin. In contrast, ARP2 was detected in both control and amylin-stimulated muscles. Amylin stimulation evoked incorporation of $[^{35}\text{S}]-\text{methionine}$ but not $^{32}\text{Pi}$ into the protein and increased its concentration significantly.

It is concluded that amylin elicits the production of ARPI through phosphorylation and increases the protein biosynthesis of ARP2; the amylin-evoked production of ARPI is insulin independent; amylin and CGRP share, at least in part, an intracellular signal transduction pathway; and ARPI and 2 may be involved in the development of insulin resistance. It is suggested that ARPI and 2 could potentially be used as molecular markers for the analysis of amylin action.
ACKNOWLEDGMENT

I would like to express my sincere thanks to all who have contributed to the study, especially:

Professor Garth JS Cooper for creating the opportunity; Mrs. Catriona Knight for performing the protein sequencing and her help in proof-reading and editing; Mrs. Judy Douglas for day to day back-up; Dr. D. Christie for his great help; Dr. M. Hubbard for being my advisor; Mrs. Cynthia Tse and Professor JAJH Critchley for their help in proof-reading and editing; and Professor J. Kistler for his comments on the manuscript submitted to the Journal of Biological Chemistry for publication.

Appreciation is also extended to Mr. S. Spence, J. Bai, A. Clarke, Mrs. S. Zhang, Mrs. C. Buchanan and other group members for friendship.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>2DE</td>
<td>Two dimensional gel electrophoresis</td>
</tr>
<tr>
<td>ARP1</td>
<td>Amylin responsive protein 1 (form 1 protein p20)</td>
</tr>
<tr>
<td>ARP2</td>
<td>Amylin responsive protein 2</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDL</td>
<td><em>Extensor Digitorum Longus</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>GCG</td>
<td>Genetics computer group</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IDDM</td>
<td>Insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>IPG</td>
<td>Immobilised pH gradient</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NIDDM</td>
<td>Non insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>PMF</td>
<td>Peptide mass fingerprinting</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl Sulfate</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of flight</td>
</tr>
<tr>
<td>Tris</td>
<td>Tri(hydroxymethyl)aminomethane</td>
</tr>
</tbody>
</table>

Abbreviations of units of measurement and of physical and chemical quantities are those recommended by the *Journal of Biological Chemistry* (*J. Biol. Chem.* 271, 1-4, 1996).
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CONTENT</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>1</td>
</tr>
<tr>
<td>ACKNOWLEDGMENT</td>
<td>2</td>
</tr>
<tr>
<td>ABBREVIATION</td>
<td>3</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>11</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>13</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>117</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>118</td>
</tr>
</tbody>
</table>

Chapter 1: Introduction

1.1. Insulin resistance                        | 14   |
1.1.1. The history and the current concept    | 14   |
1.1.1.1. History                              | 14   |
1.1.1.2. The current concept of insulin resistance | 15   |
1.1.2. Clinical and economic significance of insulin resistance | 15   |
1.1.2.1. Insulin resistance in diseases       | 15   |
1.1.2.2. Economic impact of insulin resistance| 17   |
1.1.3. Skeletal muscle is the major site of insulin resistance | 17   |
1.1.4. Non-oxidative glucose metabolism is the major metabolic pathway involved in the development of insulin resistance | 19   |
1.1.5. Molecular mechanisms of insulin resistance | 19   |
1.1.5.1. Insulin resistance as a consequence of insulin receptor abnormalities | 19   |
1.1.5.2. Insulin resistance as a consequence of post-receptor abnormalities

1.1.5.3. Changes in glucose transport and intra-cellular metabolism in insulin resistance

1.1.5.4. The glucose-fatty acid cycle

1.1.5.5. Genetic influence

1.1.6. The common form of insulin resistance

1.1.7. Insulin resistance: Summary

1.2. Amylin

1.2.1. Potential biological functions of amylin

1.2.2. Molecular mechanisms of amylin action

1.2.2.1. Amylin signal transduction

1.2.2.2. Targets of amylin action

1.2.3. Is amylin implicated in the pathogenesis of the common form of insulin resistance?

1.2.4. Amylin and the common form of insulin resistance: Understanding of the current controversy and its potential resolutions

1.2.5. Amylin: Summary

1.3. The project

1.3.1. Objectives

1.3.2. Overall experimental design

Chapter 2: Separation of rat skeletal muscle proteins by two dimensional gel electrophoresis and the technical optimisation

2.1. Two dimensional gel electrophoresis

2.2. Materials and methods

2.2.1. Chemicals, solutions and equipment

2.2.1.1. Chemicals and equipment

2.2.1.2. Solutions

2.2.2. Sample preparation and protein quantification
2.2.2.1. Tissue homogenisation 37
2.2.2.2. Protein quantification 37
2.2.3. Procedure of 2 DE 38
2.2.4. Visualisation of protein spots 39
2.2.4.1. Silver staining 39
2.2.4.2. Coomassie blue staining 40
2.2.4.3. Post-staining treatment of fels 40
2.3. Separation of rat skeletal muscle proteins using analytical 2 DE 40
2.4. Attempts made for the optimisation of the analytical 2 DE 44
2.4.1. Removal of the background plaque 44
2.4.2. Effects made for better protein focusing 44
2.5. Preparative 2 DE of EDL proteins and its technical optimisation 46
2.6. Discussion 52
2.6.1. Separation of skeletal muscle proteins using 2 DE 52
2.6.2. Determination of the electrophoresis conditions for the project 52
2.6.2.1. Analytical 2 DE 52
2.6.2.2. Preparative 2 DE 53

Chapter 3: Computer-assisted image analysis of 2D gels

3.1. Introduction 54
3.2. Image analysis using the ImageMaster 1.1: The basics 54
3.3. Analysis of gel and film Images using ImageMaster 1.1 56
3.3.1. Spot detection 55
3.3.2. Image matching 59
3.4. Strategy of 2D image matching for the project 59
Chapter 4: Analysis of EDL stimulated *in vitro* with amylin using analytical 2 DE and computer-assisted image analysis: The discovery of two amylin responsive proteins

4.1. Introduction 61
4.2. Experimental procedure 62
4.2.1. Animals 62
4.2.2. Muscle dissection and incubation 62
4.2.3. Metabolic labelling of skeletal muscle using $[^{35}\text{S}]-\text{methionine}$ 63
4.2.4. Muscle sample homogenisation and protein quantification 63
4.2.5. Analytical 2 DE 63
4.2.6. Molecular weight calibration 64
4.2.7. Image analysis 64
4.3 Results 64
4.3.1. Distribution of EDL proteins on 2D gel: An overview 65
4.3.1.1. EDL proteins on analytical 2D gels 65
4.3.1.2. Reproducibility of the 2 DE analysis of EDL sample 65
4.3.2. Identification of two EDL proteins response to amylin stimulation 65
4.3.2.1. Identification of a protein produced in response to amylin stimulation 66
4.3.2.2. Identification of a protein quantitatively increased in response to amylin stimulation 66
4.3.2.3. Results of the metabolic labelling of ARP1 and ARP2 with $[^{35}\text{S}]-\text{methionine}$ 69
4.4 Discussion 71
4.4.1. The molecular mechanism of ARP1 production: Evidence that ARP1 is produced through amylin-evoked protein post-translational modification 71
4.4.2. Amylin-stimulated protein synthesis: The molecular mechanism of the increase in ARP2 concentration 71
4.4.3. The power and limits of the current 2 DE technology 74
Chapter 5: Microcharacterisation of the amylin responsive proteins: Identification of ARP1 as a novel monomeric form of protein p20

5.1. Introduction 75
5.2. Materials and methods 77
5.2.1. Chemicals, enzyme and solutions 77
5.2.1.1. Chemicals and enzyme 77
5.2.1.2. Solutions used in the in-gel digestion 78
5.2.2. In-gel digestion and organic solvent extraction 77
5.2.3. Reversed phase HPLC 78
5.2.4. Protein microsequencing 79
5.2.5. Protein sequence analysis 79
5.3. Results 79
5.3.1. Microcharacterisation of a high concentration EDL protein (spot D, Fig. 4.1): Testing the technical procedure 79
5.3.2. Microcharacterisation of the amylin responsive proteins 81
5.3.2.1. Identification of ARP1 as protein p20 81
5.3.2.2. Approaches used to characterise ARP2 85
5.4. Discussion 85
5.4.1. ARP1 is a monomeric form of protein p20 85
5.4.2. ARP1 is a novel monomeric form (designated form 1) of protein p20 86
5.4.3. Evidence that ARP1 is produced through phosphorylation in response to amylin stimulation 87
5.4.4. Molecular characterisation of ARP2: The technical considerations 88
5.4.5. Protein microsequence analysis: My unique experience 90
5.4.6. Trypsin autodigestion during the in-gel digestion 90
Chapter 6: Insulin-independent production of ARP1: Relevance to the development of insulin resistance

6.1. Introduction 92
6.2. Experimental procedure 93
6.2.1. Sample preparation and analytical 2 DE-image analysis 93
6.2.2. Mild heat shock treatment 93
6.2.3. Metabolic labelling with $^{14}$C-D-glucose 94
6.2.4. Glycogen purification and $^{14}$C counting 94
6.3. Results and discussion 95
6.3.1. Amylin elicits insulin resistance in EDL 95
6.3.2. ARP1 is produced in skeletal muscles in response to CGRP stimulation 95
6.3.3. Insulin dose not have an effect on ARP1 production in response to amylin or CGRP stimulation 98

Chapter 7: Towards the proteome analysis of rat skeletal muscle EDL: Identification of 18 EDL proteins

7.1. Introduction 102
7.2. Experimental protocols 103
7.2.1. Separation of EDL proteins using preparative 2 DE 103
7.2.2. Protein microcharacterisation using sequence analysis 103
7.3. Results and discussion 104

Chapter 8: Discussion

8.1. A summary of the results 111
8.2. ARP1 111
8.2.1. A mini review on protein p20 and homologous HSP27/28 111
8.2.2. Protein phosphorylation: The mechanism of ARP1 production 113
8.2.2.1. ARP1 is produced through post-translational modification(s) 113
8.2.2.2. Phosphorylation is involved in amylin-elicited ARP1 production 113
8.2.3. The search for ARPI's precursor

8.2.4. ARPI can possibly be used as a molecular marker in the analysis of amylin action and the role of amylin in the development of the common form of insulin resistance

8.3. ARP2

8.4. Possible directions for future work
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 2.1</td>
<td>Separation of EDL proteins using analytical 2 DE</td>
</tr>
<tr>
<td>Fig. 2.2</td>
<td>Separation of soleus muscle proteins using analytical 2 DE</td>
</tr>
<tr>
<td>Fig. 2.3</td>
<td>Separation of rat liver proteins using analytical 2 DE</td>
</tr>
<tr>
<td>Fig. 2.4</td>
<td>Gel image after the plaque removal</td>
</tr>
<tr>
<td>Fig. 2.5</td>
<td>Separation of EDL proteins using preparative 2 DE</td>
</tr>
<tr>
<td>Fig. 2.6</td>
<td>Separation of EDL proteins using preparative 2 DE at a high sample loading using loading cup method (part of a whole image)</td>
</tr>
<tr>
<td>Fig. 2.7</td>
<td>The construction of a rehydration chamber for rehydration loading of protein samples</td>
</tr>
<tr>
<td>Fig. 2.8</td>
<td>Separation of EDL proteins using preparative 2 DE at a high level of sample loading using rehydration loading</td>
</tr>
<tr>
<td>Fig. 3.1</td>
<td>Use of the image analysis software package ImageMaster 1.1 in the detection of protein spots</td>
</tr>
<tr>
<td>Fig. 3.2</td>
<td>False positive and negative gel spot detection by ImageMaster 1.1</td>
</tr>
<tr>
<td>Fig. 3.3</td>
<td>Significant false negative detection of film spots by ImageMaster 1.1.</td>
</tr>
<tr>
<td>Fig. 3.4</td>
<td>Strategy for muscle sample preparation and subsequent 2D gel image matching</td>
</tr>
<tr>
<td>Fig. 4.1</td>
<td>Distribution of EDL proteins on the analytical gels</td>
</tr>
<tr>
<td>Fig. 4.2</td>
<td>Identification of a protein spot consistently and exclusively present in the EDL muscles stimulated <em>in vitro</em> with amylin (part of a whole image)</td>
</tr>
<tr>
<td>Fig. 4.3</td>
<td>Identification of a protein spot quantitatively increased in</td>
</tr>
</tbody>
</table>
the amylin-stimulated EDL muscles (part of a whole image)

Fig. 4.4. Autoradiography of the 2D gels of EDL muscles metabolically labelled with $[^{35}S]$-methionine

Fig. 4.5. Identification of a radioactive signal spot present only on the films of the experimental 2D gels

Fig. 5.1. Modern protein microcharacterisation techniques

Fig. 5.2. Separation of tryptic fragments of a trial protein (spot D, Fig. 4.1) by reversed phase HPLC

Fig. 5.3. Separation of tryptic fragments of the proteins separated in spots 1, 3 and 4

Fig. 5.4. Detection of a radioactive signal spot corresponding to ARP1 from EDL muscles metabolically labelled with $^{32}$Pi

Fig. 5.5. Separation of trypsin autodigests by reversed phase HPLC

Fig. 6.1. Glycogen synthesis in EDL muscles with different experimental treatments

Fig. 6.2. Partial gel image of some muscle strips prepared under different experimental conditions

Fig. 7.1. Separation of EDL proteins on preparative 2D gels for proteome analysis

Fig. 7.2. Plotting the theoretical pI values against the gel location of 11 relatively well characterised proteins
<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1. Some physiological and pathophysiological conditions associated with insulin resistance</td>
<td>16</td>
</tr>
<tr>
<td>Table 1.2. Syndrome X</td>
<td>17</td>
</tr>
<tr>
<td>Table 1.3. Some inhibitory factors of insulin receptor</td>
<td>21</td>
</tr>
<tr>
<td>Table 1.4. Potential biological functions of amylin</td>
<td>26</td>
</tr>
<tr>
<td>Table 2.1. Running conditions for the first dimensional separation</td>
<td>38</td>
</tr>
<tr>
<td>Table 2.2. Running conditions for the second dimensional separation</td>
<td>39</td>
</tr>
<tr>
<td>Table 2.3. Isoelectric focusing of EDL proteins with extended eime</td>
<td>46</td>
</tr>
<tr>
<td>Table 5.1. Microsequence analysis of the proteins separated in spots 1 (ARP1), 3 and 4 (Fig. 4.1 and 4.2)</td>
<td>83</td>
</tr>
<tr>
<td>Table 5.2. Analysis of sequence obtained from tryptic fraction 9 of ARP1</td>
<td>84</td>
</tr>
<tr>
<td>Table 6.1. Detection of ARP1 in EDL muscle with different experimental treatments</td>
<td>99</td>
</tr>
<tr>
<td>Table 7.1. Sequence analysis of 18 EDL proteins</td>
<td>106</td>
</tr>
<tr>
<td>Table 7.2. The theoretical pI value and gel location of 11 relatively well characterised proteins</td>
<td>109</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1. Insulin resistance

1.1.1. The history and the current concept

1.1.1.1. History

The emergence and development of the concept, insulin resistance, has long been closely intertwined with the discovery of insulin, and the research on diabetes and insulin action. More than a century ago, von Mehring and Minkowski observed that total pancreatectomy in experimental animals led to the development of severe diabetes mellitus, and initiated the speculation that a mysterious substance produced by the pancreas is responsible for metabolic control (von Mering and Minkowski, 1890). This substance was subsequently named “insulin”. Although some pioneering researchers were able to obtain preliminary evidence of the functional activity of insulin at the beginning of this century, it was not until 1922-3 when Banting and Best successfully showed the efficacy of pancreatic extracts in the treatment of human diabetes, that insulin’s existence was established (reviewed by Bliss, 1982; 1993). A decade later, in an era when clinicians were beginning to differentiate the ketosis-prone and -resistant types of diabetes, Himsworth (Himsworth, 1936; 1939) discussed the division of diabetic patients into two groups, the insulin-sensitive and insulin-insensitive. He further suggested that insulin-sensitive diabetic patients were ketosis-prone and of early-onset, while insulin-insensitive individuals were ketosis-resistant and of late-onset. Furthermore, most diabetic patients were of insulin-insensitive type. Thus, the concept of insulin resistance, described as insulin-insensitivity, emerged. The two types of diabetes described by Himsworth are now defined as type 1 (Insulin-dependent diabetes mellitus, IDDM) and type 2 (Non insulin-dependent diabetes mellitus, NIDDM) diabetes (National Diabetes Data Group, 1979).

Significant advances in understanding in vivo and in vitro insulin action and insulin resistance were made in the 1970s and 1980s. It was at that time that researchers firmly established the view that individuals with NIDDM and some other diseases
were resistant to insulin action, and started investigating the molecular mechanism of insulin action and the defects responsible for the development of insulin resistance (Flier, 1993).

In recent years, clinical, biochemical and genetic characterisations using modern technology have yielded a significant amount of new knowledge, however, the molecular mechanism(s) and the functional roles of insulin resistance have so far remained poorly understood.

1.1.1.2. The current concept of insulin resistance

What is insulin resistance? As stated originally by Berson and Yalow in 1970 (Berson and Yalow, 1970), ‘insulin resistance may be defined as a state (of cell, tissue, system or body) in which greater-than-normal amounts of insulin are required to elicit a quantitatively normal response’. It was often described as a diminished biological effect of insulin on glucose metabolism, as the control of glucose homeostasis is one of the major biological activities of insulin.

1.1.2. Clinical and economic significance of insulin resistance

1.1.2.1. Insulin resistance in disease

Insulin resistance is a reported feature of a number of physiological and pathophysiological states in man (Table 1.1). Awareness of the potential importance of insulin resistance was heightened by Reaven’s hypothesis (Reaven, 1988) that insulin resistance represents a fundamental link among a number of common chronic non-communicable diseases in syndrome X (Table 1.2). Resistance to insulin action is likely to be pathogenic in some major diseases. In NIDDM, for example, resistant to insulin action is believed to precede β-cell dysfunction which is another critical defect in the disease. Maintenance of glycemic control is only achieved through β-cell hyperfunction in compensating for the reduced peripheral insulin sensitivity. Overt NIDDM ol develops when β cells are no longer able to compensate for insulin resistance (Reaven, 1988; DeFronzo, 1987).
### Table 1.1 Some physiological and pathophysiological conditions associated with insulin resistance

<table>
<thead>
<tr>
<th>A: Physiological Condition</th>
</tr>
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<tbody>
<tr>
<td>Female sex</td>
</tr>
<tr>
<td>Puberty and aging</td>
</tr>
<tr>
<td>Pregnancy</td>
</tr>
<tr>
<td>Physical inactivity and/or immobility</td>
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</tbody>
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<table>
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<tr>
<th>B: Pathophysiological Condition</th>
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<tr>
<td>a. <em>Common or relative common</em></td>
</tr>
<tr>
<td>Obesity (especially of central/abdominal distribution)</td>
</tr>
<tr>
<td>Impaired glucose tolerance</td>
</tr>
<tr>
<td>NIDDM</td>
</tr>
<tr>
<td>Essential hypertension</td>
</tr>
<tr>
<td>Cancer</td>
</tr>
<tr>
<td>Stress</td>
</tr>
<tr>
<td>Polycystic ovary/hyperandrogenism syndrome</td>
</tr>
<tr>
<td>Uraemia</td>
</tr>
<tr>
<td>Cirrhosis (and other hepatic disorders)</td>
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</table>

| b. *Uncommon or rare*                                           |
| Extreme insulin resistance syndromes (type A, B, and others)    |
| Cushing’s syndrome                                              |
| Acromegaly                                                      |
| Myotonic dystrophy                                              |
| Werner’s syndrome                                               |
| Turner’s syndrome                                               |
| Duchenne muscular dystrophy                                     |
Table 1.2 Syndrome X (adapted from Reaven, 1988)

<table>
<thead>
<tr>
<th>Resistance to insulin-stimulated glucose uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose intolerance</td>
</tr>
<tr>
<td>NIDDM</td>
</tr>
<tr>
<td>Hyperinsulinaemia</td>
</tr>
<tr>
<td>Increased plasma VLDL triglyceride</td>
</tr>
<tr>
<td>Decreased plasma HDL cholesterol</td>
</tr>
<tr>
<td>Hypertension</td>
</tr>
<tr>
<td>Hyperuricaemia</td>
</tr>
<tr>
<td>Increased plasma plasminogen activator inhibitor 1</td>
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</table>

1.1.2.2. Economic impact of insulin resistance

The overall prevalence in the general population is about 7% for type 2 diabetes, 30% for obesity and 20% for essential hypertension (DeFronzo, 1992), all of which have been associated with insulin resistance (section 1.1.2.1). In the US, earlier estimates indicated that there were about 14 million diabetes patients, and this number was growing (Kovar, 1987). About US$ 9.6 billion was spent on direct diabetes care, and this cost was expected to increase to more than US$20 billion in the following years. Furthermore, the ‘hidden burden’ for treating the chronic complications of diabetes was though to be immense. The total number of NIDDM patients world-wide is estimated to be around 110 million, which was predicted to increase to 216 million by the year 2010 (Zimmet and MacCarty, 1995).

Insulin resistance is also found among 25% phenotypically “normal” (non-diabetic, non-obese) subjects in the general population (Hollenbeck and Reaven, 1987). However, the clinical implication of the condition in ‘healthy’ individuals is unknown.

1.1.3. Skeletal muscle is the major site of insulin resistance

Insulin controls glucose homeostasis through three co-ordinated mechanisms: suppression of hepatic glucose output, stimulation of glucose uptake by splanchnic tissue (hepatic and gastrointestinal), and stimulation of glucose uptake by peripheral
tissues. It has been long known that in insulin resistant states, the three major actions of insulin are all impaired. However, skeletal muscle is identified as the major site of insulin resistance (Sinacore and Gulve, 1993; DeFronzo, 1987; Kraegen, 1990).

In NIDDM patients in the post-absorptive state, there is a small but significant increase in hepatic glucose output. Although the increase may seem small, it is significant to calculate that over 24 hours, the liver of a 70 kg diabetic individual will add an additional 50 grams of glucose to the circulation. The increase in basal hepatic glucose output is closely correlated with the degree of fasting hyperglycaemia. These data suggest that an excessive rate of hepatic glucose output is a major factor responsible for the elevated fasting plasma glucose concentration in NIDDM patients. However, in response to an increment in plasma insulin concentration (100 μU/ml) above baseline, there was more than 90% suppression of hepatic glucose output in diabetic individuals, and this result was essentially identical to that observed in normal subjects. Similarly, experimental data also show that in NIDDM, extra-hepatic splanchnic tissues are sensitive to the effect by which insulin augments glucose uptake. Neither a defect in suppression of hepatic glucose output nor a defect in hepatic glucose uptake can account for the observed impairment in whole body insulin sensitivity during clamp studies (DeFronzo, 1987).

In contrast, in skeletal muscle, which comprises 40-50% of total body mass and is responsible for nearly 90% of insulin stimulated glucose uptake in peripheral tissues, the insulin stimulated glucose uptake was seen to be delayed and blunted in NIDDM patients. By careful analysis, it was found that more than 90% of the impairment in total body glucose uptake in NIDDM patients could be accounted for by insulin resistance in skeletal muscle. Thus, skeletal muscle is the primary site of insulin resistance in NIDDM. Under other pathological conditions, such as hypertension and stress (Julius, 1992; Mizock, 1995), skeletal muscle has also been identified as the primary site of insulin resistance.
1.1.4. Non-oxidative glucose metabolism is the major metabolic pathway involved in the development of insulin resistance

Glucose uptake in skeletal muscle depends on two major metabolic pathways: glucose oxidation and glucose storage. Experimental results showed that muscle glucose oxidation and energy expenditure do not appear to be stimulated significantly during hyperinsulinaemia at euglycaemia. A major portion of the insulin-stimulated glucose metabolism in skeletal muscle is due to non-oxidative processes, which comprise lipogenesis, non-oxidative glycolysis (to lactate and alanine) and glycogen synthesis. Using $^{14}\text{C}$ nuclear magnetic resonance (NMR) spectroscopy, glycogen synthesis has been proven to account for all non-oxidative glucose metabolism stimulated by insulin in the clamp method. In NIDDM, reduced glycogen synthesis in skeletal muscle is the major factor responsible for impaired glucose metabolism (Natali, 1990; Kelley, 1990; Shulman, 1990; Shulman, 1996). It should be noted that abnormal glucose transport may be another key indication of abnormalities contributing to the impairment of glucose uptake in the insulin resistant state (Rothman, 1995). But there is no consensus on this view, although defects in glucose transporters may indeed significantly contribute to pathogenesis of insulin resistance in hypertension (Livingstone, 1995).

1.1.5. Molecular mechanisms of insulin resistance

The molecular mechanisms of insulin resistance are poorly understood. In theory, functional deviation at any step in the insulin signal transduction-action cascade can potentially lead to the suppression of insulin's biological activity and the resulting development of insulin resistance. In practice, a big part of the research attempting to characterise the molecular defect(s) responsible for the pathogenesis of insulin resistance has focused on the insulin receptor.

1.1.5.1. Insulin resistance as the consequence of insulin receptor abnormalities

Various types of functional suppression of the insulin receptor's biological activity have been documented in insulin resistant states, The presence of insulin receptor inhibitors can also account for the development of
insulin resistance. In the syndrome of type B extreme insulin resistance, auto-anti-insulin receptor antibodies act as inhibitors of the insulin receptor (Gorden, 1994). The autoantibodies are usually multi-clonal, and show insulin-like biological activities. Although the insulin receptors in these patients seem to be intrinsically functionally normal, the binding of the auto-antibodies to the insulin receptors induces receptor desensitisation, which is believed to cause the severe insulin resistance seen in these patients. Besides the auto-anti-insulin receptor antibodies, a number of other molecules have also been shown to have an inhibitory effect on the insulin receptor (Table 1.3).

Mutations of the insulin receptor gene can also cause insulin resistance. Numerous insulin receptor mutants have been found. These have been categorised into five classes (Taylor and Moller, 1994; Accili, 1995):

Class I. Mutations that decrease the rate of receptor biosynthesis
Class II. Mutations that impair intracellular transport and post-translational processing
Class III. Mutations that impair the receptor’s insulin binding capacity
Class IV. Mutations that impair receptor tyrosine kinase activity
Class V. Mutations that accelerate receptor degradation

Mutations in the insulin receptor gene are estimated to occur within a frequency that is equal to or greater than 0.1% of the general population. Many patients with mutation(s) in the insulin receptor gene, particularly those with two mutant alleles, are extremely insulin resistant.

There are other proposed mechanisms of functional suppression of the insulin receptor in insulin resistance. These include changes in receptor microenvironment (Nadiv, 1994), changes in receptor isoforms (Haring, 1994), formation of hybrid receptors (Frattali, 1992), and abnormal insulin receptor mRNA metabolism (Nowak, 1995).
1.1.5.2. Insulin resistance as the consequence of post-insulin receptor abnormalities

Available data suggest that defects distal to the insulin receptor may also be present (Bak, 1994; Saad, 1994; Bonini, 1995). Recently, protein p55, sphingomyelinase and an inhibitory form of insulin receptor substrate-1 (IRS-1) have been shown to be involved in the development of tumour necrosis factor-α (TNF-α)-elicited insulin resistance (Peraldi, 1996). Functional association of proteins p85, GRB2, NCK and Syp with the development of insulin resistance has also been observed in KKAy mice (Bonini, 1995). Furthermore, in a theory proposed for the involvement of

<table>
<thead>
<tr>
<th>Factor</th>
<th>Effect on the Insulin Receptor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Extracellular</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zinc deficiency</td>
<td>Decrease in binding</td>
<td>Gomot, et al., 1992</td>
</tr>
<tr>
<td>α2-HS-glycoprotein</td>
<td>Inhibition of Kinase Activity</td>
<td>Serinivas, et al., 1993</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>Decrease in phosphorylation</td>
<td>Giorgino, et al., 1993</td>
</tr>
<tr>
<td>TNF</td>
<td>Decreased autophosphorylation</td>
<td>Hotamisligi, et al., 1994</td>
</tr>
<tr>
<td>Amylin</td>
<td>Inhibition of kinase activity</td>
<td>Bryer-Ash, et al, 1995</td>
</tr>
<tr>
<td>Glucose</td>
<td>Desensitisation</td>
<td>Ide, et al., 1995</td>
</tr>
<tr>
<td>Insulin</td>
<td>Inhibition of receptor activity</td>
<td>Kavety, et al., 1994</td>
</tr>
<tr>
<td>2. Intracellular</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosolic β fragment</td>
<td>Autophosphorylation inhibition</td>
<td>Knutson, et al., 1995</td>
</tr>
<tr>
<td>Protein tyrosine phosphatases</td>
<td>Suppression</td>
<td>Li, et al., 1996</td>
</tr>
<tr>
<td>PC-1</td>
<td>Kinase inhibition</td>
<td>Maddux, et al., 1995</td>
</tr>
</tbody>
</table>

cellular signal transduction in disease pathogenesis, known as “the up-regulated signalling model”, McCarty (McCarty, 1996) hypothesised that insulin resistance may be the consequence of increased protein kinase C-mediated signalling.

Targets of insulin action have also undergone intensive scrutiny for possible
pathogenic involvement in the development of insulin resistance. Under most conditions, glucose transportation is the rate-limiting step in glucose uptake and metabolism. Abnormal (e.g. decreased) glucose transport has been observed both in diabetic animal models and in NIDDM patients (Rothman, 1995; Foley, 1991). Protein concentrations of the GLUT4 isoform, which is predominantly expressed in skeletal muscle, decreased with the development of insulin resistance in man (Houmard, 1995). Moreover, changes in or related to glycogen synthase bioactivity are also believed to contribute to the development of insulin resistance (Bak and Federsen, 1994). Decreases in muscle glycogen content (Shulmam, 1990), insulin-stimulated glycogen synthesis (Young, 1988), glycogen synthase mRNA levels (Vestergaard, 1991), basal glycogen synthase activity (Thorburn, 1990), and insulin-stimulated glycogen synthase activity (Thorburn, 1990) were observed in insulin resistant states. Treatment of insulin resistance partially reverses impaired insulin-mediated activation of glycogen synthase (Bak, 1989).

1.1.5.3. Changes in glucose transport and intro-cellular metabolism in insulin resistance

Glucose transport is a rate-limiting step for glucose utilisation in skeletal muscle over the physiological range of glucose and insulin levels (Yki-Jarvinen, 1987; Katz, 1988). Insulin-stimulated glucose uptake by muscle is crucial for the maintenance of glucose homeostasis, in which the muscle glucose transporter isoform, GLUT4, plays an important role (Hirshman, 1990). Resistance to the stimulatory effect of insulin on glucose uptake into skeletal muscle in vivo is a universal feature of insulin-resistant animal models and human states (Peters, 1993). This may be due to the decreased plasma membrane GLUT4 concentrations as well as protein synthesis (Garvey, 1988; 1991).

Intracellular metabolism also shows significant alterations in insulin resistant states. Firstly, glucose oxidation is decreased (Butler, 1990), which may be associated with an impairment in insulin’s ability to decrease pyruvate dehydrogenase activity in skeletal muscle (Kelley, 1992). Non-oxidative metabolism of glucose in skeletal muscle is also decreased (section 1.1.4). This accounts for the major decrease in
association with glucose uptake in insulin resistance, although both decreased glucose oxidation and storage contribute to the decrease in glucose disposal (Kelley, 1992).

1.1.5.4. The glucose-fatty acid cycle

Randle cycle proposed an interaction of glucose and fatty acid metabolism (Randle, 1963). It was subsequently demonstrated that the metabolism of free fatty acids could influence glucose metabolism in various ways (Ruderman, 1969): 1. a decrease in nicotinamide; 2. an increase in NADH; 3. an increase in acetyl CoA; 4. an increase in cytosolic nitrate. Both in vitro and in vivo studies have produced evidence that elevated circulating free fatty acids could cause decrease in glucose disposal (Boden, 1991; Groop, 1989). However, conflicting results have also been obtained (Capaldo, 1991). Furthermore, caution should be taken in interpreting data about the relationship of free fatty acid metabolism, as the current techniques used for measurement of intracellular lipogenesis are not all that reliable (Alzaid, 1993).

1.1.5.5. Genetic influence

The development of insulin resistance seems to be determined by the interplay of genetic and environmental factors. There is consensus that insulin resistance is under strong genetic influence (Neel, 1962; 1982; Moller, 1996; Mitchell, 1995). However, a large number of candidate genes have been tested without any conclusive positive associations being found (Yagi, 1996; Elbein, 1995). Genome-wide scanning for localising the so called 'diabetogenes' which are logical candidate genes for insulin resistance has not so far produced conclusive results. However, a marker (D2S125) has been found to be linked with type 2 diabetes, a recognised insulin resistant state (Hanis, 1996). Thus, the genetic determinants for the development of insulin resistance remain to be identified.

1.1.6. The common form of insulin resistance

Insulin resistance can be quantitatively measured. In some cases, it is severe, while in others it is moderate. At the molecular level, different mechanisms have been
proposed for the pathogenesis of this condition (section 1.1.4). It seems that insulin resistance is a pathophysiological condition with heterogeneous expression and mechanisms. Although classification of this condition has not yet been performed, the insulin resistance characteristically observed in common diseases such as NIDDM and obesity is termed the common form, in order to differentiate it from others such as the rare forms of extreme insulin resistance.

Search for molecular primary molecular defect(s) responsible for the development of the common form insulin resistance has to date proven to be fruitless. None of the molecular changes described in the above section (section 1.1.5), at either gene or protein level, explains the reduced insulin sensitivity found in the common form insulin resistance. In contrast, environmental factors affecting insulin sensitivity seem to be relatively well documented. In addition to genetic control, environmental factors are believed to be important. These factors are often closely related to lifestyle. Diet and physical activity are two typical examples. In rats, high fat diets evoke skeletal muscle insulin resistance (Kraegen, 1986). Acute lipid withdrawal ameliorates high fat diet-induced muscle insulin resistance (Oakes, 1997). Both single bout and chronic exercise counteract the effect of high fat diets, resulting in improved insulin sensitivity in high fat fed rats (Kraegen, 1989; Oakes, 1997). In man, insulin sensitivity has also been found to be under the influence of high fat or high-energy diet (Stefikova, 1997; Bell, 1997; Kraegen, 1991) and physical activity (Lohmann, 1979; Bogardus, 1983).

It is believed that a number of hormones have regulatory effects on insulin sensitivity (Hettiarachchi, 1997a; Smith, 1997; Haffner, 1994; Fowelin, 1989). Induction of insulin resistance by human growth hormone is one of the typical examples (Hettiarachchi, 1997a). Evidence of the involvement of growth hormone in the development of insulin resistance has also been obtained (Fowelin, 1989; Chan, J., personal communication).

1.1.7. Insulin resistance: Summary

Insulin resistance, common or rare forms, exists when the normal level of insulin induces subnormal functional responses. As stated previously, it affects mainly non-
oxidative type of fuel metabolism in skeletal muscle. The common form insulin resistance is believed to be a critical component in the pathogenesis of syndrome X, but, the mechanisms of its pathogenesis remain unknown at this time.

1.2. Amylin

Amylin is a recently discovered peptide hormone that is predominantly secreted by pancreatic β cells (reviewed by Cooper, 1994).

1.2.1. Potential biological functions of amylin

Results of *in vivo* and *in vitro* studies indicate that amylin may be a hormone with multiple biological functions (Table 1.4). This is consistent with the observation that its binding sites are widely distributed (Johnson, 1988; Leffert, 1989; Ferrier, 1989; Wagner, 1995; Tamura, 1992; Bhogal, 1992; Kenney, 1993; Aiyer, 1995; Sexton, 1994; Skofitsch, 1995). For example, the hormone may be an important mechanistic link between hypertension and diabetes in Syndrome X, as it activates plasma renin (Cooper, 1995) and elicits pressor and hypertensive responses (Haynes, 1997).

However, an involvement in the regulation of fuel (carbohydrate) metabolism is so far believed to be the primary biological function of amylin. As reviewed recently by Young et al. (Young, 1995), amylin seems to regulate fuel metabolism at two levels, the control of nutrient uptake from food, and the subsequent nutrient storage and translocation.

Deposition of a nutrient load appears to be controlled by sensing mechanisms in both the gut and stomach. The presence of nutrients in the gut triggers the release of incretins. The nutrients in plasma, together with the incretins, stimulate the release of both insulin and amylin. While insulin promotes the disposal of nutrients, amylin feeds back to the stomach to inhibit further release of nutrients into gut.

Among the insulin sensitive tissues, liver and fat tissues are relatively insensitive to amylin. Skeletal muscle seems to be the major site of amylin action, where amylin inhibits glycogen synthesis, activates glycogenolysis and increases lactate production.
Lactate, as a vector of fuel translocation, is used as a substrate for liver glycogenesis and fat tissue lipogenesis.

**Table 1.4. Potential biological functions of amylin**

<table>
<thead>
<tr>
<th>Physiological Function</th>
<th>Amylin Action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Immune system</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammation (Eosinophils)</td>
<td>↑</td>
<td>Hom, et al., 1995</td>
</tr>
<tr>
<td>Inflammation (animal model)</td>
<td>↓</td>
<td>Clementi, et al., 1995</td>
</tr>
<tr>
<td><strong>2. Vertebal blood flow</strong></td>
<td>↑</td>
<td>Baskaya, et al., 1995</td>
</tr>
<tr>
<td><strong>3. Calcium and bone metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum calcium level</td>
<td>↓</td>
<td>Wimalawansa, et al., 1992</td>
</tr>
<tr>
<td>Bone resorption</td>
<td>↓</td>
<td>Pietschmann, et al., 1993</td>
</tr>
<tr>
<td>Bone formation</td>
<td>↑</td>
<td>Cornish, et al., 1995</td>
</tr>
<tr>
<td><strong>4. Gastrointestinal functions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastric emptying</td>
<td>↓</td>
<td>Clementi, et al., 1996b</td>
</tr>
<tr>
<td>Intestinal transit</td>
<td>↑</td>
<td>Clementi, et al., 1996b</td>
</tr>
<tr>
<td><strong>5. Endocrine system</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin production</td>
<td>↓</td>
<td>Degano, et al., 1993</td>
</tr>
<tr>
<td>Growth hormone release</td>
<td>↓</td>
<td>Netti, et al., 1995</td>
</tr>
<tr>
<td>Gastrin release</td>
<td>↑</td>
<td>Funakashi, et al., 1992</td>
</tr>
<tr>
<td>Islet β cell apoptosis</td>
<td>↑</td>
<td>Lorenzo, et al., 1994</td>
</tr>
<tr>
<td><strong>6. Behaviour</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Memory retention</td>
<td>↓</td>
<td>Morley, et al., 1995</td>
</tr>
<tr>
<td>Locomotor activity</td>
<td>↓</td>
<td>Clementi, et al., 1996a</td>
</tr>
<tr>
<td>Latency of passive avoidance</td>
<td>↑</td>
<td>Kovacs and Telegdy, 1996</td>
</tr>
<tr>
<td>Meal size (anorectic effect)</td>
<td>↓</td>
<td>Lutz, et al., 1995</td>
</tr>
<tr>
<td><strong>7. Cardiovascular system</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vasodilatation</td>
<td>↑</td>
<td>Muff, et al., 1995a</td>
</tr>
<tr>
<td>Arterial pressure</td>
<td>↓</td>
<td>Muff, et al., 1995a</td>
</tr>
<tr>
<td>Chronotropic action</td>
<td>↑</td>
<td>Muff, et al., 1995a</td>
</tr>
<tr>
<td>Cardiomyocyte contractility</td>
<td>↑</td>
<td>Bell and McDermott, 1995</td>
</tr>
</tbody>
</table>


8. Fuel metabolism

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle lactate release</td>
<td>Pittner, et al., 1995</td>
</tr>
<tr>
<td>Muscle glycogen synthesis</td>
<td>Leighton and Cooper, 1988</td>
</tr>
<tr>
<td>(\textit{In vivo}) glucose production</td>
<td>Molina, et al., 1990</td>
</tr>
<tr>
<td>Peripheral glucose disposal</td>
<td>Molina, et al., 1990</td>
</tr>
</tbody>
</table>

1.2.2. Molecular mechanisms of amylin action

Very limited amounts of data about the signal transduction and action of amylin have been obtained to date.

1.2.2.1. Amylin signal transduction

Complicated by the overlapping binding of amylin and homologous peptides (i.e. calcitonin gene-related peptide, CGRP), characterization of the amylin receptor has so far proven difficult and fruitless, although specific amylin receptors are believed to exist (Cooper, 1994; Huang, 1996; Muff, 1995a; 1995b; Beaumont, 1995; Aiyar, 1995). Experimental data, particularly those from studies on skeletal muscle (Pittner, 1995) and kidney (Wookey, 1996), show that amylin activates adenylate cyclase and increases cAMP concentration, indicating that G, proteins are involved in amylin signal transduction. Huang et al. (Huang, 1996) found that amylin elevated IP₃ levels in rat acinar AR42J cell line cells, and suggested that amylin signal transduction may go through a G-protein-linked receptor coupled to IP₃. Another report (Weiel, 1993) demonstrated that amylin activated cAMP-dependent protein kinase (PKA) in skeletal muscle, suggesting that PKA may also be involved in amylin signal transduction.

1.2.2.2. Targets of amylin action

The insulin receptor, glycogen synthase and phosphorylase are known to be targets of amylin action. It has been relatively well documented that amylin signalling inhibits insulin receptor activity (Bryer-Ash, 1995), inhibits glycogen synthase through phosphorylation (Leighton and Cooper, 1988; Lawrence and Zhang, 1994) and activates glycogen phosphorylase through dephosphorylation (Deems, 1991; Lawrence and Zhang, 1994). It is not clear whether glucose transporters are also
targets of amylin action, as the reported data are conflicting (Hoppener, 1994).

1.2.3. Is amylin implicated in the pathogenesis of the common form insulin resistance?

Soon after amylin was discovered, it was found to inhibit glucose incorporation into glycogen in skeletal muscle in vitro (Leighton and Cooper, 1988; 1990). Subsequently, it was further shown to promote the mobilisation of glycogen in skeletal muscle in vitro (Tabata, 1992), cause insulin resistance in perfused muscles (Hoppener, 1994), and decrease whole body insulin sensitivity when administrated in vivo (Frontoni, 1991; Sowa, 1990; Young, 1993b). Furthermore, elevated peripheral amylin levels (hyperamylnaemia) and increased peripheral amylin:insulin ratios were observed in animal models of diabetes (Pieber, 1994; Cooper, 1994) as well as in NIDDM patients (Permenter, 1994; Blackard, 1994), and an amylin antagonist was found to block the amylin-evoked impairment in glucose tolerance (Espinal, 1995). These results suggest that amylin acts as an antagonist of insulin action (Young, 1992), probably in a non-competitive manner (Young, 1992). At the molecular level, all the known actions of amylin (section 1.2.2.2) also favour the development of insulin resistance.

However, despite the existence of a strong link between amylin action and the occurrence of insulin resistance, its implication in the pathogenesis of the common forms of insulin resistance (section 1.1.6) is controversial. The opposing evidence (Nyhholm, 1996; Kolterman, 1996; Bretherton, 1992; Wilding, 1994; Rother, 1995; Erikson, 1992; Ludvik, 1994) includes: 1. amylin evokes insulin resistance at pharmacological concentrations, which are much higher than physiological levels of amylin; 2. patients with insulin-dependent diabetes are not necessarily hypersensitive to insulin, although they are considered to be deficient in amylin; 3. hyperamylinnaemia is not a consistent finding in the common forms of insulin resistant states; 4. endogenous hyperamylinnaemia. In patients with end stage of renal failure, hyperamylinnaemia occurs, possibly due to the reduced renal clearance of plasma amylin. Insulin action was found to remain unchanged in the patients with this type of hyperamylnaemia (Ludvik, 1994); and 5. in vivo administration of amylin in humans
does not necessarily impair insulin sensitivity.

1.2.4. Amylin and the common form of insulin resistance: understanding the current controversy and its potential resolution

It is unknown why the strong link between amylin action and the development of insulin resistance can’t be consistently demonstrated in man. Difference in biological function(s) of amylin seems to exist between rodents and humans (Cooper and Tse, 1996). Furthermore, the lack of data about amylin structure may also hinder the understanding of the hormone’s function. Although human amylin is a small peptide, its solutions show a high degree of structural heterogeneity (Shiomi, 1992; Miyazato, 1992; Beeley and Prickett, 1996; Sacks, 1996; Goldsbury, personal communication). This necessitates the consideration of specific aspects of amylin structure in the analysis of its biological function.

A clear view is that technical problems are believed to contribute significantly to the controversy. The rat amylin which was used in some infusion experiments was found to contain one mercury atom per amylin molecule (Edmonds, 1990). Although the biological activity of the mercury containing- and free-amylin sample has not been directly compared, it is logical to suspect that the mercury atom, which was incorporated into amylin during chemical synthesis, may interfere with amylin action. In addition, amylin has a tendency to stick to glass surfaces. Unless it is monitored properly, the true concentration of amylin may be different from what is actually present in a solution (Cooper, personal communication).

More importantly, current amylin assays may not be sufficiently well developed to provide accurate data, as they differ significantly in sensitivity (Pieber, 1994). This has been further stressed recently by the finding that a significant association between elevated plasma amylin concentrations and glucose intolerance may or may not be detected, depending on the different assays used for plasma amylin quantification (Percy, 1996).

Thus, a conclusion that amylin does not play a role in the pathogenesis of the common form of insulin resistance may not be necessarily follow from the previous studies.
1.2.5. Amylin: Summary

There is a moderate consensus that amylin plays a role in the regulation of fuel metabolism and the action of amylin favours the development of insulin resistance. However, its implication in the pathogenesis of the common form insulin resistance remains controversial. It was believed that examination of the amylin signalling-action cascade in the common form insulin resistance might shed light on the controversy by detecting one or more components of the amylin signal transduction pathway that are specific for amylin action.

1.3. The project

1.3.1. The objectives

The primary aim of this project was to identify, purify and characterise new proteins involved in amylin signal transduction in rat skeletal muscle. The protein(s) specifically responsive to amylin are likely to be useful molecular probes or markers for future studies on amylin action.

There are two main factors that need to be considered when analysing the possible involvement of a hormone in the pathogenesis of a disease. There are changes in the hormone’s concentration and changes in the responsiveness of target tissue(s) to stimulation by the hormone. The fact that physiological studies produce conflicting evidence as to whether amylin plays a role in the development of insulin resistance (sections 1.2.3 and 1.2.4) makes the analysis of the molecular mechanisms of muscle response to amylin attractive to researchers. Through this direction of research, it was hoped that evidence might be obtained to address the following important questions: (1). is amylin implicated in the development of insulin resistance, (2). does amylin-elicted insulin resistance occur as a consequence of abnormal responsiveness of skeletal muscle?

There are several currently known amylin responsive proteins, which are either involved in signalling (such as Gs proteins, section 1.2.2.1) or serve as targets for the hormone’s action (such as glycogen synthase, section 1.2.2.2). However, these are
believed to be a small part of the amylin signal transduction pathway. Recent technical developments in the method of two dimensional gel electrophoresis, coupled with computerised gel image data analysis have occurred (section 2.1, Chapter 2; Chapter 3); these allow thousands of proteins from a sample tissue to be separated and the proteins of interest localised. At least two advantages of using this method are apparent: (1) the scope of study can be expended enormously compared to analyse limited to only a few known proteins; (2) protein associated with either amylin action and/or insulin resistance may be found, whether or not there is previous evidence for a functional association.

1.3.2. The overall experimental design

Rat skeletal muscle was chosen as the tissue for study, as this tissue is the major sit of insulin resistance (reviewed in section 1.1.3). In particular, the skeletal muscle extensor digitorum longus (EDL) was selected, as in it non-oxidative carbohydrate metabolism, a major metabolic pathway for the development of insulin resistance (section 1.1.4), is dominant. In vitro muscle strip incubation (Cooper, 1994) was employed for preparing the experimental samples. Two dimensional gel electrophoresis (2 DE, Chapter 2) and computer-assisted gel data analysis (Chapter 3) were utilised for locating differences occur due to experimental treatments (e.g. amylin stimulation). Proteins of interest were then isolated and characterised using an integrated protein microcharacterisation procedure (Chapter 5).

A simplified experimental procedure was designed to search for amylin responsive proteins following the establishment and optimisation of 2 DE and computer-assisted two dimensional (2D) gel data interpretation (section 3.4, Chapter 3).
Chapter 2: Separation of rat skeletal muscle proteins by two dimensional gel electrophoresis and the technical optimisation

2.1. Two dimensional gel electrophoresis

Two dimensional gel electrophoresis (2 DE) is usually performed according to the procedures described by O'Farrell (O'Farrell, 1975) and Klose (Klose, 1975). The technique uses two sequential gel electrophoresis steps: isoelectric focusing as the first dimension and SDS polyacrylamide gel as the second. The separation parameter of the first dimensional run, the pI, is independent of the molecular weight, which is the separation parameter of the second dimension.

With this technique, it is possible to separate large numbers of cellular proteins with a high level of sensitivity. The amounts of the most abundant proteins in a 2 DE separation are at very low picomole to femtomole levels, corresponding to amount ranging from a few micrograms to hundreds of nanogrmes. Most of the practical problems in 2 DE analysis originate in the first dimensional separation. The recent introduction of the wide-range immobilised pH gradients (IPGs) as the first dimensional gel has greatly improved the technique, as IPGs ensure high reproducibility, focus proteins over a wide pH range, and allow direct comparisons between two or more two dimensional (2D) gel images (Gianazza, 1986; Gorg, 1988; Bjellqvist, 1993).

Widespread interest in the technique (Tsoni, 1995, Guy, 1994, Okazaki, 1995) has led to the establishment of a number of specialised academic and/or commercial organisations, such as the World-wide 2 DE Federation (Appel, 1996). In addition to the increasing amounts of information being published in the scientific literature, there has been a rapid increase in 2 DE databases and services on the Internet. For example, the World 2 DE PAGE, which is organised by the World 2 DE Federation (http://expasy.hcuge.ch/ch2d/2d-index.html), contains dozens of 2 DE databases, which cover a wide range of biological samples including sub-cellular organelles.
(mitochondria), prokaryotic cells (bacteria), eukaryotic cells (yeast, fibroblast cells, etc.), and animal and human tissues (liver, heart, plasma, etc.). Services on the Internet, such as computer-assisted image matching, systematic technical guidance and protein identification analysis are also provided by a number of institutions.

The technique can be used for either analytical or preparative purposes, depending on the investigator's interests. Analytical 2 DE is often used to identify changes in cellular protein composition that occur under different experimental conditions. In practice, each first dimensional gel is loaded with a limited amount of protein (e.g. 100 μg per gel), and a silver staining method is utilised for visualising the protein spots previously separated on the second dimensional gels. Preparative 2 DE is performed in order to obtain a sufficient amount of a protein of interest to enable further molecular characterization. For this purpose, a large amount of protein (e.g. 1 mg per gel) is loaded, and Coomassie blue staining is widely used for localising the separated proteins, since this stain is compatible with protein sequencing by Edman chemical degradation.

In this chapter, I describe my experience in establishing and optimising 2 DE, particularly for the analysis of rat skeletal muscle proteins. Although the technique has now been performing well for a variety of biological samples in our laboratory, enormous effort was invested in its establishment and optimisation during the initial stage of this project.

2.2. Materials and methods

2.2.1. Chemicals, solutions, and equipment

2.2.1.1. Chemicals and equipment

Chemicals used for general purposes were purchased from Sigma (St. Louis, MO, USA). The equipment and consumable items for 2 DE were purchased from Pharmacia-Biotech (Uppsala, Sweden). The major hardware parts included the electrophoresis tank (Multiphor III), cooler (Multitemp III) and power pack (ESP 3500 XL). The major consumable items included the first and second dimensional
gels and buffer strips. Immobiline DryStrip (non-linear, pH3-10) and SDS PAGE (ExcelGelXL SDS 12-14) were used as the first and second dimensional gels respectively.

2.2.1.2. Solutions

A. Solutions for muscle sample homogenisation and 2 DE

1. Lysis buffer

Urea 13.5 g  
Triton X-100 0.5 ml  
DTT 500 mg  
Pharmalyte 3-10 0.5 ml  
PMSF 35 mg

Make up to 25 ml with Mini-Q water

2. Sample loading solution

Urea 13.5 g  
DTT 250 mg  
Pharmalyte 3-10 0.5 ml  
Triton X-100 0.13 ml  
Bromophenol blue a few grains

Make up to 25 ml with Mini-Q water

3. Rehydration solution

Urea 12 g  
Triton X-100 0.13 ml  
Pharmalyte 3-10 0.13 ml  
DTT 50 mg

Make up to 25 ml with Mini-Q water

4. Equilibration solution 1

Tris-HCl (1 M, pH 6.8) 2.5 ml  
Urea 9 g
10% SDS 2.5 ml
Glycerol 7.5 ml
DTT 75 mg
Make up to 25 ml with Mini-Q water

5. Equilibration solution 2
Tris-HCl (1 M, pH 6.8) 2.5 ml
Urea 9 g
10% SDS 2.5 ml
Glycerol 7.5 ml
Iodoacetamide 1 g
Bromophenol blue a few grains
Make up to 25 ml with Mini-Q water

B. Bradford reagent for protein quantification

1. Stock solution
Serva blue R 100 mg
Absolute ethanol 50 ml
Phosphoric acid (85%) 100 ml
NaOH 2 g

2. Working solution
Stock solution 15 ml
Mini-Q water 85 ml
Filtered and then stored at 4 °C

C. Solutions for Coomassie blue staining

1. Coomassie blue staining solution
Serva blue R 1.5 g
Methanol 550 ml
Mini-Q water 550 ml
Acetic acid 110 ml
2. Coomassie blue de-staining solution
Methanol 200 ml
Acetic acid 70 ml
Make up to 1000 ml with distilled water

D. Solutions for silver staining

1. fixation solution
Methanol or Ethanol 100 ml
Acetic acid 25 ml
Make up to 150 ml with distilled water

2. Sensitising solution
Ethanol 75 ml
Glutardialdehyde (25% v/v) 1.25 ml
Sodium thiosulphate 0.5 g
Sodium acetate 17 g
Make up to 250 ml with distilled water

3. Silver reaction solution
Silver nitrate solution (2.5%) 25 ml
Formaldehyde (37%) 0.1 ml
Make up to 250 ml with distilled water

4. Developing solution
Sodium carbonate 6.25 g
Formaldehyde (37%) 0.05 ml
Make up to 250 ml with distilled water

5. Stopping solution
EDTA (0.5 M) 20 ml
Distilled water 230 ml
6. Preserving solution
Glycerol (87% v/v) 25 ml
Distilled water 225 ml

2.2.2. Sample preparation and protein quantification

2.2.2.1. Tissue homogenisation

Freshly dissected rat skeletal muscle samples, EDL or soleus, were homogenised in lysis buffer in a Duncan homogeniser. The ratio of muscle wet weight to lysis buffer was 1:3 (mg/μl). Homogenates were transferred to fresh eppendorf tubes, and centrifuged for 20 min at 4 °C (10000 g, Jouan Centrifuge, Model MR1822). Supernatants but not the pellets were then collected for protein quantification and subsequent 2 DE analysis. Homogenisation was performed on ice, without occurrence of obvious urea precipitation.

Rat liver tissue was homogenised using the same procedure. Samples were not treated with DNAse or RNAse, as the treatments were not recommended in the protocol provided by the manufacturer.

2.2.2.2. Protein quantification

The Bradford assay (Bradford, 1976) was employed for protein quantification. BSA was used to establish standard curves. Briefly, 10 μl of a sample was aliquoted into a fresh eppendorf tube and diluted with lysis buffer using a ratio of one to twenty (v/v), then 2 μl of the diluted sample were mixed with 1 ml Bradford reagent. After 5 min of incubation at room temperature, absorbency of the mixture was measured at a wavelength at 590 nm (OD590) in polystyrene cuvettes. A mixture of 2 μl of lysis buffer and 1 ml of Bradford reagent was used to zero the spectrophotometer. Protein concentration was determined by plotting the obtained OD590 measurement against BSA concentrations on a simultaneously prepared standard curve. Protein concentrations of the samples prepared using the described protocol (section 2.2.1) usually fell within a range of 10 to 15 mg/ml.
2.2.3. Procedure of 2 DE

The electrophoresis procedure of 2 DE recommended by the manufacturer (Pharmacia-Biotech) was followed. This procedure consisted of 4 steps:

1. Re-hydration of the first dimensional gels:
The first dimensional gels were re-hydrated in a cassette (Pharmacia-Biotech) filled with re-hydration solution. Re-hydration was performed overnight at room temperature.

2. Isoelectric focusing:
Protein samples were loaded onto the re-hydrated first dimensional gels using a sample-loading cup (Pharmacia-Biotech). For analytical 2 DE, samples were usually diluted with loading solution before loading to a final volume was adjusted to 80 μl. Isoelectric focusing was performed at 20 °C under the conditions described in Table 2.1. The focused first dimensional gels were frozen at -80 °C until analysis.

3. Equilibration:
Prior to the second dimensional run, the focused gels were equilibrated with SDS in equilibration solutions 1 and 2 respectively, at room temperature, each for 10 min.

4. Second dimensional (SDS PAGE) separation:
This was performed at 15 °C under the conditions described in Table 2.2.

| Table 2.1. Running conditions for the first dimensional separation. These were programmed in the gradient running mode of the powerpack. |
|---|---|---|
| Phase 1 | 500 | 0.01 |
| Phase 2 | 500 | 5 |
| Phase 3 | 3500 | 5 |
| Phase 4 | 3500 | 9.5 |

38
Table 2.2. Running conditions for the second dimensional separation. These were programmed in the step running mode of the powerpack. *: approximate running time. Electrophoresis of this phase was terminated when the bromophenol blue approached the bottom edge of the second dimensional gel.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Current (mA)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase 1</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>Phase 2</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>Phase 3</td>
<td>40</td>
<td>150*</td>
</tr>
</tbody>
</table>

2.2.4. Visualisation of protein spots

2.2.4.1. Silver staining (Westermeier, 1993)

Silver staining was performed at room temperature. The procedure consisted of the following steps:

1. Fixing of protein spots in fixation solution for 30 min,
2. Sensitisation of spots in sensitising solution for 30 min,
3. Washing of gels in water for three times, each for 5 min,
4. Binding of silver onto protein spots in silver reaction solution for 20 min,
5. Washing of gels in water twice, each for 1 min,
6. Visualisation of the silver stained spots in development solution for 5-10 min,
7. Termination of the developing in stopping solution for 10 min,
8. Washing of gels in water three times, each for 5 min,
9. Treatment of gels for preservation in preserving solution for 20 min,
10. Washing of gels in distilled water for 10 min.
2.2.4.2. Coomassie blue staining (Westermeier, 1993)

Coomassie blue staining was carried out at room temperature. The staining procedure consisted of the following steps:

1. Staining: 4 hours or more in Coomassie blue staining solution,
2. De-staining: 4 x 1 hours or more in De-staining solution,
3. Preservation: 20 min in preserving solution.

2.2.4.3. Post-staining treatment of gels

Stained gels were covered with cellophane, air dried over night and then stored in the dark.

2.3. Separation of rat skeletal muscle proteins using analytical 2 DE

Separation of EDL proteins by analytical 2 DE was initially attempted, as EDL was selected as the primary tissue for examination (section 1.3.2). Although the overall separation was satisfactory, two major shortcomings were observed (Fig. 2.1). One was spot streaking, either horizontal (e.g. spots A, B, D and E) and/or vertical (e.g. spot C, V indicates the vertical tail). Another was the presence of a large dark background plaque (indicated by F). When soleus, another type of skeletal muscle, was analysed, its 2D pattern (Fig. 2.2) was found to be very similar to that of EDL, but a few spots such as S (Fig. 2.2) were different. This allowed image comparisons between EDL and soleus (Chapter 6) to be performed easily.

Subsequently, separation of rat liver proteins was achieved without obvious spot streaking (Fig. 2.3). This indicated that the technical procedure was adequate for liver protein separation, and led to the optimisation of the technique for better separation of skeletal muscle proteins.
Fig. 2.1. Separation of EDL Proteins by Analytical 2 DE. The 2 DE was performed using the procedure described in section 2.2.3. Approximately 100 µg of total EDL proteins was loaded on each gel. The overall separation result was considered to be satisfactory. A, B, C D and E indicate some of the spots with horizontal streaking. Horizontal streaking was also present (e.g. spot C), although to a less degree. V indicates the vertical streaking tail of spot C. F indicates the location of the dark background plaque. The gel was stained using silver staining method (section 2.2.4.1).
Fig. 2.2. Separation of Soleus Muscle Proteins by Analytical 2 DE. The 2 DE was performed using the procedure described in section 2.2.3. Approximately 100 µg of total soleus proteins was loaded. The overall separation result was considered to be satisfactory. Spot streakings (indicated by A, B and C) and the background plaque (indicated by F) were present. A high degree of 2D pattern similarity between EDL and soleus was observed. However, obvious differences were also found. For example, a high concentration soleus protein (spot S) was not detected in EDL muscles (Fig. 2.1). The gel was stained using silver staining method (section 2.2.4.1).
Fig. 2.3. Separation of Rat Liver Proteins by Analytical 2 DE. The 2 DE was performed using the procedure described in section 2.2.3. Approximately 100 μg of total liver proteins was loaded. Although the background plaque (indicated by F) was also present, the liver proteins were separated without obvious spot streakings. This led to the technical optimization for better separation of skeletal muscle proteins. It was also noticed that no high concentration proteins such as spots A, B and C in skeletal muscle (Fig. 2.1 and 2.2) were found in liver tissue. A large proportion of liver proteins were seen to be acidic species. The gel was stained using silver staining method (section 2.2.4.1).
2.4. Attempts made for the optimisation of the analytical 2DE

2.4.1. Removal of the background plaque

Most of the chemicals initially used in the analysis of muscle samples had been subjected to long term storage, while those in analysing the liver tissues were freshly purchased. Thus, it was suspected that the background plaque (indicated by F, Fig. 2.1, 2.2 and 2.3) was resulted from unknown contaminant(s), which was subsequently proven to be true. The plaque was largely removed when fresh batches of the chemicals were used for the analysis (Fig. 2.4), and the area where the plaque used to be became clear (indicated by F, Fig. 2.4) with the spots much more easily seen. However, there was still some remaining residue background (indicated by A, Fig. 2.4) after the removal of the plaque, which may be caused by ampholytes or by other unknown factors.

2.4.2. Efforts made for better protein focusing

Significant spot streaking of the horizontal type (Fig. 2.1) is said to be due to poor protein focusing during the first dimensional run (Westermeier, 1993). Therefore, attempts were made to improve the isoelectric focusing of proteins. Firstly, de-salting of EDL samples prior to 2DE was performed using either Microcon-3 or Microcon-10 concentrators (Amicon, Beverly, USA), as excess salt is one of the common factors that causes poor protein focusing (Westermeier, 1993). Although no salt(s) known to interfere with protein isoelectric focusing were introduced during the sample preparation, it was hoped that removal of endogenous salt(s) from skeletal muscle tissue might enable better focusing of the proteins. However, no benefits were observed. Secondly, effects of changes in the method of sample loading site were tested. Instead of loading at the acidic end of the pH gradient (2-D electrophoresis Protocol, Pharmacia-Biotech), samples were loaded separately at the basic end, or in the middle, or some other sites over the pH gradient. It was found that sample application at the acidic end resulted in the best protein focusing. Application of proteins at other sites of the pH gradient only resulted in worse spot streaking.
Fig. 2.4. Gel Image after the Plaque Removal. The background (indicated by F in Figs. 2.1, 2.2 and 2.3) was not present on the gels when the chemical of long term storage were not used in the experiment. Protein spots in the area where the plaque used to appear (indicated by F) were to be seen and analysed. There was remaining residue background (indicated by A) even when the plaque had been removed, which may be caused by ampholytes or unknown factor(s). This did not affect the overall image analysis, as the weak remaining background was localised in a small area with very few spots in it.
Finally, the electrophoresis conditions were modified by separately extending the time of each phase along the electrophoresis procedure (Table 2.3). This also failed to produce any improvement in protein focusing. A reduction in the time for Phase 4 of the procedure from 9.5 to 6.5 hours neither reduced nor intensified the spot streaking. Thus, changing the running did not seem to be an effective way of achieving better protein isoelectric focusing.

**Table 2.3. Isoelectric focusing of EDL proteins with extended time.** The time of each phase of isoelectric focusing was modified separately and to a different degree. Improvement in protein focusing was not observed. The original isoelectric focusing procedure was also described in section 2.2.3.

<table>
<thead>
<tr>
<th>Running Phase</th>
<th>Original Time (hour)</th>
<th>Extended Time (hour)</th>
<th>Improvement in Focusing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase 1</td>
<td>0.01</td>
<td>0.5</td>
<td>No</td>
</tr>
<tr>
<td>Phase 2</td>
<td>5</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Phase 3</td>
<td>5</td>
<td>8</td>
<td>No</td>
</tr>
<tr>
<td>Phase 3</td>
<td>5</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Phase 4</td>
<td>9.5</td>
<td>12</td>
<td>No</td>
</tr>
<tr>
<td>Phase 4</td>
<td>9.5</td>
<td>24</td>
<td>No</td>
</tr>
<tr>
<td>Phase 4</td>
<td>9.5</td>
<td>36</td>
<td>No</td>
</tr>
</tbody>
</table>

**2.5. Preparative 2 DE of EDL samples and its technical optimisation**

As shown in Fig. 2.5, EDL proteins were well separated on the preparative 2D gels. It appears that 1 mg is likely to be close to the maximum loading capacity of the system, as an increase in sample loading from 1 mg to 1.5 or 1.8 mg per gel generally resulted in very poor focusing (Fig. 2.6). In addition, it was noted that increasing the loading from the analytical (100 µg per gel) to the preparative (1 mg per gel) level changed the electrophoretic behaviour of some proteins, which led to differences between the analytical and preparative 2D profiles. These differences were
Fig. 2.5. Separation of EDL Proteins by Preparative 2 DE. The 2 DE was performed using the procedure described in section 2.2.3. Approximately 1 mg of total EDL proteins was loaded. It was observed that preparative 2D profile was not identical to the analytical counterpart. Increase in sample loading was believed to affect the electrophoretic behaviour of some protein species. Different types changes due to the increased sample loading were observed and summarized into three categories (section 2.4). Some proteins spots which were visualised on the preparative but not analytical (Fig. 2.1) 2D gels were indicated by A. The gel was stained by Coomassie blue staining method (section 2.2.4.2).
Fig. 2.6. Separation of EDL Proteins by Preparative 2 DE at a High Sample Loading Using Loading Cup Method (Part of a whole Image). The 2 DE was performed using the procedure described in section 2.2.3. Approximately 1.8 mg of total EDL proteins was loaded. An increase in sample loading from 1 to 1.8 mg resulted in general poor protein focusing. The gel was stained using Coomassie blue staining staining method.
summarised into three categories:

1. a spot present on analytical gels is not separated on preparative gels;
2. a spot not present on analytical gels is separated on preparative gels (Fig. 2.4);
3. a spot present on analytical gels is not quantitatively separated on preparative gels in proportion to the increase in sample loading.

During the optimisation of preparative 2 DE, two changes were found to be beneficial. One was the introduction of an additional 0.1% of carrier ampholyte (Pharmalyte 3-10) into the solutions employed for 2 DE. This was found to assist proteins, particularly the basic species, to be better focused.

Another was the use of re-hydration loading (Rabilloud, 1994) to replace the sample loading cup method (section 2.2.3) for application. This was performed in a very simple and effective re-hydration chamber (Fig. 2.7) developed during the course of this thesis project. The device was made from two siliconised glass plates and several plastic spacing strips. For use, the spacers were attached with a thin layer of Vaseline, then were placed on a plate (bottom plate) with the desired distance in between. The first dimensional gels were placed between the spacers. When the second plate (top plate) was placed to cover the spacers and was held by bull dog clips, the first dimensional gels were sandwiched in the chambers thus created. The device was placed on the bench tilted to an angle of approximately 15 degrees, and the protein samples were introduced through a needle from the bottom of the Vaseline-sealed chamber until the gels were covered. During overnight re-hydration, proteins were 'absorbed' into the first dimensional gels presumably through diffusion. The device was much simpler and more flexible than that of Rabilloud’s (Rabilloud, 1994). The size of re-hydration chamber was adjustable to suit different applications.

Following the re-hydration loading, first dimensional gels were briefly rinsed with the re-hydration solution, blotted with damp filter paper, then directly subjected to isoelectric focusing. Using this method, effective separation of EDL proteins was consistently obtained at high sample loading levels (e.g. up to 1.8 mg total protein per gel, Fig. 2.8).
Fig. 2.7. Construction of a Rehydration Chamber for Rehydration Loading. a: Parts; b: Integrated view. First dimensional gels were placed in the chambers. Protein solutions were injected into the chambers to cover the gels.
Fig. 2.8. Separation of EDL Proteins by Preparative 2 DE at a High Level of Sample Loading Using Rehydration Loading. Approximately 1.8 mg of total tissue proteins was loaded onto each gel during the first dimensional gel rehydration (section 2.4). Apparently, compared with sample loading cup method (Fig. 2.4), more proteins were loaded onto gels using this sample application procedure with satisfactory separation. The gel was stained using Coomassie blue staining method (section 2.2.4.2).
2.6. Discussion

2.6.1. Separation of skeletal muscle proteins using 2 DE

It seems that spot streaking, in particular the horizontal type, is a common finding in 2D analysis of highly differentiated cells (http://expasy.hcuge.ch/cgi-bin/map1). Many factors such as salt and collapse of pH gradients are known to affect the isoelectric focusing of proteins (O'Farrell, 1977; O'Farrell, 1975; Chrambach, 1973). It is also possible that spot streaking is an electrophoretic feature of some protein species (Wrigley, 1993). In 2 DE analysis of skeletal muscle, in particular, it may be due to unique muscle protein structures, such as the rod-like shape of myosin (Gazith, 1970, Gershman, 1969).

It is believed that skeletal muscle proteins can be focused without obvious spot streaking, as this was accidentally achieved at times without any specific changes in the technique. Apparently, factors causing spot streaking of EDL proteins, although unknown, are presumably simple. It is my perception that this problem can be minimised by including a limited number (e.g. 1 or 2) of the first dimensional gels in a single experiment.

Including a larger number of gels in a single isoelectric electrophoresis (e.g. more than 6) universally resulted in poorer focusing, although up to 12 gels can theoretically be focused simultaneously using the equipment. In addition, focusing of EDL proteins seemed to be improved when the gels and two electrode paper strips were blotted intensively to remove excess liquid before focusing. These observations seem to suggest that the amount of water present in a focusing experiment is one of the causative factors leading to EDL spot streaking.

2.6.2. Determination of the electrophoresis conditions for the project

2.6.2.1. Analytical 2 DE

It was decided that the 2 DE procedure recommended by the manufacturer (section 2.2.3) would be followed for the subsequent analytical 2 DE in this project, as no
worthy modification was found during the optimisation.

2.6.2.2. Preparative 2 DE

The same procedure would also apply to the subsequent preparative 2 DE, except that re-hydration loading was used for sample application, and the carrier ampholyte (Pharmalyte 3-10) concentration in lysis buffer and loading solution was increased by 0.1%, which brought its final concentration to 0.12% (v/v).
Chapter 3: Computer-assisted image analysis of 2D gels

3.1. Introduction

With the development of specialised computer software such as Melanie (Melanie II, internet address: http://expasy.hcuge.ch/melanie/melanie-top.html), QUEST (QUEST II: http://sunspot.bioc.cam.ac.uk/ANALYSIS.html) and ImageMaster (ImageMaster 1.1), laborious image analysis can be performed much more efficiently. In this study, only ImageMaster 1.1 was utilised for 2D gel image data interpretation, as it was the only package available for use at the time of my thesis work.

In this chapter, I describe my experience of using the software ImageMaster in the analysis of the 2D gel images.

3.2. Image analysis using ImageMaster 1.1: The basics

The basic and operational protocols for image analysis using ImageMaster 1.1 were provided in the manual (the software package for the analysis of 1-D and 2-D gels and films, Pharmacia-Biotech).

The major 2D image analysis functions in ImageMaster 1.1 include:

1. Protein spot detection:
Protein spots are detected and recorded, and the quantity of each recorded spot is mathematically modelled. The spot detection can be carried out using either the default (auto-detection) or customised (manual detection) parameters.

2. Image matching:
Up to 5 individual 2D images can be included into a matchset for image comparison. One of them serves as the reference to which others (members) are compared. Unmatched protein spots can be identified immediately after the image matching using the function of 'mark unmatched spot'.
3. Quantitative analysis:
Quantitative changes occurring under different experimental conditions can be detected and measured using quantitative analysis functions in the software.

3.3. Analysis of gel or film images using ImageMaster 1.1

3.3.1. Spot detection

Fig. 3.1 shows the spot detection (Fig. 3.1b) of an analytical EDL 2D gel (Fig. 3.1a) performing using ImageMaster 1.1. The default detection parameters were used. Each circle corresponded to the recording of a single protein spot. Clearly, the software was able to perform effective spot detection, as nearly 100% of the spots visible to the naked eye were detected by the software. However, false detection occasionally occurred, which mainly involved the high concentration protein species.

As shown in Fig. 3.2, spot A (Fig. 3.2a), when separated on the gel was not detected by the ImageMaster 1.1 (Fig. 3.2b). This apparently resulted from a false negative detection. In contrast, spot B on the gel (Fig. 3.2a) was mistakenly detected as a group of separate spots, as indicated by B in Fig. 3.2b. This is a typical example of false positive detection. These false detection is believed to be caused by density saturation, or silver over-staining of high concentration proteins.

ImageMaster 1.1 was proven to be inadequate for the analysis of Coomassie blue stained (preparative) gels and films of autoradiography. In addition false positive detection, significant false negative detection was observed on the abundant protein species. For example, the film spots C and D (Fig. 3.4a) were not detected by the software using the default detection parameters (Fig. 3.4b). Although the false negative detection could largely be overcome by using customised detection parameters, the creation of a set of parameters for an appropriate spot detection of films and Coomassie blue stained gels was found to be difficult. I was unable to determine why significant false negatives occur on the Coomassie blue stained gels.
Fig. 3.1. Use of Image Analysis Software Package ImageMaster 1.1 in the Detection of Protein Spots.
Protein spots separated on an analytical 2D gel (Fig. 3.1a) were detected, mathematically modelled and recorded (Fig. 3.1b). Each circle corresponded to a spot detected (Fig. 3.1b). Using the default detection parameters, almost all the protein spots visible to the naked eyes (Fig. 3.1a) were detected by the software.
Fig. 3.2. False Positive and Negative Gel Spot Detections by ImageMaster 1.1. Both false positive and negative detections by ImageMaster 1.1 occurred mainly on high concentration protein species. As a result of false negative detection, a high concentration protein spot (indicated by A, Fig. 3.2a) was not detected (3.2b). In contrast, as a result of false positive detection, a spot (indicated by B, (Fig. 3.2a) was detected as separate groups of spots (indicated by B, Fig. 3.2b).
Fig. 3.3. Significant false negative detection of film spots by ImageMaster 1.1. Visible film spots C and D (Fig. 3.3a) were not detected (Fig. 3.3b) by the software due to false negative detection. Although the false negative detection could be reduced using customised spot detection parameters, it was very difficult to create a set of parameters for appropriate detection of film spots. a: autoradiographic film image from \(^{35}\)S]-methionine metabolic experiments (part of a whole image); b: film spot detection of a. Gel spots A and B (Fig. 3.3a) correspond to film spots A and B (Fig. 3.3b).
3.3.2. Image matching

The most important aspect of 2D image analysis, as far as many researchers are concerned, is probably the image comparison, which is usually performed using analytical gels. In my study, I found that the software was adequate only for simple image matching such as the comparison of up to 4 individual images with a standard reference gel (section 3.2). Neither the integration of more than 5 individual images into a composite (master) image nor the comparison among master images could be performed due to limitations of the software. Thus, the experiments of the study have to be designed to ensure simple image comparison is adequate for image data interpretation within the limitations of this software package (section 3.4).

3.4. Strategy of 2D image matching for the project

The above observations show that ImageMaster 1.1 can be an adequate 2D gel image analysis tool under the conditions that:

1. a user is able to establish criteria for editing high concentration protein spots where false detection often occurs;
2. a user is able to build up a simple strategy of image comparison that ImageMaster 1.1 can perform.

For the project, a simple experimental procedure was designed (Fig. 3.4). Image data interpretation was completed in two steps, the computer-assisted image matching (step 1) and the manual post-image matching data analysis (step 2). In practice, two EDL muscle strips from the same rat were incubated as an experimental set, one as the control (no amylin) and the other as the test strip (amylin-stimulated). The two corresponding 2D images were compared using the ImageMaster 1.1. The differences, regardless of whether specific to experimental treatment or due to technical variation, were recorded as a “data group”. In step 2, consistency of the differences among several data groups (I aimed at 6 in this study) from the computer-assisted image evaluation was assessed visually. Consistent differences were regarded as specifically in relation to amylin stimulation.
Step 1: Two muscle strips from a same animal were incubated as control and amylin-stimulated samples respectively. ImageMaster 1.1 was used to analyse the 2D gels, and the differences were recorded as a data group.

Step 2: The data groups were analysed manually for the consistent differences.

**FIG. 3.4. Strategy for Muscle Sample Preparation and Subsequent 2D Gel Image Matching.** Two muscles from a same rat were incubated within an experimental group as control and amylin-stimulated samples respectively. The differences in 2D pattern were examined by computer-assisted image matching, and were recorded as a data group. The consistent differences among the data groups were obtained by manual comparisons of the data groups.
Chapter 4: Analysis of EDL stimulated \textit{in vitro} with amylin using analytical 2 DE and computer-assisted image analysis: The discovery of two amylin responsive proteins

4.1. Introduction

Amylin is a newly discovered peptide hormone (section 1.2). Experimental results have shown that the hormone is predominantly produced by the pancreatic \( \beta \) cells, and plays a role in the regulation of fuel metabolism (section 1.2). However, its implication in the development of the common form of insulin resistance is controversial (section 1.2). The available evidence from animal models has consistently demonstrated a strong link between amylin and the common form insulin resistance (section 1.2.3). However, the experimental data obtained from humans does not generally support this view (section 1.2.3).

Analysis of the amylin signalling-action cascade might provide informative data that could enable researchers to have a better view of amylin’s possible role in the pathophysiology of insulin resistance (section 1.2.4), at least the amylin-elicited form of insulin resistance. For this, molecular probes (markers) are required.

The molecular mechanisms of the amylin signalling-action are poorly characterised. Amylin signal transduction is likely to be coupled with \( G_\alpha \) proteins, as it activates adenylate cyclase and increases cAMP content in skeletal muscle (section 1.2.2). Action of amylin evokes functional inhibition of the insulin receptor, inhibition of glycogen synthase through phosphorylation, and activation of glycogen phosphorylase through dephosphorylation (section 1.2.2). However, none of these molecules serves as a specific molecular marker to enable the analysis of amylin action, as all of these enzymes are also regulated by insulin (section 1.1.5; Lawrence and Zhang, 1994; Bryer-Ash, 1995). Hyperinsulinaemia occurs in common forms of insulin resistance, which potentially complicates the analysis of the amylin signalling-action when
molecules under the regulation of both amylin and insulin are used as markers.

The successful establishment of the 2 DE and image analysis system (Chapter 2; Chapter 3) has provided the technical basis for the search for new molecules specific to amylin action. In this chapter, the experience of the discovery of two amylin responsive proteins using 2 DE is described.

4.2. Experimental procedure

4.2.1. Animals

Male Wistar rats, 30 ± 2 days old and 200 ± 30 g in weight, were supplied by The Animal Research Unit, University of Auckland. Rats were maintained on a 12:12 hour light:dark cycle until the experimental day. They were freely fed with standard rat chow (86 Diet, NRM, Auckland New Zealand), and watered ad libitum.

4.2.2. Muscle dissection and incubation

EDL dissection was carried out in Dulbecco’s Modified Eagle Medium (DMEM, Sigma, #D-3916) supplemented with methionine (0.03 g/l) and cysteine (0.0626 g/l). The medium was pre-gassed with a mixture of 95% O₂ and 5% CO₂ for approximately 20 min prior to muscle dissection. Dissected EDL strips were stored in the medium with constant gassing before use for period of not more than 20 min.

As described in section 3.4, two EDL strips from each rat were included within an experimental group, one as the control (no amylin) and the other as the test (amylin-stimulated). This was designed to better control the experiment and to allow for image data interpretation to be performed using the 2D image analysis software package ImageMaster 1.1 (section 3.4).

The dissected EDL strips were put into siliconised 50 ml Erlenmeyer flasks containing 10 ml of pre-gassed medium. Incubation was performed at 30 °C with constant gassing (2 ml/min) and gentle shaking (170 rpm) for 5 hours. The incubation was set for 5 hours, as muscle strips must remain vital during the time of incubation. This was
the previous experience of my co-workers (G. Cooper, Personal communication). Rat amylin (H-9475, Bachem, California) was added into the experimental flasks at the beginning of incubation to give a final concentration of 100 nM which had previously found to result in the maximal stimulation (G. Cooper, 1994).

In this study, six groups of incubation were performed (section 3.4). Each of the groups had one control and one amylin-treated EDL muscle strip (section 3.4). Image data interpretation was performed according to the procedure specifically designed for this study (section 3.4).

4.2.3. Metabolic labelling of skeletal muscle using \(^{35}\)S-methionine

For the \(^{35}\)S-methionine metabolic labelling experiments, DMEM supplemented only with cysteine (0.0626 g/l) was used during EDL dissection and incubation. One mCi of \(^{35}\)S-methionine (#51001H, ICN Costa Mesa, USA) was added to each flask at the beginning of the incubation, which was performed under the same conditions as described in the above section. Autoradiography was performed at room temperature for 72 hours using medical X-ray film (Fiji Photo Film Co. Ltd., Japan) and a cassette with an intensifying screen on one side (Fuji FG-8, Fuji Photo Film Co. Ltd., Japan).

Four pairs of samples (each consisting of one control and one amylin-treated EDL muscle strip) were metabolically labelled and subsequently analysed.

4.2.4. Muscle Sample homogenisation and protein quantification

Muscle homogenisation and protein quantification were performed using the protocols described in section 2.2.2.

4.2.5. Analytical 2 DE

The basic 2 DE procedure (sections 2.2.3 and 2.5.2) was followed in performing the analytical 2 DE.
4.2.6. Molecular weight calibration

Molecular weight determination was performed by external calibration (2-D gel electrophoresis protocol, Pharmacia-Biotech) employing molecular weight standards. In practice, a piece of sample loading paper (approximately 0.3 x 0.5 cm, Pharmacia-Biotech) was positioned at each end of the first dimensional gel before it was run. Molecular weight standards were loaded onto the sample loading pieces. After the second dimensional separation, standard molecular weight ladders were formed at each of the vertical sides of a 2D image. By plotting the R_f values against the known molecular weights of the standard proteins. The molecular weight of an unknown EDL protein could be determined by interpolation.

Internal calibration was attempted for more accurate molecular weight calibration by including molecular weight standards in the isoelectric focusing. However, this procedure was found to be difficult, as it was hard to distinguish the standards from the endogenous muscle proteins in the 2D gels.

4.2.7. Image analysis

Gel image analysis by the ImageMaster 1.1 was performed according to the procedure designed for this project (section 3.4), which included the localisation of the corresponding gel and film spots and spot quantification. In quantitative image analysis, the level of significance was set at 3 (Miller, 1992). That is, 3-fold or greater difference in optical density of a protein spot (the software detects and compares the optical density as a method of analysing the differences in protein concentrations) was accepted as a statistically significant change. Differences of less than 3-fold were considered not significant, as they could be the consequence of technical or random variations.
4.3. Results

4.3.1. Distribution of EDL proteins on 2D gels: An overview

4.3.1.1. EDL proteins on analytical 2D gels

At the sample loading level of 100 μg, approximately 3000 EDL protein spots were resolved on each analytical 2D gel (Fig. 4.1). Two groups of high concentration protein species (represented by spots A, B and C and spots D, E and G respectively) were localised towards both ends of the pH gradients. The majority of EDL proteins were of low to moderate concentration and were separated between the two groups of high concentration species. Despite the fact that the high concentration proteins were small in number, they seemed to comprise a significant percentage of total EDL protein mass, although the mass distribution of these proteins was not quantitatively analysed. In addition, most EDL proteins detected by the method had molecular weights of greater than 20 kDa. The majority of the rat liver proteins seemed to be acidic species (Fig. 2.3), whereas, there was a similar abundance of the acidic and basic protein species in the EDL (Fig. 4.1).

4.3.1.2. Reproducibility of the 2 DE analysis of the EDL samples

Gel images of the control EDL samples from the six experimental groups (section 4.2.2) were compared each to the other for an assessment of experimental variation. It was found that the difference in spot number between any two of the 6 control gels was less than 50, which is less than 1.7% of the total separated proteins. The number of spots that showed quantitative differences was not more than 7, which is less than 0.23%. Thus, the variation in the 2 DE analysis of the EDL proteins is considered to be small.

4.3.2. Identification of two EDL proteins responsive to amylin stimulation

Through comparisons of the experimental 2D gels against the controls, two amylin responsive proteins were discovered.
4.3.2.1. Identification of a protein produced in response to amylin stimulation

One amylin responsive protein spot was found to be consistently present only on the experimental gels (spot 1, Figs. 4.1 and 4.2a); it was never detected in the control gels (Fig. 4.2b). It was designated “amylin responsive protein 1” (ARP1). More original gel images of muscle samples in which ARP1 was found are shown in Fig. 6.2.

As indicated in Fig. 4.1, ARP1 was visualised as one of the proteins with a low to moderate concentration. Its molecular weight was estimated to be close to 20 kDa by external molecular weight calibration using molecular weight standards (section 4.2.6). It was also observed that the protein was separated in a gel area corresponding to a low to moderate acidic pH value (Fig. 4.1), suggesting that it is likely to be an acidic protein. However, the pI of this protein was not calibrated. The pH gradient of the first dimensional gels used in this study was non-linear, with an unknown pH scale (Pharmacia-Biotech). Thus, calibration was required for the estimation of pI values. Unfortunately, I was unable to achieve satisfactory pI calibration using pI standard due to technical difficulties. Even when using external calibration, more spots than expected were visualised on the gels, possibly due to unknown contaminants. In fact, it was difficult to verify the spots corresponding to the standards. When internal calibration was used, it was also difficult to locate the spots of the added pI standards.

ARP1 was the most easily visualised and seemed to be the only protein that consistently appeared in the presence of amylin during the incubation under the experimental conditions. Qualitative changes in other spots (section 4.3.1.2) were likely to be the result of unknown technical and/or random variations.

Consistent disappearance of protein spots resulting from amylin stimulation was not observed.

4.3.2.2. Identification of a protein quantitatively increased in response to amylin stimulation

Another amylin responsive protein, designated amylin responsive protein 2 (ARP2) was found to be present in both the control (spot 2, Fig. 4.3a) and amylin-stimulated
Fig. 4.1. Distribution of EDL Proteins on the Analytical Gels. At the loading level of 100μg of total EDL proteins, approximately 3000 EDL proteins were separated on an analytical 2D gel. Two groups of high concentration EDL proteins, as represented by spots A, B and C and spots D, E and G respectively, were localised towards both ends of the pH scale. Proteins with low to moderate concentrations were distributed mainly between the two groups of high concentration species. The majority of EDL proteins seemed to have moderate to high molecular weights (eg. greater than 22 kDa). There was a similar abundance of acidic and basic proteins. Spots 1, 2, 3, and 4 were subsequently characterised using an integrated molecular characterization procedure (Chapter 5). This is a silver stained gel has also been shown in Chapter 2 (Fig. 2.1).
Fig. 4.2. Identification of a Protein Spot Consistently and Exclusively Present in the EDL Muscle Stimulated in vitro with Amylin (Parts of Whole Gel Images). Both amylin-stimulated (Fig. 4.2b) and control (Fig. 4.2a) EDL samples were analysed by analytical 2 DE. A protein spot (spot 1) was found to be present only in the amylin-stimulated EDL strips. This protein was designated amylin responsive protein 1 (ARPI). Spots A and B correspond to those shown in the whole EDL image (Fig. 4.1). The gel was stained using silver staining method (section 2.2.4.1).
EDL samples. With amylin stimulation, its concentration was increased by 4.5-fold on average (Fig. 4.3), ranging from 3 to 6 folds.

ARP2 was also seen as a low concentration protein in stimulated EDL. However, it is likely to be a basic protein, as it was separated within a gel area corresponding to moderate to high pH value (Fig. 4.1). The pI value of this protein was not calibrated for the same reason as stated above (section 4.3.2.1). The molecular weight of ARP2 was estimated to be between 30 and 33 kDa using external calibration (Fig. 4.1).

It should be stressed that ARP2 was the most easily detectable and possibly the only protein that showed a consistent increase in protein concentration with amylin stimulation under the experimental conditions. Others quantitative changes (section 4.3.1.2) were inconsistent and were likely to be either technical or random variations.

4.3.2.3. Results of the metabolic labelling of ARP1 and ARP2 with $[^{35}\text{S}]$-methionine

Approximately 300 radioactive signal spots, which correspond to nearly 10% of the total EDL proteins separated on the analytical gels, were detected on each of the X-ray films following autoradiography (Fig. 4.4). Spot matching was performed between the analytical gels and their corresponding X-ray film images to determine whether the amylin responsive proteins produced corresponding radioactive signal spots in autoradiography. The ARP2 separated from the amylin-stimulated EDL samples (spot 2, Fig. 4.5b) was matched to a radioactive signal spot on the corresponding X-ray films (Fig. 4.5b). In contrast, none of the radioactive signal spots was matched to ARP2 separated from the control EDL strips in the image matching (Fig. 4.5a).

No radioactive signal spot corresponding to ARP1 was found, no matter whether ARP1 was separated from the amylin-stimulated or control EDL samples.
Fig. 4.3. Identification of a Protein Spot ARP2) Quantitatively Increased in the Amylin-stimulated EDL Muscles (parts of whole images). Comparing control gels (Fig. 4.3a) and experimental (Fig. 4.3b) 2D gels, a protein spot (spot 2) that showed significant increase in concentration was found. The average increase was 4.5 fold, ranging from 3 to 6. This protein was designated amylin responsive protein 2 (ARP2). Spot D corresponds to that shown in the whole EDL image (Fig. 4.1). The gel was stained using silver staining method (section 2.2.4.1).
4.4. Discussion

4.4.1. The mechanism of ARP1 production: evidence that ARP1 is produced through amylin-evoked protein post-translational modification

ARP1 was detected only in the EDL samples stimulated \textit{in vitro} with amylin (Fig. 4.2), which indicates that it is specifically produced in response to amylin stimulation. The molecular mechanism of the production of this protein remains unknown. It could be a product of either amylin-activated gene expression or amylin-induced protein post-translational modification. Metabolic labelling was performed in an attempt to label ARP1 with radioactive \[^{35}S\]-methionine. The assumption was that if it showed amylin-induced \[^{35}S\]-methionine incorporation, then this would be evidence of a mechanism of amylin-activated gene expression. However, this was not established. This was not considered to be a consequence of any technical failure, as the metabolically labelling of ARP2 with \[^{35}S\]-methionine was achieved under the same experimental conditions (see the section below, section 4.4.2). Assuming that ARP1 has methionine residue(s), and is not produced as a consequence of amylin-evoked proteolysis, then protein modification seems to be the most likely mechanism for its production by amylin.

4.4.2. Amylin-stimulated protein synthesis: the molecular mechanism for the increase in ARP2 concentration

ARP2 is the first known protein that is quantitatively increased in response to amylin stimulation. Unlike ARP1, I found that ARP2 was present in both control and amylin-stimulated samples (Fig. 4.3), which shows that it is a normal component of EDL protein composition. To test the hypothesis that amylin stimulation increases the biosynthesis of ARP2, metabolic labelling of the protein with \[^{35}S\]-methionine was performed. The assumptions were:

ARP2 is likely to contain methionine residue(s). It is a consensus that the average molecular weight of amino acids is 110 Da, and the frequency of methionine in the general protein population is 1%. Thus, ARP2 (30-33 kDa in size) was estimated
Fig. 4.4. Autoradiography of the 2D Gels of EDL Muscles Metabolically Labelled with \[^{35}\text{S}]\text{methionine.} \] Approximately 300 radioactive signal spots (10% of the gel spots) were detected on each autoradiographic film. The spot detection was performed by ImageMaster 1.1 using the default parameters. The film spots A, B, C and D correspond to those shown in Fig. 4.1.
Fig. 4.5. Identification of a Radioactive Signal Spot Present Only on the Films of the Experimental (Amylin-treated) 2D Gels. The control (Fig. 4.2a) and experimental (Fig. 4.2b) gels were subjected to autoradiography. A radioactive signal spot (spot 2) corresponding to ARP2 was found on the autoradiographic films (Fig. 4.5b) of the experimental gels but not on the films (Fig. 4.5a) of control gels. This shows that ARP2 incorporated [35S]-methionine in the metabolic labelling experiments only in the presence of amylin stimulation. Spot D corresponds to that shown in Fig. 4.1.
1. to have 270 to 300 amino acid residues, which might include about 3 methionine residues;
2. \([^{35}\text{S}]\)-methionine would likely to be incorporated into ARP2 as a consequence of protein mass increase in response to amylin stimulation;
3. The amylin-elicited incorporation of \([^{35}\text{S}]\)-methionine into ARP2 could afterwards be detected using autoradiography.

The results showed that ARP2 separated from the amylin-stimulated but not from the control muscles was matched to a radioactive signal spot in autoradiography (section 4.3.2.3). This indicated that the protein was labelled with \([^{35}\text{S}]\)-methionine during amylin stimulation. These findings further suggests that: 1. ARP2 has one or more methionine residues; and 2. its concentration is increased in response to amylin stimulation.

ARP2 is not considered to be an actively metabolising protein species in resting EDL muscles. In contrast to the amylin-stimulated muscles in which the protein biosynthesis was significantly increased, the 5 hours of in vitro incubation without amylin did no cause \([^{35}\text{S}]\)-methionine incorporation neither change its concentration as quantitatively assessed using the ImageMaster 1.1. (Fig. 4.4a). These observations demonstrate nicely the potent effect of amylin on rat skeletal muscle.

### 4.4.3. The power and limits of the current 2 DE technology

The identification of ARP1 and 2 has clearly demonstrated that 2 DE is a powerful tool in protein biochemistry research. It is sensitive, easy and cost-effective. However, further improvements in this technique may provide higher resolution and sensitivity. For example, development of first and second dimensional gels to separate proteins over smaller pH and/or molecular weight ranges will enable more protein species to be visualised. Highly populated gel areas are not uncommonly seen in gels of different type of tissues (http://expasy.hcuge.ch/ch2d/2d-index.html). It is difficult to analyse most of the proteins clustered within a relatively small area, unless the pI and/or molecular weight scales covering the area are amplified.
Chapter 5: Microcharacterisation of the amylin responsive proteins: Identification of ARP1 as a novel Monomeric form of protein p20

5.1. Introduction

Although 2 DE is a powerful technique for protein separation, it does not provide much insight into the identity of the proteins of interest. Therefore, supplementary techniques are required for molecular characterization of these proteins.

The simplest method for the analysis of protein identity is to perform electrophoretic co-migration on SDS PAGE of a sample protein and standards with known identity. Equivalence in electrophoretic mobility of a sample protein and a candidate protein implies that they are identical (Giometti, 1979). This method is clearly primitive and has very limited practical applications. Today, there are better techniques for protein microcharacterisation (Fig. 5.1). Amino-terminal sequence analysis, for example, has come to a level where a few picomoles of a protein can be sequenced. The two other most commonly used methods, amino acid composition analysis and mass spectrometry, also work within this range of sensitivity. Amino acid sequence, composition and accurate mass data of a large number of proteins have been deposited in various databases accessible to researcher word-wide. Identity of a sample protein can be found by searching the databases using any of these kinds of data, providing it has previously been characterized.

An integrated protein microcharacterisation procedure was employed for the molecular characterisation of the amylin responsive proteins that I discovered in this thesis project. In this procedure, specific gel spots of a protein were excised from preparative 2D gel(s) and combined for in-gel protein fragmentation by trypsin. Tryptic fragments were then eluted from the polyacrylamide gel matrix by organic solvent extraction, and separated by reversed phase HPLC. Sequences obtained from microsequencing the tryptic fragment(s) were then used to determine the protein’s identity through database searching.
Fig. 5.1. Modern Protein Microcharacterization Techniques (adapted from Lottspeich, 1994). Protein spots on 2D gels can be extracted from the polyacrylamide gel matrix either through transferring onto membranes or in-gel digestion followed by organic solvent extraction. Peptides from enzymatic cleavage of a protein are separated by reversed phase HPLC, and are finally subjected to sequencing, or amino acid analysis, or mass spectrometry. The resulting protein sequence or amino acid composition or accurate mass data are used for database searching for protein identity.
5.2. Materials and methods

5.2.1. Chemicals, enzyme and solutions

5.2.1.1. Chemicals and enzyme

Chemicals were purchased from Sigma or Applied Biosystems. Trypsin (#1047841) was provided by Boehringer Mannheim (Mannhem, Germany). The enzyme was dissolved in 0.01% TFA to give a final concentration of 1 µg/ml, and aliquoted into eppendorf tubes (5 µl each tube) before freezing at -80°C until use.

5.2.1.2. Solutions used in the in-gel digestion

1. Washing solution
   - Acetonitrile: 5 ml
   - Tris-HCl (1 M, pH 8.5): 1 ml
   - Milli-Q water: 4 ml

2. Digestion buffer
   - Tris-HCl (1 M, pH 8.5): 1 ml
   - Acetonitrile: 1 ml
   - Triton X-100, reduced (10%),: 0.02 ml
   - Milli-Q water: 8 ml

3. Extraction solution
   - TFA: 0.01 ml
   - Acetonitrile: 6 ml
   - Triton X-100, reduced (10%),: 0.02 ml
   - Milli-Q water: 4 ml

5.2.2. In-gel digestion and organic solvent extraction

Rosenfeld’s in-gel digestion procedure (Rosenfeld, 1992; Hubbard, 1995) was followed. For high concentration protein spots, such as spot D (Fig. 4.1), a gel spot was used for in-gel digestion, while 10 to 20 gel spots were required for those with a
low to moderate concentration. Taking spot C as an example, an excised gel spot was washed several times (e.g. 4 x 1 hours) in Rosenfeld washing solution at 30 °C until the colouring from Coomassie blue was no longer visible. The washed spot was cut into fine pieces (approximately 1x1 mm or smaller), transferred to a fresh eppendorf tube, and air dried at 37 °C for 30 min. After cooling to room temperature, 0.5 µg of trypsin and an amount of digestion buffer sufficient just to cover the gel pieces were added. The mixture of gel pieces and trypsin was vortexed followed by centrifuging at 10,000 g for 30 seconds (Jouan Centrifuge). In-gel digestion was performed by an overnight incubation at 37 °C. Usually, after the first 30 min of the incubation, when the gel pieces had completely swelled, sufficient amount of additional digestion buffer was added to keep all the gel pieces immersed. The digested samples were then frozen at -80 °C until analysis.

Digests were incubated with 5 µl of TFA, and then 100 µl and then 300 µl of extraction solution in sequence at 37 °C. Each of these organic solvent extractions was performed for 30 min. The resulting supernatants were collected after brief centrifugation (10,000 g, 30 seconds, Jouan Centrifuge), combined and subjected to volume reduction (50-60%) using a speed vac concentrator.

5.2.3. Reversed phase HPLC

Tryptic fragments were separated by reversed phase HPLC at room temperature using an Applied Biosystems solvent delivery system (140A), a diode array detector (1000s), and a Brownlee RP-700 microbore column (220 x 2.1 mm, 7 µm bead diameter). Solvent A was 0.1% TFA, while solvent B was 0.0825% TFA in 80% acetonitrile. Before each HPLC run, the column was equilibrated for 10 min with the initial solvent A:B ratio of 9:1. Following equilibration, the sample was injected onto the column. Elution was performed using a linear gradient of solvent B from 10% to 70% over 45 min (ramped to 80% solvent B over an additional 5 min) at 200 µl/min. A blank run was completed prior to sample injection. The optical density was monitored at 214 and 280 nm and the peptide peaks were collected manually.
5.2.4. Protein microsequencing

Microsequencing (Edman chemical degradation) was carried out using a Procise Sequencer, model 492, which was manufactured by Applied Biosystems ABI. Sequencing was performed by Mrs. C. Knight, School of Biological Sciences, The University of Auckland.

5.2.5. Protein sequence analysis

The FASTA computer program of the Genetics Computer Group (GCG) and The OWL Composite Database on the World Wide Web were used for sequence analysis. The address of The OWL software on the internet was: http://www.biochem.ucl.ac.uk/bsm/ddbrowser/OWL/QuizOWLSQ.html.

5.3. Results

5.3.1. Microcharacterisation of a high concentration EDL protein (spot D, Fig. 4.1): testing the technical procedure

Protein microcharacterisation using the integrated procedure (sections 5.2) was firstly attempted on spot D (Fig. 4.1), a high concentration EDL protein

One spot D from a preparative gel was subjected to in-gel digestion, organic extraction and reversed phase HPLC separation. As shown in Fig. 5.2, a number of tryptic fragments were separated by the HPLC, which showed that the previous in-gel digestion and organic extraction were successfully carried out.

A microsequence, GGDDLDPNYV, was obtained from HPLC fraction number 13. This sequence was matched completely and exclusively to creatine kinase M chain by microsequence analysis, corresponding to creatine kinase M chain amino acids 117 to 126. Thus, the protein separated in the spot D was identified as creatine kinase M chain. The molecular weight of this protein in spot D was estimated to be between 40 and 45 kDa, which was in agreement with the identification of the protein as creatine kinase M chain (43 kDa).
Fig. 5.2. *Separation of Tryptic Fragments of a Trial Protein (Spot D, Fig. 4.1) by Reversed Phase HPLC.* A single gel spot of spot D was used for the in-gel digestion by trypsin. The tryptic fragments were extracted from the polyacrylamide gel matrix by organic solvent extraction, and separated by reversed phase HPLC. The HPLC fraction 13 (marked with *) was subsequently subjected to sequencing. The full scale of absorbance monitoring was 0.1.
These results show that the integrated procedure is effective for the microcharacterisation of proteins separated on the preparative 2D gels. It was estimated that a quarter of the gel from spot D was adequate for the microcharacterisation. This provides a valuable guide for sample collection for the characterization of other proteins. For example, if a protein spot is 40 times less in concentration than spot D, as assessed easily by ImageMaster 1.1, 10 gel spots of this protein will then be adequate for completing the microcharacterisation comfortably using this technical procedure.

5.3.2. Microcharacterisation of amylin responsive proteins

Following the successful identification of the protein in spot D as creatine kinase M chain, molecular characterization of ARPI and 2 was started.

5.3.2.1. Identification of ARPI as protein p20

Microcharacterisation on ARPI was firstly attempted. Twenty gel spots of ARPI were collected from preparative 2D gels, as estimated to be adequate for the molecular characterization (section 5.3.1). Subsequent to in-gel digestion and organic solvent extraction, the tryptic fragments of ARPI were separated by reversed phase HPLC (Fig. 5.3c). Microsequencing gave the following sequences: VGDHVEVHAR, LFDQR, HEERPDEHGFIA, R(H)A(F)S(S)A(P)P(E)L(E)P(I)G(S)F(V)S(K)TPGR, VPVQPSWLR and PSVALPTAQVPTDPGYFSVLLDVK from HPLC fractions 8, 9, 10, 12, 14 and 21 respectively (Table 5.1). Sequence analysis using The OWL database perfectly matched the sequences obtained from fractions 8, 10 and 21 to those of rat protein p20, a recently discovered protein without known biological function (Kato, 1994a; 1994b). The sequence from fraction 14, VPVQPSWLR, was also matched to rat protein p20 as well as homologous human protein p20. Considering the muscle samples were obtained from rat rather than humans, the protein in the spot 1 (Fig. 4.1) from which this sequence was obtained was rat but not human protein p20. This analysis is hereby termed as “species exclusion”. Using this, the protein was again identified as rat protein p20 using the microsequence derived from the HPLC Fraction 9 (LFDQR), despite the fact that it was matched to 26 different macromolecules (protein, RNA or DNA) (Table 5.2).
Fig. 5.3. Separation of Tryptic Fragments of the Proteins Separated in Spots 1, 3 and 4 (Figs. 4.1 and 4.2). Spots 1 (20 gel spots, chromatogram c), 3 (25 gel spots, chromatogram b) and 4 (15 gel spots, chromatogram a) were digested in-gel by 0.5 μg trypsin. The tryptic fragments were extracted from the gel matrix, and were separated by HPLC. The HPLC fractions marked with * were selected for sequencing. Despite the fact that all these three proteins were subsequently identified as protein p20 (Table 5.1), their HPLC profiles were not identical, although similar to certain degree. This may serve as indirect evidence that the protein may be multiply modified.
Table 5.1. Microsequence analysis of the proteins separated in spots 1 (ARPI), 3 and 4 (Figs. 4.1 and 4.2). From protein microsequencing, 7, 1 and 2 sequences were obtained respectively from the proteins separated in spots 1 (ARP1), 2 and 3 (Figs. 4.1 and 4.2). All these sequences were matched perfectly to rat protein p20 by sequence analysis alone, except that the sequences from HPLC fraction 9 (LFDQR) and 14 (VPVQPSWLR) were matched to rat protein p20 by microsequence analysis in coupling with species exclusion (Table 5.2).

<table>
<thead>
<tr>
<th>Spot</th>
<th>Fraction</th>
<th>Microsequence</th>
<th>Matched protein(s)</th>
<th>Residues in rat p20</th>
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<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>VGDHVEVHAR</td>
<td>Rat p20</td>
<td>93-102</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>LFDQR</td>
<td>Rat p20</td>
<td>28-32</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>HEERPDEHGFIA</td>
<td>Rat p20</td>
<td>103-114</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>R(H)A(F)S(S)A(P)(E)</td>
<td>Rat p20</td>
<td>14-27, 82-91</td>
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<td></td>
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<td></td>
<td></td>
<td>L(E)P(I)G(S)F(V)S(K)T</td>
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<td></td>
<td>PGR</td>
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<tr>
<td>1</td>
<td>14</td>
<td>VPVQPSWLR</td>
<td>Rat p20</td>
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<td>21</td>
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<td>4</td>
<td>7</td>
<td>HFSPEEIS</td>
<td>Rat p20</td>
<td>82-89</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>APSVALPTAQVPT</td>
<td>Rat p20</td>
<td>57-69</td>
</tr>
</tbody>
</table>
The sequence from HPLC fraction 9, LFDQR, was matched to 26 known proteins or nucleic acid sequences that may encode this amino acid sequence. An identification as rat protein p20 was concluded by species exclusion, that was, on this list, protein p20 was the only rat protein. Molecular weight exclusion was also found to be useful. Only the molecular weight, 20 kDa, matches to the molecular weight observed for ARP1 (around 20 kDa, Fig. 4.1 and section 4.3.2.1).

Table 5.2 Analysis of the microsequence obtained from tryptic fraction 9 of ARPI.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Nucleic Acid Sequence</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puff scientific</td>
<td>Fruit fly</td>
<td>alg 8 and 44 genes</td>
<td>A. Vinelandii</td>
</tr>
<tr>
<td>Protein p59</td>
<td>Human</td>
<td>Campestris DNA</td>
<td>Xanthomonas</td>
</tr>
<tr>
<td>p59 protein</td>
<td>Mouse</td>
<td>alg44 gene</td>
<td>Bacteria</td>
</tr>
<tr>
<td>p59 protein</td>
<td>Rabbit</td>
<td>mSin3A mRNA</td>
<td>House mouse</td>
</tr>
<tr>
<td>Proline</td>
<td>Xanthomonas</td>
<td>hrc genes</td>
<td>Erwinia</td>
</tr>
<tr>
<td>inminopeptidase</td>
<td>Virus</td>
<td>DNA sequence</td>
<td>Xanthomonas</td>
</tr>
<tr>
<td>28kDa protein</td>
<td>Virus, different strain</td>
<td>mRNA sequence</td>
<td>Arabidopsis thaliana</td>
</tr>
<tr>
<td>70.2 protein</td>
<td>Fission yeast</td>
<td>Asp45 gene</td>
<td>Fission yeast</td>
</tr>
<tr>
<td>YOP protein</td>
<td>Y. Pseudotuberculos</td>
<td>Cosmid SCY06G11</td>
<td>Bacteria</td>
</tr>
<tr>
<td>Protein p20</td>
<td>Human</td>
<td>Polyprotein gene</td>
<td>Virus</td>
</tr>
<tr>
<td>Transition protein2</td>
<td>Mouse</td>
<td>RNA sequence</td>
<td>Virus</td>
</tr>
<tr>
<td>Ig p59</td>
<td>Mouse</td>
<td>IVa2 protein gene</td>
<td>Ovine</td>
</tr>
</tbody>
</table>


Two sequences were obtained from Fraction 12. Using the previously determined rat protein p20 sequence (Kato, 1992) as reference, they were confirmed to be HFSPEEISVK and RASAPLPGFSTPGR, which also matched perfectly with corresponding parts of the rat protein p20 sequence.

5.3.2.2. Approaches used to characterise ARP2

Unlike ARP1 that was separated in an area of low spot density, ARP2 was surrounded by other proteins (Fig. 4.1), and did not clearly separate on the preparative 2D gels. In order to characterise ARP2, several approaches were used. These included: 1. Modifying the sample loading quantity. The amount of sample loading was lowered from 1.5 to 1.0, 0.8 and then to 0.6 mg per preparative gel with the hope that a distinguishable ARP2 spot might emerge. However, this was not the case; 2. Searching the literature for a staining method that is both sensitive enough to visualise the spot on analytical gels and compatible with the subsequent protein microcharacterisation. Unfortunately, no suitable staining method was found in literature. Although Shevchenko’s silver staining is said to be sensitive and compatible with PMF, the method did not work well in our trial on sample proteins (Nickson, 1997); and 3. In this study, I also tried the metabolically labelled muscle samples for 2 DE followed by autoradiography without gel staining. It was hoped that the film spot corresponding to ARP2 could serve as a marker to localise the ARP2 protein spot. But, this did not work in two experiments. Alternatively, fractionation of the whole muscle homogenate might be attempted in future. This pre-running treatment of samples might reduce the number of protein spots surrounding ARP2 on the resulting gels, and may allow ARP2 be separated more clearly for sample collection. This approach was not attempted in my study, as was not considered to be cost-effective.

5.4. Discussion

5.4.1. ARP1 is a monomeric form of protein p20

As shown by Kato et al. (Kato, 1994a and b), rat protein p20 exists as dimers and may aggregate under normal physiological conditions. The monomeric unit of protein p20, which is produced when the disulfide bond in a dimer is broken (e.g. by reducing
reagent), showed a molecular weight of 20 kDa, as calibrated on SDS PAGE against molecular weight standards. ARP1 is considered to be a monomeric form of protein p20, as its molecular weight was close to 20 kDa on the 2D gels (Fig. 4.1).

5.4.2. ARP1 is a novel monomeric form (designated form 1) of protein p20

It was suspected that other monomeric form(s) of protein p20 might have been co-separated on the 2D gels with ARP1, as the reducing reagent, DTT, was present in the sample preparation and 2 DE analysis. To investigate this, two other 20 kDa proteins (spots 3 and 4) were selected for molecular microcharacterisation. As shown in Fig. 5.3a and b, the proteins were digested by trypsin into smaller peptides. Through microsequencing, three sequences were obtained, which were HFSPEEIS from the HPLC Fraction 21 of spot 3 (Fig. 5.3b), and PSVALPTAQVPTDPGYFS and APSVALPTAQVPT from the HPLC Fractions 7 and 13 of spot 4 (Fig. 5.3a). Each of these sequences gave a perfect match with rat protein p20 (Table 5.1). Thus, the proteins present in spots 3 and 4 are also monomeric forms of rat protein p20.

The discovery of these three forms of monomeric protein p20, which are hereby designated forms 1 (ARP1), 2 (spot 3) and 3 (spot 4) respectively, shows that at least two post-translational modifications can occur on rat protein p20. Forms 2 and 3 were found to be present in both of the control and amylin-stimulated EDL samples, and are considered to be normal components of the EDL proteins. In contrast, ARP1 is a novel monomeric form (form 1) of protein p20 that is specifically produced in response to amylin stimulation. On the 2D gel (Fig. 4.1), the form 3 protein p20 monomer was separated in the central area of the pH gradient, indicating that its pI value is close to neutral. Both of the other forms were localised within an area of low to moderate pH value, which suggests that they are acidic proteins. Among the three monomeric forms of protein p20, ARP1 had the lowest pI value.

Despite the fact that the proteins separated in spots 1, 2 and 3 were all identified as protein p20 monomers, their profiles of HPLC separation after the tryptic digestion (Fig. 5.3) were not identical, although similar to a certain extend. The reasons for
these differences are unknown. It is likely that different post-translational modifications such as phosphorylation (section 5.4.3; Fig. 5.4) may be the cause of these differences. But, I also noted that experimental variations could be another factor. Differences in trypsin:sample protein ratio in in-gel digestions may result in different degree of partial digestion, which affects HPLC results.

To investigate further the behaviour of protein p20 in muscles stimulated with amylin, requests for a copy of the cDNA of the protein and its specific antibody were made to relevant workers in other countries at different times during the project. Unfortunately, there was no reply. I was reluctant to start preparing the cDNA clone and antibody myself, as it is very time consuming.

5.4.3. Evidence that ARP1 is produced through phosphorylation in response to amylin stimulation

As discussed in 8.2.2., there are reasons to speculate that ARP1 could be produced by amylin-evoked protein phosphorylation. To test this possibility, metabolic labelling of EDL protein with $^{32}$P$_i$ was performed. The first group of experiments using H$_2$$^{32}$PO$_4$ failed in general, as little radioactivity incorporation was detected. This may be due to changes in the pH of the incubation solutions when the $^{32}$P$_i$ was introduced. However, despite adjusting the pH of the incubation solutions to around 7.3 using NaOH in the second group of experiments, effective metabolic labelling was still not achieved.

Metabolic incorporation of radioactive $^{32}$P$_i$ into ARP1 was detected in another set of experiments, in which a mixture of Na$_2$H$^{32}$PO$_4$ NaH$_2$$^{32}$PO$_4$ was used. In those experiments, EDL strips were incubated with 4:1 v/v of Na$_2$H$^{32}$PO$_4$ (#64051, ICN) and NaH$_2$$^{32}$PO$_4$ (#6405, ICN) for 4 hours (equilibration of the muscles with $^{32}$P$_i$), and then for 1.5 hours with amylin. The total incubation length was set at 5.5 hours, as it was found that muscle strips prepared under these conditions remained physiologically vital (G. Cooper, personal communication). The muscle samples were analysed by 2DE in combination with autoradiography for 48 hours at -80 °C. A radioactive signal spot (on the films) corresponding to the ARP1 protein spot (on the 2-D gels) was consistently detected in all three gels (Fig. 5.4). This suggests that ARP1 is produced by amylin-elicited phosphorylation of a protein p20 precursor. Surprisingly, a
radioactive signal spot corresponding to form 2 protein p20 (spot 3, Figs. 4.1 and 4.2) was also detected (Fig. 5.4), suggesting this protein is also phosphorylated. It is unknown whether form 2 protein serves as a precursor for the production of ARP1. It is also possible that in vitro incubation itself acts as a kind of stress treatment, which cause the phosphorylation on form 2 protein p20, as protein phosphorylation in response to stress has been observed in homologous small heat shock proteins (Kato, 1994 a and b). Unfortunately, control incubations were not included in this group of experiments. The main reasons were: 1. it was thought that ARP1 would not be produced without amylin stimulation; and 2. the experimental conditions then did not seem to allow higher amounts of radioactive materials be used. Further experiments are required to test whether form 2 protein p20 is produced during incubation, and whether it is due to phosphorylation with amylin stimulation.

5.4.4. Molecular characterization of ARP2: The technical considerations

ARP2 was not characterized using the integrated protein microcharacterisation procedure because it was not resolved on the preparative 2D gels. Different approaches had been used in attempting to separate this protein on the preparative gels in order to collect sufficient protein for microcharacterisation but failed (section 5.3.2.2). Several techniques may to used for this in the future, which include:

1. searching or developing a staining method that is of high sensitivity and compatible with the current technology of protein sequencing;
2. use of the differential detergent fractionation method (Ramsby and Kreutzer) for the muscle samples preparation;
3. use of the molecular genetic methods such as subtraction cloning or differential display.
4. optimisation of the experimental conditions of PMF using silver stained protein samples (Nickson, 1997).
Fig. 5.4. Detection of a Radioactive Signal Spot Corresponding to ARPI from Amylin-stimulated EDL Muscles Metabolically Labelled with $^{32}$Pi. Electrophoresis was performed according to the described protocols (Chapter 2). On the autoradiographic films, a radioactive signal spot (spot 1) corresponding to ARPI (Form 1 protein p20) gel spot (spot 1, Figs. 4.1 and 4.2) was detected. A radioactive signal (spot 3) corresponding to form 2 protein p20 gel spot (spot 3, Figs. 4.1 and 4.2) was also detected. Spots A and D correspond to those shown in Figs. 4.1, 4.2 and 4.3.
5.4.5. Protein microsequence analysis: My unique experience

It may be worth of presenting my personal experience with protein microsequence analysis, although I have not answered all the questions that had been raised. I hope that my experience may benefit the local researchers and, in particular, the students who may be involved in the identity analysis using protein sequences.

Microsequence analysis of ARPI was initially performed using the FASTA program on the local GCG service, as it is commonly utilised for this type of study. These analyses had shown that ARPI was homologous to a variety of different proteins, particularly small heat shock proteins. However, no conclusive identification was made. This once led us to speculate that ARPI was a previously unidentified skeletal muscle protein.

However, I had been cautious on this, and had a perception that additional assessments were required. Since 2D databases on the Internet were frequently consulted in the study for practical and theoretical guidance (section 2.1), it thus looked for protein sequence analysis databases that might be available on the net. The searching on the internet by key word “protein” plus “database” using AltaVista (internet address: http://altavista.digital.com/) has located some useful protein sequence analysis services, which included THE OWL, a composite database for sequence comparison. Using the OWL program, ARPI was immediately identified as protein p20 (Table 5.1). It is uncertain why the GCG service failed in the microsequence analysis of ARPI, when it was successful in identifying many others (Chapter 7). It seemed that the database(s) included in the GCG station for protein sequence comparison may not be updated or timely updated. It is my advice that before announcing the identification of a new protein, the candidate protein has to be examined using a wide range of procedures.

5.4.6. Trypsin autodigestion during the in-gel digestion

In the characterization of ARPI, 0.5 μg trypsin was used for the in-gel digestion, despite the fact that others recommend smaller amounts (Hubbard, 1995). In a control experiment, where the amount of trypsin was incubated with some gel pieces
containing no protein samples, trypsin autodigests were found (Fig. 5.5). This is likely to be the reason that occasionally trypsin fractions were mistakenly collected as experimental samples for microsequencing (C. Knight, personal communication). Moreover, this observation may be helpful in the analysis of protein identity using PMF, in which one may find that not all the mass data can be matched to a same protein (Nickson, 1997). The unmatched fragments may include the trypsin autodigests, and thus it seems to be the priority to exclude the mass data of trypsin autodigests in PMF data interpretation.

![Retention time (mins)](image)

**Fig. 5.5. Separation of Trypsin Autodigests by Reversed Phase HPLC.** Approximately 0.5 µg of trypsin was incubated in the digestion buffer with gel prices containing no protein samples. Autodigestion of trypsin was evidenced as several peaks of autodigests were revealed on the HPLC trace.
Chapter 6: Insulin-independent production of ARP1: Relevance to the development of insulin resistance

6.1. Introduction

Following the identification of ARP1 as form 1 protein p20 (Chapter 5), experiments were designed to investigate two important issues with priority: the possible effects of insulin and calcitonin gene-related peptide (CGRP) on ARP1 production, and that of amylin stimulation on the insulin sensitivity of the treated EDL muscles.

Amylin and CGRP are homologous peptides. They have in common an N-terminal ring structure and an amidated C-terminus, and exhibit considerable amino acid sequence similarity. The two hormones also show overlapping biological functions including the regulation of carbohydrate metabolism (Cooper, 1987; Westermarck, 1987; Cooper, 1988; Leighton and Cooper, 1988; Molina, 1990; Cooper, 1994). Amylin, CGRP and their antagonists have been intensively investigated as these two hormones have been implicated in the development of insulin resistance, and both have potential pharmaceutical applications (sections 1.2.3 and 1.2.4; Leighton and Cooper, 1988; Molina, 1990; Cooper, 1991 and 1996; Muff, 1995a and b). However, few studies have focused on the intracellular changes in response to amylin or CGRP, despite such information having the potential to provide valuable insights into the physiology and pathophysiology of the two hormones.

Although the available evidence indicates that there are amylin and CGRP binding proteins, molecular characterization of the putative receptors for CGRP and amylin has not yet been achieved (Foord and Craig, 1987; Dotti-Sighton, 1988; Chantry, 1991). Evidence has accumulated both for and against the hypothesis that amylin and CGRP share the same signal transduction pathway(s). Both amylin and CGRP activate adenylate cyclase and elevate intracellular concentrations of cAMP (section 1.2.2.1; Chantry, 1991; Pittner, 1995), suggesting that the signal transduction of the two hormones may involve G_s proteins. Moreover, amylin displaces the binding of
CGRP from CGRP binding sites in a dose dependent manner (Chantry, 1991), and the competitive antagonist, CGRP<sub>8-37</sub>, blocks both the amylin and CGRP regulation of fuel metabolism in skeletal muscle (16). Recently, data have emerged to support the view that amylin and CGRP have separate receptors. In cultured rat mesangial cells, rat pancreatic acinar AR42J cells, isolated rat thymocytes, and COS-7 cells expressing adrenomedullin, there was a clear distinction between the biological actions of amylin and CGRP (Osajima, 1996; Huang, 1996; Kapas, 1995; Kurz, 1995). Separate binding sites for amylin and CGRP in the nucleus accumbens and the lung have also been demonstrated (Aiyer, 1995). At the molecular level, the insulin receptor, glycogen phosphorylase and glycogen synthase have been shown to be targets of amylin action (section 1.2.2.2; Lawrence and Zhang; 1994; Bryer-Ash, 1995).

The aims of this group of studies included: 1. to compare the insulin sensitivity of EDL muscles with or without amylin stimulation; 2. to compare the signal transduction of amylin and CGRP using ARP1 as a marker; and 3. to test the possibility that insulin stimulation and mild heat shock treatment evoke the production of ARP1.

6.2. Experimental procedure

6.2.1. Sample preparation and analytical 2 DE-image analysis

Dissection, incubation, homogenisation and protein quantification of EDL muscle samples were performed following the procedures described in section 2.2. Both rat cyclic CGRP (#PCAL60, Bachem, California, USA) and human insulin (monocomponent, Novo Nordisk Pharmaceuticals, Denmark) were used at a final concentration of 100 nM. Analytical 2 DE and image analyses were carried out following the procedures described in sections 2.2.3, 2.2.4.1 and 2.5.2.1.

6.2.2. Mild heat shock treatment

Mild heat shock treatment of muscle samples was performed at 42 °C for 20 min (Kato, 1994a) in the incubation medium (section 4.2.2) which was pre-gassed with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> for 20 min (Kato, 1994a and b). The heat shock
treated muscle samples were then immediately frozen in liquid nitrogen and stored at -80 °C until analysis. The influence of heat shock on the production of ARPI by amylin was studied, as the treatment causes protein re-distribution and modification of protein p20 homologous small heat shock proteins (Kato, 1994a and b).

6.2.3. Metabolic labelling of [14C]-D-glucose

[14C]-D-glucose labelling was performed to evaluate the rates of glucose incorporation into glycogen in EDL muscles with different treatments. For this, 0.5 μCi of [14C]-D-glucose (#11048.2, ICN) was added into both experimental and control flasks at the beginning of incubations. Muscle strips were incubated in different experimental groups, which included control (C, n=5), amylin (A, n=5), insulin (I, n=6), CGRP (CGRP, n=6), amylin plus insulin (A+I, n=6) and CGRP plus insulin (CGRP+I, n=6).

All hormones were used at a final concentration of 100 nM (Cooper, 1994).

6.2.4. Glycogen purification and 14C counting

Glycogen concentrations as well as the glycogen accumulation rates were determined using the methods previously described (Cooper, 1988; Leighton and Cooper, 1988). Muscle strips incubated with [14C]-D-glucose were freeze dried, weighed and subsequently dissolved in 400 μl of 60% KOH solution (70 °C, 45 min). Briefly, glycogen from each muscle strip was precipitated by 1.5 ml absolute ethanol (-20 °C, overnight). Pelleted glycogen precipitates (10,000 g, 4 °C, 15 min, Jouan MR1822 Centrifuge) were washed twice in 1.5 ml ice cooled absolute ethanol, freeze dried, re-dissolved in 500 μl of Mini-Q water, and finally mixed with 4 ml of scintillation fluid (EcoLite, ICN) for 14C measurement. [14C]-D-glucose incorporation into glycogen was calculated by plotting the obtained counts per minute against glucose concentrations on a standard curve. Rates of glycogen accumulation (an indication of glycogen synthesis) were expressed as the rate of accumulation of glucosyl units (μmol of glucosyl units per mg of muscle wet weight per hour, μmol/mg/h), and used for statistically analysis using ANOVA on the computer software package STATISTICA (Statsoft, Tulsa, USA). The significance level was set at p<0.05.
The rate of glycogen accumulation = (concentration of $^{14}$C-glucosyl units in glycogen) x (concentration of $[^{14}$C]-D-glucose in medium + concentration of glucose in the medium)/[$^{14}$C]-D-glucose concentration in the medium.

### 6.3. Results and discussion

#### 6.3.1. Amylin elicits insulin resistance in EDL

As shown in Fig. 6.1, the glycogen accumulation rate was 1.77 +/- 0.23, 1.27 +/- 0.32, 5.11 +/- 1.38 and 1.88 +/- 0.26 (µmol/mg/h) for EDL samples incubated in groups C, A, I and A+I (section 6.2.3) respectively. Insulin stimulation was found to significantly increase the glycogen accumulation rate (290%, p<0.001). This shows that insulin stimulates glycogen synthesis in EDL, and in turn demonstrates that the EDL muscle strips are physiologically active under the *in vitro* incubation environment. The glycogen accumulation rate in the muscles of group A+I was found to be comparable to that of muscles in the group C (p<0.05). This shows amylin completely inhibits the stimulatory effect of insulin on glycogen synthesis. Thus, amylin stimulation elicits *in vitro* insulin resistance in EDL muscle. Amylin was also found to decrease the glycogen synthesis by 28% (group A, p>0.05). A significant inhibition of basal glycogen synthesis is likely to be demonstrable if the analysis is performed on a larger sample size.

#### 6.3.2. ARPI is also produced in skeletal muscles in responsive to CGRP stimulation

To investigate the effects of amylin on the protein composition of soleus muscle, computer-assisted image analysis (chapter 3) was performed using the gels of the amylin-stimulated EDL (Fig. 4.1) as the reference images for localising ARPI spot. It was found that a protein spot corresponding to ARPI was separated from the amylin-stimulated soleus muscles (Fig. 6.2k) but not from the control soleus muscles (Fig. 6.2l). This indicates that amylin also elicits the production of ARPI in soleus muscles. Similarly, protein spots corresponding to ARPI were detected from the CGRP-stimulated EDL (Fig. 6.2h, Table 6.1) and soleus (Fig. 6.2r, Table 6.1) muscles.
Fig. 6.1. Glycogen Synthesis in EDL Muscles with Different Experimental Treatments. C: control; A: stimulated with amylin; I: stimulated with insulin; and A+I: stimulated with amylin plus insulin. *: Statistical significance detected. Glycogen accumulation rate was used as an indication of glycogen synthesis in the muscle samples. Insulin significantly stimulated the glycogen synthesis in EDL (I, p<0.01). This effect was completely inhibited by amylin (A+I, p>0.05). Amylin was found to decrease basal glycogen synthesis by 28% (A), However, this was not significant under the current experimental protocol (p>0.05) The data were expressed in mean ± s.e.m.
Fig. 6.2. Gel Images of some Muscle Strips Prepared under Different Experimental Conditions (Partial). a, b and c: amylin-treated EDL muscles with d, e and f as the paired controls respectively; g, EDL treated with insulin; h, EDL treated with CGRP; i, EDL treated with CGRP+insulin; j, EDL treated with amylin+insulin; o, EDL with heat shock treatment; k, soleus muscle treated with amylin; l, soleus muscle without any treatment; m, soleus muscle treated with insulin. n, soleus muscle treated with CGRP. An image of soleus muscle treated with CGRP+insulin was not given, as the gels were damaged during storage. Spots 1, 3 and 4 correspond to those in Fig. 4.1, which were identified as forms 1 (ARP1), 2 and 3 protein p20. A and S indicate the proteins spots separated from the soleus but not EDL muscles. Spot 1 (ARP1) in Fig. 6.2j (amylin+insulin) was faint on the original gel, and seemed to became invisible on the zoom-out image. All the gels were stained using the silver staining method.
These results suggest that:

1. CGRP also elicits production of ARP1;
2. the two types of rat skeletal muscle, EDL and soleus, respond to amylin and CGRP in a similar manner.

**6.3.3. Insulin dose not have an effect on ARP1 production in response to either amylin or CGRP**

Further experiments were carried out to investigate the effect of insulin on ARP1 production in response to either amylin or CGRP. No protein spot corresponding to ARP1 was detected in the homogenates of the insulin-stimulated EDL or soleus (Figs. 6.2g and m; Table 6.1), indicating that insulin does not evoke the production of this novel form of protein p20. The results from the quantitative image analysis using the ImageMaster 1.1 suggest insulin neither inhibits nor promotes the production of ARP1, as the presence of insulin did not quantitatively alter the concentrations of ARP1 in the amylin- or CGRP-stimulated EDL and soleus (Figs. 6.2i and j; Table 6.1). Thus, the production of ARP1 in response to amylin or CGRP in skeletal muscle appears to be insulin independent.

The proteins previously known to be under amylin regulation, that are the insulin receptor, glycogen synthase and phosphorylase, are all co-regulated by insulin (section 4.1). ARP1 is the first protein to be identified for which the regulation by amylin and insulin is separate. The finding that both amylin and CGRP elicit the production of ARP1 in an insulin-independent manner suggests that the two hormones share, at least to some degree, the same intracellular machinery for signal transduction and action.

Biological functions of protein p20 remain unknown. Data from the quantification of total protein p20 in skeletal muscle during rat development with or without denervation have suggested that protein p20 may play a role in skeletal muscle physiology (Inaguma, 1996). In my study, the data showed the appearance of ARP1 (form 1 of protein p20) in EDL and soleus muscles stimulated by either amylin or CGRP is associated with the inhibition of insulin-stimulated glucose incorporation into glycogen (Table 6.1; Cooper, 1988; Leighton. and Cooper, 1988; Leighton,
Table 6.1. Detection of ARPI in EDL and Soleus Muscles with Different Experimental Treatments. The gel of amylin-stimulated EDL muscles (Fig. 4.1), where ARPI was initially discovered, was used as a standard reference. All 2D gels of other samples were matched to the references using ImageMaster 1.1. in the search for a protein spot corresponding to ARPI. The high degree of 2D image similarity between EDL and soleus muscles (Figs. 2.1 and 2.2) allowed the image matching between the two types of skeletal muscle to be easily and accurately performed. C: control (with treatment); A: amylin stimulation; I: insulin stimulation; CGRP: CGRP stimulation; A+I: stimulation with amylin plus insulin; CGRP+I: stimulation with CGRP plus insulin; Y: ARPI was detected; N: ARPI was not detected. ARP2 was not separated well on these gels, which did not allow us to perform equivalent analysis to that done for ARPI. Some of the original gel images covering the area of interests were presented in Fig. 6.2.

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<tr>
<th></th>
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<tr>
<td></td>
<td>Experimental treatment</td>
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<td>I</td>
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<tr>
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<td></td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
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<td>Y</td>
</tr>
<tr>
<td>Soleus</td>
<td></td>
<td>N</td>
<td>Y</td>
<td>N</td>
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<td>Y</td>
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</table>
These data suggest that: 1. Protein p20 may be involved in the regulation of the fuel metabolism; and 2. ARP1 is likely to play a role in the development of insulin resistance, at least the amylin-evoked form.

It was interesting to observe that a protein spot corresponding to ARP1 was detected in mild heat shock treated skeletal muscles (Fig. 6.2a; Table 6.1), which is consistent with the previous observation that protein p20 responded to heat stress (Kato, 1994a). If the appearance of ARP1 is an indication of the existence of an insulin-resistant state, then insulin resistance should be demonstrable in mild heat shock-treated muscles. This was not investigated in the present study. However, insulin resistance is related to several other stress states (section 1.1.2.1; Mizock, 1995), which implicates that mild heat shock, as a typical type of stress, might cause insulin resistance. Additional indirect evidence comes from the observation that mild heat shock treatment delayed the development of insulin responsiveness in cultured fetal hepatocytes (Benatmane, 1996; Zachayus and Plas, 1995), which implies that heat stress has an effect on insulin sensitivity. Insulin sensitivity of EDL strips after heat shock was not assessed, as it was suspected that muscle strips might not be vital at this temperature sufficiently long to enable the completion of such experiments.

It should be noted that the identifications of ARP1 spots from the muscle samples with different treatments (Fig. 6.2sa, b, c, h, i, j, k, n and o; Table 6.1) were performed by image analysis only. Further characterization(s) may be required to confirm these identifications. The necessary evidence to prove the identity of these spots may include: 1. data to show that they are protein p20. This may be achieved by immunoassay using specific antibody or by protein microcharacterisation techniques such microsequencing or PMF; 2. Data to show that these spots are phosphorylated. This may be achieved using radioactive 32P labeling (section 8.2.2.2) or PMF; 3. Data to show that these spots are phosphorylated at the same site(s). This might be achieved by PMF, or by immunoassay using antibody specific to certain phosphorylated amino acid residues.

In conclusion, these results show that both amylin and CGRP elicit the production of ARP1 (form 1 protein p20, Chapter 5) in an insulin-independent manner. The
appearance of this novel monomeric form of protein p20 is associated with the development of insulin resistance. It is suggested that: 1. amylin and CGRP share, at least in part, a common signal transduction pathway; and 2. ARP1 is functionally involved in the signaling-action of these two hormones and the subsequent development of insulin resistance.
Chapter 7: Towards the proteome analysis of rat skeletal muscle EDL: Identification of 18 EDL proteins

7.1. Introduction

The growing interest in proteome projects comes as genome scientists are producing sequence data on more genes than they can put a function to, and with its rapid advance the limitations of genome analysis have become obvious. Neither DNA sequence of a gene nor its mRNA level completely describes the structure and function of an encoded protein. After synthesis, proteins often undergo post-translational modifications (e.g. addition of phosphate groups or removal of amino acids) and change their activities. Proteome projects primarily target the analyses of identification, concentration and structure of the cellular proteins in various types of tissues. These two types of projects are "two sides of a same coin". Data from proteome analysis, when linked to nucleic acid data from the related genome analyses, will provide powerful information for understanding the biological functions and their molecular mechanisms of cellular proteins.

Practically in a proteome analysis, 2 DE is performed for separating the cellular proteins, and the modern protein microanalysis techniques are used for the molecular characterisation of the proteins of interest (section 4.1). The final result is the construction of a reference map of cellular protein. Under different experimental conditions, either changes in expression level or occurrence of post-translational modifications on a protein can be identified through comparisons between a sample image data set and the corresponding reference.

Although microsequence analysis is a powerful tool in protein microcharacterisation, peptide mass fingerprinting (PMF) is an alternative option (Fig. 5.1). With the improvement in mass spectrometry (MS) technology, masses of tryptic fragments of a protein can now be accurately determined, and this can be used in database searching for the protein identification. Currently there are two modern MS technologies, laser
desorption MS developed by Frans Hillenkamp in Germany and electrospray MS devised by John Fenn in USA. Both have been used for accurate determinations of peptide mass. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry is a newly developed version of the laser desorption method (Hillenkamp, 1991), and it is usually coupled to time-of-flight (TOF) mass analysis. MALDI-TOF MS is often used for PMF, as it shows high degree of accuracy (0.001% for proteins up to 30,000 Da, Beavis, 1989 and 1990) and sensitivity (1 to 10 fmol, Strupat, 1991; Karas, 1989). Thus it can be used for structural analysis (Bahr, 1994).

Following the establishment of the necessary techniques (Chapters 2, 3 and 5), the project of proteome analysis of EDL was initiated with the aim of understanding better the molecular basis of amylin action and for serving broader research interests in skeletal muscle physiology. This chapter describes the microcharacterisation of 18 EDL proteins at the initial stage of the analysis. In addition, the applications of microsequence analysis and PMF for protein microcharacterisation are discussed.

7.2. Experimental protocols

7.2.1. Separation of EDL protein using preparative 2 DE

EDL dissection, homogenisation and protein concentration determination and preparative 2 DE were performed according to the protocols described in section 2.2.

7.2.2. Protein microcharacterisation using sequence analysis

EDL protein separation by 2 DE, in-gel digestion, reversed phase HPLC purification of tryptic peptides and protein sequencing were performed according to the protocols described in section 5.2. Microsequencing was terminated at 8 to 10 amino acid residues. Protein sequencing analysis was performed by database searching using the FASTA program of GCG and the OWL service on the Internet (section 5.2.6).
7.3. Results and discussion

At this initial stage of the proteome analysis of rat EDL muscle, 18 protein spots (Fig. 7.1) over a range of molecular weights, pI values and concentrations were selected for molecular characterization using sequence analysis.

As shown in Table 7.1, using single microsequence analysis alone, protein identification was obtained for 12 out of the 18 proteins (66.7%). These included parvalbumin α, ATP synthase coupling factor 6 (mitochondria precursor), superoxide dismutase, myosin light chain 2 (skeletal muscle isoform), protein p20 in spots 6 and 7, myosin light chain 1 (skeletal muscle isoform) in spots 8 and 9, triosephosphate isomerase, fructose-bisphosphate aldolase, creatine kinase M in spot 15, and ATP synthase β chain (mitochondria precursor). Useful information was obtained for the remaining 6 (33.3%), as the proteins in spots 3, 11, 14, 15, 16 and 18 appeared to belong to the haemoglobin, tropomyosin, actin, creatine kinase, enolase and heat shock protein families respectively. Using a single microsequence analysis in combination with tissue exclusion (section 5.3.2.1), proteins in spots 11 and 15 were confirmed to be tropomyosin and creatine kinase M chain respectively. Although the proteins in spots 14 and 18 were finally identified as α-actin and heat shock cognate 71 kDa protein respectively, multiple microsequence analyses was required. No previously unknown skeletal muscle proteins were found among the 18 spots subjected for analysis. Protein sequencing of these proteins was terminated at 8 to 10 amino acid residues. It is logically expected that a higher percentage of cellular proteins could be identified by microsequence analysis if more than 10 amino acid residues were obtained.

Cautions must be taken regarding the interpretation of these data. Firstly, these are partial sequences. Only a complete sequence gives conclusive identification. Secondly, more than one protein species or their fragments may be co-separated in the same spot. In an analysis involved limited pieces of microsequences, biased identification may be derived.
Fig. 7.1. Separation of EDL Proteins on Preparative 2D Gels for Proteome Analysis. Approximately 1.5 mg of total EDL proteins were loaded. A total of 18 protein spots (numbered 1 to 18) that cover a range of pI values, molecular weights and concentrations were selected for molecular characterisation. The protocols described in chapters 2 and 5 were followed for 2 DE and microcharacterisation. The gel was stained using Coomassie blue (section 2.2.4.1). ARP2 was not resolved on the preparative gel, as it is difficult to separate under higher loading (>0.4 mg per gel, section 5.3.2.2).
Table 7.1. Sequence Analysis of 18 EDL Proteins. Note: smi, skeletal muscle isoform; mp, mitochondrial precursor; HS, heat shock.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Sequence</th>
<th>Best Match</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GFIEEDELG</td>
<td>Parvalbuminα</td>
<td>Parvalbuminα, P02625</td>
</tr>
<tr>
<td>2</td>
<td>LASGGPVD</td>
<td>ATP synthase coupling factor 6 (mp)</td>
<td>ATP synthase coupling factor 6 (mp), P21571</td>
</tr>
<tr>
<td>3</td>
<td>MFAAFPTT</td>
<td>Hemoglobin α1 and α2</td>
<td>Hemoglobin family</td>
</tr>
<tr>
<td>4</td>
<td>GDGPVQGV</td>
<td>Superoxide dismutase</td>
<td>Superoxide dismutase, P07623</td>
</tr>
<tr>
<td>5</td>
<td>EAFTVIDQ</td>
<td>Myosin light chain 2, (smi)</td>
<td>Myosin light chain 2 (smi), P04466</td>
</tr>
<tr>
<td>6</td>
<td>PSVALPTAQV</td>
<td>Protein p20</td>
<td>Protein p20, A53814</td>
</tr>
<tr>
<td>7</td>
<td>APSVALPTAQ</td>
<td>Protein p20</td>
<td>Protein p20, A53814</td>
</tr>
<tr>
<td>8</td>
<td>HVLATLGEK</td>
<td>Myosin light chain 1 (smi)</td>
<td>Myosin light chain 1 (smi), P02600</td>
</tr>
<tr>
<td>9</td>
<td>VFDKEGN</td>
<td>Myosin light chain 1 (smi)</td>
<td>Myosin light chain 1 (smi), P02600</td>
</tr>
<tr>
<td>10</td>
<td>HIFGESD</td>
<td>Triosephosphate isomerase</td>
<td>Triosephosphate isomerase, P48500</td>
</tr>
<tr>
<td>11</td>
<td>AEDADVAS</td>
<td>Tropomyosin (smi)</td>
<td>Tropomyosin (smi), P04692</td>
</tr>
<tr>
<td>12</td>
<td>GILADESTG</td>
<td>Fructose-bisphosphate aldolase</td>
<td>Fructose-bisphosphate aldolase, P05065</td>
</tr>
<tr>
<td>13</td>
<td>GGDDLDPNYV</td>
<td>Creatine kinase M</td>
<td>Creatine kinase family</td>
</tr>
<tr>
<td>14</td>
<td>SYELPDGQ</td>
<td>Actin-α</td>
<td>Actin-α, P02568</td>
</tr>
<tr>
<td></td>
<td>and EITAL</td>
<td>Actin-β</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Actin-γ</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>GYSFV</td>
<td>Actin-α</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>FEEILTR</td>
<td>Creatine kinase M</td>
<td>Creatine kinase M, P00564</td>
</tr>
<tr>
<td>17</td>
<td>GKYDLDKF</td>
<td>Enolase-α</td>
<td>Enolase family</td>
</tr>
<tr>
<td>18</td>
<td>HGGYSVFAG</td>
<td>ATP synthase β chain (mp)</td>
<td>ATP synthase β chain (mp), P10719</td>
</tr>
<tr>
<td>19</td>
<td>IINEDTAAA</td>
<td>HS 70 kDa protein 1/2</td>
<td>HS cognate 71 kDa protein, P08109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HS 70 kDa protein 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HS 70 kDa protein 3, etc.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VNHF</td>
<td>HS cognate 71 kDa protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>hsp-72-psl protein</td>
<td></td>
</tr>
</tbody>
</table>
These EDL proteins were also analysed by PMF (Nickson, 1997). It was found that PMF identified 13 out of the 18 EDL proteins (72.2%), and localised 2 (11.1%) in spots 3 and 18 as members of the haemoglobin and heat shock protein families respectively. However, it failed to provide any informative data on 3 (16.7%), which were α-actin (spot 14) and forms 2 and 3 protein p20 (Chapter 5) in spots 6 and 7.

The data from the molecular weight estimation (Fig. 7.1) by external calibration (section 4.2.6) generally support the identification of these EDL proteins. However, exceptions were observed. One was spot 15, which was identified as creatine kinase M chain by microsequence analysis (Table 7.1). The size of this spot was estimated to be smaller than 21.5 kDa (Fig. 7.1), which is far less than the molecular weight of the creatine kinase M chain (43 kDa). It is possible that the protein in spot 15 is a fragment of creatine kinase M chain, which is produced from protease cleavage during the sample preparation. Another was spot 1, which was identified as parvalbumin. Despite that fact that the molecular weight of parvalbumin (11.8 kDa) is smaller than that of ATP synthase coupling factor 6 (12.5 kDa), it separated on the 2D gels in an area of higher molecular weight than ATP synthase coupling factor 6 (Fig. 7.1). The mechanism of this remains unknown. The parvalbumin in spot 1 may be coupled to other molecules such as calcium, which results in a slower migration rate. Also, protein migration rates on SDS PAGE are not accurately proportional to their molecular weights.

Clearly, neither single microsequence analysis nor PMF alone can identify all the cellular proteins. A critical shortcoming of PMF as a tool for protein microcharacterisation is that it fails completely for some proteins. In this study of 18 samples, PMF completely failed for 3 spots (16.7%), such that, neither conclusive nor suggestive identifications were obtained. This is slightly higher but still in agreement with the previously finding that PMF failed to identify 10% of yeast cellular proteins (Shevchenko, 1996b). In these cases, sequence analysis is required. In addition, mass signal suppression during MS occurred from time to time. This may be reduced through optimisation of the sample preparation method (Shevchenko, 1996b). On the other hand, PMF has distinct advantages (Nickson, 1997). The most impressive aspect of PMF was that it distinguishes individual proteins from homologous members.
more effectively than does microsequence analysis. For example, while single microsequence analysis could only suggest that the proteins in spots 15 and 16 belong to the creatine kinase and enolase families respectively, PMF revealed that they were creatine kinase M chain and α-enolase respectively.

In the earlier experiments, calibration of pI values was not successful (section 4.3.2), although others have shown that pI calibration is possible (Bjellqvist, 1993). With the characterisation of these protein species, I tested further the possibility of constructing a pI scale using the theoretical pI values of these proteins. For this, the theoretical pI values of 11 proteins separated in different gel areas were calculated using the program Isoelectric+ of the GCG, and the distance of each spot from the left end (lowest pI point in theory) of the gel image (Fig. 7.1) was measured as the location variant. When the theoretical pI values were plotted against the location variants, a trend of positive correlation was noted (Table 7.2; Fig. 7.2a and b). This indicates that a reference pI scale can be established using the theoretical pI values of proteins that are relatively well characterised. However, this type of reference pI scale is far from accurate; exceptions have been observed in this study (Figs. 7.1 and 7.2; Tables 7.1 and 7.2). Several factors may cause the inconsistency between the theoretical and observed pI values of a protein, which may include:

1. protein fragmentation during sample preparation. The molecular characterisation of the spot 15 as creatine kinase M may serve as an example. The molecular weight of rat creatine kinase M is close to 43 kDa (Section 5.3.1). The molecular weight of the protein in spot 15 was found to be between 14 and 21 kDa (Fig. 7.1). This indicates that the protein in spot 15 is likely a fragment of the intact rat creatine kinase M.

2. post-translational modifications. Cellular proteins are often post-translationally modified under physiological conditions (Guy, 1994); this can greatly change the pI values of a protein. A typical example is the characterisation of the three forms of protein p20 (Chapter 5). They are located on the gels several millimeters apart from each other, despite the fact that three spots have got the same protein;

3. technical variations.
It is suggested that in large-scale protein microcharacterisation, PMF can be used as a primary technique, as it is efficient and can potentially be automated. However, sequence analysis is necessary when PMF fails. Also, in more specific microcharacterisation where a limited number of proteins are involved and accurate identification is a priority, protein sequence analysis is recommended.

Proteome analyses of a variety of biological samples have been initiated world-wide. However, to my knowledge, rat skeletal muscle has not yet been included. Characterization of these EDL proteins in my project signals the initiation of the proteome analysis of rat skeletal muscle. It was noted that these high concentration proteins from rat EDL muscle, such as myosin light, creatine kinase M chain and α-enolase, showed a 2D separation pattern similar to those of rabbit skeletal muscle (Giometti, 1979). This indicates that these high concentration proteins in skeletal muscle are highly conserved, at least between the rat and rabbit.

Table 7.2. The theoretical pI values and gel locations of the 11 relatively well characterised proteins. The gel location was expressed as the horizontal distance between (cm) the spot and the left end of the gel. Cyto: cytosolic form; mt: mitochondria form.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Theoretical pI</th>
<th>Gel Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin-α</td>
<td>5.15</td>
<td>1.6</td>
</tr>
<tr>
<td>Myosin light chain 2</td>
<td>4.62</td>
<td>2.0</td>
</tr>
<tr>
<td>ATP synthase β chain</td>
<td>4.95</td>
<td>2.0</td>
</tr>
<tr>
<td>Myosin light chain 1</td>
<td>4.82</td>
<td>2.4</td>
</tr>
<tr>
<td>Myosin light chain 1</td>
<td>4.82</td>
<td>2.6</td>
</tr>
<tr>
<td>Heat shock cognate 71 kDa protein</td>
<td>5.26</td>
<td>3.0</td>
</tr>
<tr>
<td>Parvaalbumin α</td>
<td>4.86</td>
<td>3.3</td>
</tr>
<tr>
<td>ATP synthase coupling factor 6</td>
<td>5.45</td>
<td>3.3</td>
</tr>
<tr>
<td>Superoxide dismutase (cyto.)</td>
<td>5.89</td>
<td>4.9</td>
</tr>
<tr>
<td>(mito.)</td>
<td>7.94</td>
<td>4.9</td>
</tr>
<tr>
<td>Triosephosphate isomerase</td>
<td>6.84</td>
<td>7.4</td>
</tr>
<tr>
<td>Creatine kinase M</td>
<td>7.07</td>
<td>8.2</td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase</td>
<td>8.06</td>
<td>8.8</td>
</tr>
</tbody>
</table>
Fig. 7.2a. Correlation between the Theoretical pI Value and the Gel Location (Assuming the Superoxide Dismutase is the Cytoplasmic Form)

Fig. 7.2b. Correlation between the Theoretical pI Values and the Gel Location (Assuming the Superoxide Dismutase is the Mitochondria Form)
Chapter 8: Discussion

8.1. A summary of the results

The examination of \textit{in vitro} amylin-stimulated rat skeletal muscle EDL by the analytical 2 DE has led to the discovery of two amylin responsive proteins, designated ARP1 and ARP2 respectively (Chapter 4). The subsequent protein microcharacterisation revealed that ARP1 was one of the three monomers of protein p20 that were identified during this thesis project (Chapter 5). ARP1 was produced by amylin-evoked phosphorylation on protein p20 (Chapter 4). ARP2, proven to be a methionine-containing protein, underwent active biosynthesis with amylin stimulation (Chapter 4).

I have also obtained indirect evidence that CGRP but not insulin evoked the production of ARP1, and that insulin did not affect the ARP1 production by either amylin or CGRP (Chapter 6). This indicates that the production of ARP1 is insulin-independent, and amylin and CGRP share, at least partially, the same signal transduction pathway. As stimulation of the skeletal muscles with either amylin or CGRP resulted in the suppression of the insulin-stimulated glucose incorporation into glycogen (Chapter 6), ARPs are thus likely to be association with the development of insulin resistance. In thesis project, I also made my initiative efforts for the construction of a 2D map of rat EDL muscles; molecular characterisation was performed on 18 EDL proteins.

8.2. ARP1

8.2.1. A mini review of protein p20 and homologous HSP27/28

Since ARP1 has been identified as a novel monomeric form (form 1) of protein p20, a brief review of protein p20 now follows. As reported by Kato et al. (Kato, 1992; 1994a and b) and Inaguma et al. (Inaguma, 1996), protein p20 was accidentally co-purified in large aggregates with small heat shock proteins HSP 28/27 and \( \alpha \),B-crystallin from skeletal muscle. It has 160 (rat) or 162 (human) amino acid residues,
with a single methionine residue at its N-terminus. Protein p20 is considered as a member of the small heat shock proteins, as they show a high degree of amino acid sequence similarities. In rat, protein p20 is expressed widely in different tissues at different concentrations, ranging from 3.1 ng/mg total tissue protein in the cerebral cortex to 12,800 ng/mg in soleus muscle. Although skeletal muscle is the major tissue of protein p20 expression, its concentration varies among different types of skeletal muscles. The concentration of this protein in soleus is approximately 10 times of that in EDL, and 6 times of that in diaphragm. Under normal physiological conditions, it exists as dimers (40 kDa) and large aggregates. Whether it exists in monomeric forms or not is unknown. Monomeric units of protein p20 can be found in homogenates with the presence of reducing agent, as a consequence of the breakdown of the disulfide bond.

As protein p20 is a member of the small heat shock protein family, the available data from the studies of other members such as HSP 27/27 may serve as informative template for analysing the biological functions of ARP1. As described by Kato and Inaguma and their co-workers., (Kato, 1994a and b; Inaguma, 1996), HSP27/28 can be resolved into different forms, and the aggregated form of HSP28 can be converted to a disassociated form through phosphorylation in response to stimuli. This implicates that ARP1 may be derived from amylin-elicited phosphorylation on its protein p20 precursor (discussed below, section 8.2.2). The fact that protein p20 predominantly presents in skeletal muscle, increases its concentration during skeletal muscle development, and changes its concentration in muscle greatly after denervation (Kato, 1994a and b; Inaguma, 1996) suggest that the protein may play a role in muscle physiology. This is in agreement with the data obtained in this study that ARP1 is associated with the development of skeletal muscle insulin resistance (Chapter 6). Furthermore, protein p20 re-distributes from cytosolic to nuclear fraction in response to stress (Kato, 1994a and b; Inaguma, 1996). It is unknown whether ARP1 behaves in the similar manner in response to amylin stimulation. If it does, ARP1 may likely serve as a messenger between the cytosolic and nuclear components, and thus may be involved in the regulation of gene expression by amylin.
8.2.2. Protein phosphorylation: The mechanism for ARPI production

8.2.2.1. ARPI is produced through post-translational modification(s)

ARPI is produced in skeletal muscles in response to both amylin and CGRP stimulation (Chapters 4 and 6). The experimental results indicate that it is produced through a hormone-evoked protein post-translational modification. The evidence includes:

1. three monomeric forms of protein p20 have been identified (Chapter 5). Other forms of protein p20 have also observed by Kato, et al (Kato, 1994a and b). This shows that multiple post-translational modification(s) can occur in this protein;
2. the chromatograms of forms 1, 2, and 3 protein p20 appear similar but not identical (Chapter 5). These differences may be the consequence of different protein modifications;
3. ARPI does not incorporate methionine during its production (Chapter 4), although it has a methionine residue (Kato, 1994b). This indicates that it is not produced through hormone-activated protein bioynthesis;
4. ARPI can be produced in short period of time such as 20 min of mild heat treatment (Chapter 6);
5. its homologous small heat shock proteins undergo phosphorylation in response to stimuli (Kato, 1994 a and b; section 8.2.1).

8.2.2.2. Phosphorylation is likely to be involved in ARPI production

There were reasons that post-translational modification, in particular phosphorylation, might be responsible for the production of ARPI. In addition to those given in the section 5.4, evidence also same from the structure analysis. A strong structural background for post-translational modification in protein p20 has been observed through protein motif analysis using the software ScanProsite-Protein/PROSITE on the Internet (http://expasy.hcuge.ch/sprot/scnpsit1.html). Firstly, two putative phosphorylation motifs were found. They are 13-16 RRAS for cAMP and cGMP-dependent protein kinase mediated phosphorylation and 84-87 SPEE for casein kinase II mediated phosphorylation. A third phosphorylation site, Ser84 by protein kinase C, was also found (Inaguma, 1996) using another software, GENETYX. In protein p20
sequence (Kato, 1994a), there are 23 amino acid residues that can potentially be phosphorylated.

This view was strongly supported by the data from the $^{32}$P$_{i}$ metabolic labelling experiments (section 5.4.3, Chapter 5). With the presence of $^{32}$P$_{i}$ and amylin, a radioactive protein spot corresponding to ARP1 was detected (Fig. 5.4; section 5.4.3), indicating that ARP1 is likely to be phosphorylated with amylin stimulation. It was a pity that control muscles were not included during this group of $^{32}$P$_{i}$ labelling experiment (section 5.4.3), which remains for further exploration.

**8.2.3. The search for ARP1's precursor**

Among the three monomeric forms of protein p20, ARP1 has the lowest pI. It was initially suspected that either forms 2 or 3 might serve as the precursor for the production of ARP1. Potentially, multiple phosphorylations from spot 4 to spot 3 and then to spot 1 (ARP1) might occur, in which spot 3 serves as an intermediate molecule with spot 4 as the primary precursor for the production of ARP1. However, this does not seem to be supported by the subsequent optical density quantification on the three spots using the software ImageMaster 1.1. With or without amylin stimulation, the concentrations of forms 2 and 3 protein p20 were found to remain basically unchanged. This shows no mass shift from either spot 3 or 4 occurs during the production of ARP1, albeit the mass of ARP1 was estimated on average to be greater than 50% of that of spot 4. Thus, I concluded that ARP1 is more likely to be produced from the protein p20 pool (Kato, 1994a).

The precursor of ARP1 remains to be identified. If the proposed active metabolic phosphorylation-dephosphorylation cycle on form 2 p20 (8.2.2.2) does indeed occur, then it may serve as an intermediate protein for ARP1 production.

**8.2.4. ARP1 can potentially be used as a molecular marker in the analysis of amylin action in the common form of insulin resistance**

Although evidence suggests that the $G_{s}$ proteins and PKA may be involved in the signal transduction of amylin (section 1.2.2.1, Chapter 1), the intracellular signalling
cascade of amylin is poorly understood. Whether or not amylin is involved in the development of the common form of insulin resistance and/or type 2 diabetes is controversial (sections 1.2.3 and 1.2.4, Chapter 1). With the isolation and characterisation of ARP1, a molecular probe is now available for further analysis of the amylin signal transduction and its potential involvement in the pathogenesis of diseases such as type 2 diabetes.

A successful example has already been demonstrated in my study. A protein spot corresponding to ARP1 was separated from both amylin- and CGRP-stimulated muscles (Table 6.1, Chapter 6). This strongly suggests that both hormones evoke the production of ARP1, and supports the view that amylin and CGRP are likely to share (at least in part) a same signal transduction pathway (section 6.1, Chapter 6).

Evidence from my study suggests for first time a possible association of protein p20 with insulin resistance. This indicates that a new component in the metabolic cascade involved in the development of insulin resistance may be present, and could be revealed using ARP1 as the molecular probe or marker.

8.3. ARP2

ARP2 responds to amylin by exhibiting an increase in protein synthesis. As shown in Chapter 4, it was identified among some 3,000 proteins separated on a single analytical 2D gel. The molecular weight of ARP2 was estimated to be between 30 and 33 kDa.

Increased gene expression has been observed in type 2 diabetes, which is well recognised as being associated with the common form insulin resistance. Using subtractive cloning, overexpression of the rad gene (ras associated with diabetes) in skeletal muscle samples from type 2 diabetes patients was identified among 4000 cDNA clones screened. This gene was predicted to encode a protein of approximately 29 kDa in size (Reynet and Kahn, 1993).

It is logical that increased biosynthesis of human ARP2 in response to amylin evidences a functional role of amylin in the pathogenesis of the common form of
insulin resistance. This (as well as the case of ARP1) was not investigated due to a lack of clinical samples. Alternatively, inspired by the "similarities" between ARP2 and rad, expression of rad in amylin-stimulated muscle is being investigated using a cDNA probe. Observation of over-expression of rad in amylin-stimulated skeletal muscles would indicate that ARP2 and rad may be identical, which would in turn imply that ARP2 may show an overexpression in human in association with the common form of insulin resistance.

8.4. Possible directions for future work

1. Is ARP1 produced and ARP2 overexpressed in vivo in insulin resistant animals and humans such as the patients with type 2 diabetes?
2. What are the effects of amylin blockers on the amylin-elicited production of ARP1 and overexpression of ARP2?
3. What does ARP1 do? Does it shuttle between cytosolic and nuclear fractions in response to amylin stimulation?
4. What does ARP2 do? Is ARP2 truly the rad protein?
5. Can transgenesis and/or targeted gene destruction animal models provide informative insights into the biological functions of the ARP1 and ARP2?
6. What will occur if ARP1 production and/or ARP2 overexpression is blocked? Can this reverse a insulin resistance state such as the insulin resistance elicited by amylin?
7. Can detection of the phosphorylation on ARP1 and/or quantification of ARP2 be useful indices of insulin resistance?
APPENDIX

A. The written reports based on the data obtained from this study

1. S.C. Lee, C. Knight and G.J.S. Cooper
Proteome analysis of rat skeletal muscle: characterisation of a novel insulin resistance associated form of protein p20
FASEB J. 11, A254, 1997

2. S.C. Lee, C. Knight, J. Douglas and G.J.S. Cooper
Amylin and calcitonin gene-related peptide evoke production of a specific phosphorylated form of protein p20 and insulin resistance in rat skeletal muscle
Submitted to the J. Biol. Chem., 1997

B. Other scientific credits obtained based on the achievements of this project

1. Fellowship of the 17th International Congress of Biochemistry and Molecular Biology, The International Union of Biochemistry and Molecular Biology, 1997

2. Invited for oral presentation at the Young Scientists Program of the 17th International Congress of Biochemistry and Molecular Biology, San Francisco, 1997

3. Honorary member of the American Association for the Advancement of Science (AAAS), 1997
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DeFronzo, R.A. (1992) *J. Cardiovascular Pharmacol.* **20 (suppl 1)**, S1-S16
Foley, JE., Young, DA. and Bell, PA. (1991) The Diabetes Annual/6, pp. 591-610
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120
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126