# The differentiation of chick embryonic myoblasts in primary culture

by Caroline Akrill Evans

Thesis submitted to the Faculty of Science, University of Glasgow
for the degree of Doctor of Philosophy

July 1991



ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Dedicated to my parents and to my sister Susan
with love and thanks for their encouragement and support.

## Acknowledgements

I would like to acknowledge the financial support from the MRC and to thank Prof. M. Houslay and the late Prof. R.M.S. Smellie for the making the facilities of the biochemistry department available.

Thank you to my supervisor, Dr Michael Wakelam for his help and advice during the course of the project. I would also like to thank Dr Graeme Milligan for his advice on the G-protein experiments, Dr Sue Palmer for her constructive comments about Chapter 3 and Dr John Gordon and Dr John Kusel for their help with the FRAP experiments.

Thank you as well to all those people, past and present in labs A20 and A3 for their friendly discussions especially Dr Fiona Black, Fiona Mitchell (for her help with all things G-protein), Dr Robin Plevin, Dr Sue Palmer Andrew Paterson and Sandra Gardner.

I would also like to thank Amy, Teresa Catia and Hassan for their valuable friendship.

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## Abbreviations

| | |
|---|---|
| AF C-18 | 5-N-(octadecanoyl) aminofluorescein |
| cyclic AMP | Cyclic adenosine 5' monophosphate |
| CGRP | Calcitonin Gene Related Peptide |
| *sn*-1,2-DAG | *sn*-1,2-diacylglycerol |
| DMEM | Dulbecco's Modified Eagles Medium |
| FRAP | Fluorescence Recovery After Photobleaching |
| FSF | Fusion stimulating factor |
| G-protein | Guanine nucleotide regulatory binding protein |
| Ins $(1,4,5)P_3$ | Inositol (1,4,5) trisphosphate |
| PAGE | Polyacrylamide gel elctrophoresis |
| PBS | Phosphate buffered saline |
| PDBu | Phorbol 12,13 dibutyrate |
| PI-PLC | Phosphoinositide specific phospholipase |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PtdIns | Phosphatidylinositol |
| PtdIns (4) P | Phosphatidylinositol 4 phosphate |
| PtdIns $(4,5)P_2$ | Phosphatidylinositol 4,5 bisphosphate |
| PtdOH | Phosphatidic acid |
| SDS | Sodium dodecyl sulphate |
| TPA | 12-O-tetradecanoyl-13-acetate |

## Summary

The differentiation of chick embryonic skeletal ~~myoblasts~~ myoblasts results in the formation of myotubes which are the precursors of muscle fibres. The fusion of mononucleated myoblasts represents an apparent switching point in differentiation since it results in both the formation of multinucleated myotubes and the stimulation of muscle specific protein synthesis. The aim of this project has been to examine the biochemical events involved in this process of terminal differentiation by using primary cultures of chick embryonic myoblasts as a model system.

Myoblast fusion is a receptor mediated event apparently coupled to inositol phospholipid breakdown. This results in the generation of the second messenger molecule *sn*-1,2-diacylglycerol. Its role(s) in the process of fusion was investigated.

Many agonist activated cell surface receptors interact with their effector enzymes and ion channels via intermediary G-proteins. The G-proteins present in myoblast membranes were characterised and the involvement of these proteins in fusion was investigated. The differentiation related changes in G-protein levels were also determined.

Chick embryo spinal cord extract is able to stimulate the fusion of chick embryonic myoblasts in primary culture. The nature of these fusion stimulating factor(s) was investigated by biochemical fractionation of this extract. A protocol for the partial purification of the low molecular weight fusion stimulating factor was determined and an alternative method for assaying fusion stimulating ability was investigated.

# Chapter 1
# Introduction

## 1.1 Myoblast differentiation and skeletal muscle development.

Skeletal muscle fibres are terminally differentiated, post-mitotic, multinucleated cells, which are formed during embryonic development as the result of the myoblast differentiation. During this process, myoblasts proliferate, withdraw from the cell cycle, align and subsequently fuse to form multinucleated myotubes which are the precursors of muscle fibres (Holtzer *et al.*, 1958; reviewed in Wakelam, 1985). To date, most of the information about the biochemical events involved in cell fusion processes has come from studies using model membrane systems, such as phospholipid vesicles. However, these are not truly representative of cell membranes in that, for example, they do not have protein components. For this reason, they do not therefore have the potential to yield the complete information about physiological fusion processes. The fusion of myoblasts represents a naturally occuring model system in which to study membrane fusion. The fusion of myoblasts is accompanied by the synthesis of muscle specific proteins which are required for mature muscle cell function. Such proteins include components of the contractile apparatus, for example, actin, myosin and troponin, and the nicotinic acetylcholine and β–adrenergic neurotransmitter receptors (Buckingham, 1977). The apparent temporal correlation between myoblast fusion and muscle specific protein synthesis indicates that fusion represents a defined switching point in the myoblast differentiation and suggests that the initiation of these complementary aspects of terminal differentiation may be co-ordinately regulated during embryonic development. The differentiation of skeletal myoblasts thus provides a model system in which to study the events underlying the regulation of muscle specific protein synthesis.

## 1.2 Systems of study.

The differentiation of groups of myoblasts occurs asynchrously *in vivo* which hinders analysis of the events involved in this process. Most of the available information has therefore come from studies using primary cultures of myoblasts, which are prepared by mechanical or enzymic disaggregation from various embryonic

sources, most commonly chick, quail or rat.(Konigsberg, 1978). These cells undergo reproducible differentiation *in vitro* and are amenable to experimental manipulation. In addition, various cell lines have been established from rat and mouse embryos, the most extensively studied being the L6 and L8 lines which were isolated by carcinogen treatment of rat muscle tissue (Yaffe, 1968; Yaffe and Saxel, 1977). Fusion-negative mutants derived from these cell lines have been used in an attempt to elucidate the events that are involved in myoblast differentiation. However, these cell lines are transformed, and as such, may not truly represent the situation *in vivo*.

Primary cultures of myoblasts have been studied using two types of culture system: stationary and suspension. In stationary culture, which is the standard and most commonly used system, myoblasts are cultured as a monolayer attached to a substratum of gelatin or collagen. Following proliferation and withdrawal from the cell cycle, the myoblasts migrate, align in arrays and fuse to form multinucleate myotubes.

The fusion of myoblasts is not dependent upon their attachment to a substratum and this has allowed myoblast differentiation to be studied using cultures of myoblasts in suspension. In this system, myoblasts aggregate and fuse to form spherical multinucleate cells termed 'myoballs' (Lass and Fischbach., 1976; Knudsen and Horwitz, 1977; Hausman *et al.*, 1986). This is proposed to more truly reflect the situation *in vivo* than the stationary culture, since the myoblasts are able to interact in 3 dimensions, rather than the 2 dimensions available to cells cultured in a monolayer.

A modification of these culture methods allows the synchronisation of fusion, to facilitate the analysis of the events involved in terminal differentiation. Myoblast fusion is a $Ca^{2+}$-dependent event and can be made synchronous by depleting the culture medium of $Ca^{2+}$ (van der Bosch *et al.*, 1972). In this $Ca^{2+}$ depletion method, myoblasts are cultured in a medium containing $Ca^{2+}$ at a low concentration (0.1mM) for 50 hours, during which time they achieve fusion-competence. They are however unable to fuse because the $Ca^{2+}$ concentration is not permissive for fusion. Raising the concentration of $Ca^{2+}$ in the medium results in rapid and synchronous fusion (Wakelam, 1983). Studies using such a system have demonstrated that the optimum

$Ca^{2+}$ concentration for fusion is 1.2mM (van der Bosch *et al.*, 1972; Schudt and Pette, 1975).

## 1.3. Stages in myoblast differentiation.

### 1.3.1. Myoblast interactions prior to fusion.

#### 1.3.1.1. Myoblast-myoblast interaction

Freshly prepared myoblasts proliferate in culture and go through at least one round of DNA replication (Yaffe, 1971). Proliferation ceases when the culture medium is depleted of mitogens (Konigsberg, 1971) and the myoblasts then withdraw from the cell cycle. Subsequent myoblast activities are important in bringing their plasma membranes into contact, in order to facilitate the close apposition, which is required for fusion.

In stationary cultures, myoblasts become aligned in arrays prior to fusion. This is due to myoblast specific cell-cell interaction (Bischoff, 1978) and is termed 'recognition'. This confers the specificity of fusion which is tissue but not species specific. Heterotypic fusion can occur between myoblasts from different species (Yaffe, 1969) but does not occur between myoblasts and other cell types (Holtzer and Bischoff, 1970). In suspension cultures, this recognition event results in the formation of myoblast aggregates in which specific myoblast-myoblast adhesion occurs with the exclusion of fibroblasts (Knudsen and Horwitz, 1977,1978). The recognition process has been studied using fusion-competent myoblasts obtained by the method of $Ca^{2+}$-depletion. These cells were suspended in a balanced salt solution in the presence of either 1.5mM $Ca^{2+}$ or 1mM EGTA (to chelate and thus deplete the medium of $Ca^{2+}$) and examined for aggregate formation.. Myoblast aggregates were obtained in both the presence and absence of extracellular $Ca^{2+}$ (Knudsen and Horwitz, 1978), demonstrating that the adhesion of myoblasts, in common with other cell types, occurs via both $Ca^{2+}$-dependent and $Ca^{2+}$-independent mechanisms. Similar observations have subsequently been made by Gibralter and Turner (1985).

Using this system, the process of recognition and adhesion have been demonstrated to be distinct from the fusion event itself, since differential inhibition of these processes is obtained using various inhibitors of myotube formation (Knudsen and Horwitz, 1978; Knudsen, 1985). For example, treatment of fusion-competent myoblasts with colchicine and cytocholasin B (inhibitors of microtubule disassembly) and elaidic acid or sodium butyrate (short chain fatty acids which may perturb membrane structure) resulted in the inhibition of fusion with no effect on aggregation. Treatment with energy poisons, trypsin and inhibitors of protein synthesis however inhibited both aggregation and subsequent fusion (Knudsen and Horwitz, 1978). These authors thus concluded that myoblast recognition and adhesion is mediated by a protein that is synthesised during the development of fusion-competent myoblasts. It was subsequently proposed to be mediated by a glycoprotein due to its inhibition by tunicamycin, an inhibitor of dolichol phosphate mediated glycosylation of proteins at asparagine linked sites (Knudsen, 1985). This confirmed a previous observation of tunicamycin inhibition of fusion (Gilfix and Sanwal, 1980). This inhibition was partially reversed in the presence of protease inhibitors suggesting that the carbohydrate portion of the molecule does not directly mediate adhesion but acts instead to stabilise it against proteolytic degradation (Olden *et al.*, 1981).

The neural cell adhesion molecule (N-CAM) is a potential candidate for the glycoprotein involved in myoblast recognition-adhesion. It is expressed by muscle cells (Grumet *et al.*, 1982; Rutishauser, 1983) and has been implicated to play a role in myoblast development because there are changes in its isoforms during differentiation (Moore *et al.*, 1987). It is found at the neuromuscular junction (Reiger *et al.*, 1985) where it is thought to mediate the initial interaction between spinal cord neurones and muscle cells in culture (Rutishauser *et al.*, 1983).

N-CAM possesses a covalently attached glycosyl-phosphatidylinositol moiety which serves to anchor the polypeptide portion of the molecule in the plasma membrane (Hemperley *et al* ., 1986; He *et al.*, 1986). Evidence for the involvement of a phosphatidylinositol-glycan linked protein in recognition comes from the observation of

Nameroff *et al.*, (1973) that treating myoblasts in culture with phospholipase C (PLC) inhibited fusion. This was suggested to be due to interference with the process of recognition, which is supported by the finding that the treatment of fusion-competent myoblasts with a specific phosphatidylinositol PLC, inhibited both $Ca^{2+}$-dependent and $Ca^{2+}$-independent aggregation in a dose-dependent manner (Knudsen *et al.*, 1989) and caused the release of an N-CAM immunoreactive cell surface glycoprotein which was prevented in the presence of D-myo-inositol 1 monophosphate. These observations are consistent with the known existence of phosphatidylinositol-glycan linked N-CAM in mouse and human muscle cells (Moore *et al.*, 1987; Barton *et al.*, 1988). Further support for N-CAM involvement comes from the demonstration that anti-N-CAM antibodies inhibit myoblast aggregation. N-CAM, in human muscle cells, posseses a muscle specific sequence in the extracellular domain (Dickson *et al.*, 1987). N-CAM molecules are homophilic and function by binding to N-CAM molecules on other cells (Edelman *et al.*, 1983). The presence of similar muscle specific sequences in the myoblast N-CAM of each species remains to be demonstrated , but if they occur, this homophilic binding could potentially account for the cell type (but not species type) specificity of myoblast -myoblast interaction.

The inhibition of myoblast aggregation by phosphatidylinositol specific PLC is however incomplete (Knudsen *et al.*, 1989) suggesting that recognition and adhesion may involve forms of N-CAM that are resistant to this enzyme. Such isoforms of N-CAM do exist but the result could also indicate that the presence of an additional mechanism for myoblast aggregation. Hausman *et al.*,. (1986) have suggested that myoblast aggregation is mediated by the interaction of a prostaglandin with its receptor. It is accompanied by changes in membrane order, as measured by changes in membrane conductivity and by electron paramagnetic resonance (e.p.r.) using spin-labelled membrane probes (Bonincontro *et al.*, 1987; Santini *et al.*, 1988). The mechanism by which these are achieved, and how cell specificity is conferred remain to be determined.

### 1.3.1.2 Myoblast-substrate interaction.

The myoblast-myoblast cell recognition phenomenon is distinct from the myoblast-substratum interaction observed in stationary culture, in which myoblasts and myotubes adhere and elongate upon the gelatin or collagen substrates. This is mediated by fibronectin, and only occurs in the presence of exogenous fibronectin derived, in culture, from serum or fibroblasts (Chiquet *et al.*, 1981). Myoblast-substratum attachment is not a prerequisite for differentiation since myoblasts in suspension culture fuse and synthesize muscle specific proteins in fibronectin-free medium (Puri *et al.*, 1979, 1980). In addition, this process is unaffected by the presence of either soluble fibronectin or anti-fibronectin antibodies (Puri *et al.*, 1980). This conflicts with previous observations that a cell-free matrix containing fibronectin stimulates the fusion of rat L8 myoblasts (Chen *et al.*, 1978), whilst the addition of exogenous fibronectin led to delayed myotube formation in rat L6 myoblast cultures (Podleski *et al.*, 1979). Chiquet *et al.*,. (1981) concluded that these results were probably due to indirect effects of fibronectin, and suggested that the report of fibronectin stimulation was probably due to its enhancement of myoblast-substratum attachment and migration and thus myoblast-myoblast interaction in the monolayer culture. The fibronectin induced delay observed by Podleski *et al.*, (1979) was demonstrated to occur, not by inhibition of the fusion event itself, but as the result of increased proliferation. This reduced the number of myoblasts emerging from the proliferative population and thus there were fewer post-mitotic fusion-competent myoblasts available for fusion.

Fibronectin has instead been postulated to be involved with the morphogenesis of muscle *in vivo* rather than fusion (Chiquet *et al.*, 1981; Ehrismann *et al.*, 1981). This was based on the observation that myoblasts adhere, align and migrate along oriented streaks of fibronectin *in vitro* . In addition, the myotubes formed upon fusion were unbranched , as is typical *in vivo* but infrequent *in vitro* . They also demonstrated that fibronectin is present in developing chick muscle fibres and propose that it, and perhaps other matrix components, may be involved in contact guidance of developing muscle to direct elongation in the correct orientation.

### 1.3.2. Fusion of myoblasts

Fusion of post-mitotic, fusion-competent, mononucleated myoblasts results in the formation of multinucleated myotubes. It requires the entry of $Ca^{2+}$ (David *et al.*, 1981) and involves transient changes in membrane structure together with rearrangements of cytoskeletal and membrane proteins (Kalderon and Gilula, 1979; Fulton *et al.*, 1981).

#### 1.3.2.1 Assays of fusion.

The fusion of myoblasts to form myotubes may be definitively determined ultrastructurally using transmission electron microscopy (T.E.M.). The criteria for fusion are the presence of clearly resolved U shaped membranes that are reflected at either edge of the site and a lack of amorphous material in the region of cytoplasmic continuity (Bischoff, 1978). However, this method is time consuming and thus impractical for routine use.

For this reason, the cells are usually examined by light microscopy and fusion assayed by visual scoring of the multinucleated myotubes present in the sample . Plasma membranes cannot be resolved at this level of magnification, so instead, the fused cells are defined in terms of the cell shape, size and arrangement of nuclei that are characteristic of myotubes. The percentage of fused cells is defined as the number of nuclei in cells with 3 or more nuclei divided by the total number of nuclei present in myoblasts in the culture (contaminating fibroblasts, identifiable by their characteristic triangular morphology, are excluded), and multiplied by 100. Cells with 3 or more nuclei are taken to be fused. Although cells with 2 nuclei may represent the product of the fusion of 2 mononucleated myoblasts, they could also be replicating myoblasts. These binucleated cells are therefore excluded to avoid errors due to such a misinterpretation. Cultures of myoblasts and myotubes are fixed and dehydrated using ethanol or methanol, and stained with, commonly, Giemsa or acetic orcein to visualise the nuclei. This assay is suitable for use in both monolayer and in suspension cultures.

The myoballs formed in suspension cultures are defined as fused when they are resistant to dispersion by both trypsin and EDTA. These are distinct from aggregated but unfused cells which can be dispersed by treatment with EDTA to remove $Ca^{2+}$ (Knudsen and Horwitz, 1977). They are also defined as fused when they exhibit a smooth edge morphology, which is achieved as a consequence of fusion, and is not observed in aggregated, unfused cells (Lass and Fischbach, 1976; Gibralter and Turner 1985).

### 1.3.2.2. $Ca^{2+}$-dependence of fusion.

Myoblast fusion, in common with other fusion events such as endocytosis and exocytosis (Poste and Allison, 1973) is a $Ca^{2+}$-dependent process. This was first described by Shainberg *et al.*, (1969) who demonstrated that reducing the $Ca^{2+}$ concentration of the culture medium, from 1.4mM to 270$\mu$M, prevented the fusion of rat myoblasts. The same result was obtained in the presence of EGTA to chelate and thereby effectively remove $Ca^{2+}$ from the medium (Paterson and Strohman, 1972). This requirement for $Ca^{2+}$ in fusion is specific and out of other ions tested, such as $Mg^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Ba^{2+}$, $Cu^{2+}$, $Cd^{2+}$, $Sr^{2+}$, $La^{2+}$ and Li+, only $Sr^{2+}$ can replace $Ca^{2+}$ *in vitro* (Schudt *et al.*, 1973).

The $Ca^{2+}$ ionophore, A23187 induces precocious fusion of myoblasts in the period of 1-9 hours before fusion would normally occur in culture (David *et al.*, 1981). $Ca^{2+}$ entry occurs just prior to fusion and is an essential requirement for fusion since it is inhibited by $Ca^{2+}$ channel blockers. It is sensitive to $La^{2+}$ (Entwistle *et al.*, 1988), D600 (David *et al.*, 1981; Rapuano *et al.*, 1990), nitrendipine and the $Ca^{2+}$ channel antagonist BAY K844 (Rapuano *et al.*, 1990) which is consistent with the entry of $Ca^{2+}$ via a voltage dependent $Ca^{2+}$ channel. The exact role of $Ca^{2+}$ in fusion remains to be fully determined but is further discussed in section 1.3.3.5.

### 1.3.2.3. Requirements for fusion.

Plasma membranes are stable non- interacting structures that form a semi-permeable barrier separating the aqueous contents of the cell from the surrounding medium. The integrity of the membrane is maintained by the bilayer structure. This exists as a consequence of the amphipathic nature of the component phospholipids which possess hydrophobic hydrocarbon chains and hydrophilic polar head groups. In the bilayer strucure, the polar phospholipid head groups are in contact with the cytosol and external medium which are present on either side of the bilayer. The hydrocarbon chains oppose each other inside the bilayer and are thus sequestered away from the aqueous phases. As a consequence of this structure, short range interaction between membranes results in membrane-membrane repulsion, which presents a barrier to fusion. Fusion of myoblasts thus represents a thermodynamically unfavourable event, since it requires close apposition of the plasma membranes followed by mixing of their components, at the point of contact, so that these originally separate membranes become continuous and allow cytoplasmic confluence to be established. The nature of the intermembrane repulsive forces and a possible fusion mechanism are discussed more fully in Chapter 3.

### 1.3.2.4. Ultrastructural analysis of fusion.

Ultrastructural examination of fusing myoblasts has provided information about the nature of the membrane changes that occur during the fusion event.. T.E.M. has been used to detemine the changes in plasma membrane morphology associated with myoblast-myotube fusion (Lipton and Konigsberg, 1972). Each fusion site was analysed in serial sections and a tilting stage was used to prevent membrane discontinuity due to an oblique plane of section. The earliest fusion site detected consisted of a cytoplasmic bridge of 120nm in diameter, present at the point of contact,which enlarged in the later stages of fusion. These results are consistent with the initiation of fusion at a single site, which enlarges to allow cytoplasmic continuity and permit mixing of the cytosolic contents of the fused cells.

Further information about the fusion site has been obtained by combining electro-physiological with ultrastructural techniques (Rash and Fambrough, 1972). In these experiments, the time of electrical coupling between adjacent cells was taken as the onset of fusion and samples were fixed at various times subsequent to this. Serial sections of the cultures were then prepared and examined by T.E.M. using a tilting stage. Areas of close membrane apposition (3-5nm), similar to gap junctions, were found in all cells that were electrically coupled but not fused. This electrical coupling was followed by cytoplasmic confluence as fusion proceeded. The authors proposed that these structures act either as the fusion initiation site or to mediate the transfer of information between cells that is necessary for fusion. They also demonstrated that there is no detectable ionic leakage during fusion which indicated that the permeability barrier remains intact during rearrangement of the membrane structure of fusing cells.

### 1.3.2.5. Rearrangements of membrane and cytoskeletal proteins during fusion.

Studies on the surface biochemistry of myoblasts during fusion have demonstrated that there are changes in their distribution and relative amounts. One experimental approach to examine this has been to try and identify surface antigens that are unique to fusion. This has been achieved by raising mouse monoclonal antibodies against either whole muscle cells or membranes prepared from different stages in their differentiation. Antibodies raised against other cell type surfaces, that cross-react with myoblasts, have also been used. For example, 40 monoclonal antibodies against the L8E63 rat myoblast cell line were raised and identified for use as probes for quantitative changes in the antigenic determinants on the myoblast surface during differentiation (Kaufman and Foster, 1984) . The monoclonal antibodies studied fell into 5 distinct temporal classes which probably define different stages in differentiation. Whilst this and other studies demonstrate that there are changes in antigenic expression during differentiation, the determination of their physiological significance awaits the identification of these antigens.

An examination of the variations in the iodination patterns of surface proteins using lacto -peroxidase-catalysed iodination (Moss *et al.*, 1978; Pauw and David, 1979) has revealed that there changes in the relative amounts of high and low molecualr weight proteins during fusion. Decreased amounts of high molecular weight and increased amounts of low molecular weight proteins were detected. This has been proposed to be due to limited proteolysis during fusion (Couch and Strittmatter, 1983), which may be the result of metalloendoprotease activity, since inhibitors of these enzymes, such as, 1,10 phenanthroline, inhibit fusion. It was subsequently demonstrated that these inhibitors act upon a cytosolic protein of 80kDa molecular weight (Couch and Stritmatter, 1984). This may correspond to the $Ca^{2+}$-activated neutral protease which appears in myoblasts at the time of fusion (Kaur and Sanwal, 1981), in which case, the entry of $Ca^{2+}$ into myoblasts prior to fusion may represent a signal for limited proteolysis.

Cytoskeletal rearrangements occur during fusion. These have been examined by detergent extracting myoblasts at different stages of fusion, to reveal the cytoskeletal framework (Fulton *et al.*, 1981). Lacunae were observed during fusion and these were suggested to be regions of the lipid bilayer that are glycoprotein-free. The redistribution of glycoproteins during fusion has been demonstrated using resonance energy transfer (Herman and Fernandez, 1982). These 'glycoprotein-free' regions may correspond to the lipid-rich and particle-free fusion sites observed by Kalderon and Gilula (1979). Such sites could potentially be created by the proteolytic degradation of anchoring cytoskeletal proteins to allow the migration and thus lateral movement of intramembranous proteins away from the site of fusion. The complex carbohydrate portions of glycoproteins present steric hindrance to close membrane apposition, and thus their clearance from the fusion site removes a barrier to fusion. This may involve the $Ca^{2+}$-activated protease that is present in myoblasts at the time of fusion. Following fusion, a stable cross-linked structure that has characteristic myotube morphology is formed. These observations of cytoskeletal rearrangements are consistent with the inhibition of fusion that is obtained with the microtubule + microfilament

disassembly inhibitors, cytocholasin B (Holtzer *et al.*, 1975; Knudsen and Horwitz, 1978), taxol (Antin *et al.*, 1981) and colchicine (Knudsen and Horwitz, 1978). The calmodulin antagonist, trifluoperazine, has been suggested to mediate its inhibition of fusion by preventing calmodulin stimulated cytoskeletal rearrangements (Bar-Sagi and Prives, 1983). This inhibitor may however be affecting the activity of more than one enzyme.

### 1.3.2.6. Involvement of lipids in the fusion process.

The fusion of myoblasts requires that there are transient changes in plasma membrane structure. Lipids are considered to be involved because there are changes in the physical properties of the plasma membrane lipids at the time of fusion. There is, for example, an increase in membrane fluidity prior to fusion (Weidekamm *et al.*, 1976; Herman and Fernandez, 1978) which probably correlates with the decrease in membrane microviscosity that occurs at the onset of fusion (Prives and Shinitsky, 1977). Evidence for the requirement of increased membrane fluidity for fusion also comes from the demonstration that fatty acids such as stearic and elaidic acid, which are predicted to reduce membrane fluidity, delayed fusion whilst those expected to enhance fluidity, such as oleic and linoleic acids brought forward the time of fusion onset (Prives and Shinitsky, 1977; Horwitz *et al.*, 1978).

Further support for lipid involvement comes from experiments where perturbation of the membrane structure modulates fusion. For example, enzymic modification of membrane lipids by treatment of chick myoblasts with phospholipases A or C inhibits fusion (Schudt and Pette, 1976). Such treatment with phospholipase A might be expected to promote fusion, since it results in the generation of lysolecithin which is fusogenic in erythrocyte membranes (Lucy, 1974). In this regard, agents such as retinol, some fatty acids such as myristic acid and arachidonic acid and glycerol mono-oleate, whilst inducing fusion of other cell types, inhibit myoblast fusion (Nakornchai *et al.*, 1981). Fusion is therefore probably mediated by discrete biochemical changes in membrane structure at the site of fusion, which are prevented or

interfered with by the addition of exogenous fusogens that cause general membrane perturbation.

The effects of fusion stimulation on the phospholipid metabolism of fusion-competent myoblasts have been examined using the $Ca^{2+}$-medium depletion system and. Fusion was stimulated by the addition of 1.4mM $Ca^{2+}$ to fusion -competent myoblasts and the incorporation of [$^{32}$P] Pi into phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and sphingomyelin was determined. An increase in [$^{32}$P]Pi was only obtained for phosphatidylinositol (Wakelam and Pette, 1982) and this probably resulted from increased breakdown followed by compensatory resynthesis since there is a loss of radiolabel from phosphatidylinositol (PtdIns) and phosphatidylinositol 4,5 bisphosphate (PtdIns 4,5 $P_2$) on fusion stimulation (Wakelam, 1983). This results in the generation of *sn* -1,2- diacylglycerol and the product of its phosphorylation, phosphatidic acid.

PtdIns 4,5 $P_2$ is located on the inner leaflet of the plasma membrane and is formed by a two step phosphorylation of PtdIns. This involves phosphorylation at postion 4 of the inositol head group to form PtdIns 4 P, followed by phosphorylation at the 5 postion to form PtdIns 4,5 $P_2$. There are corresponding phosphomonoesterases which convert PtdIns 4,5 $P_2$ back to PtdIns. This constitues a futile cycle. Agonist occupation of certain receptor types results in the diversion of PtdIns 4,5 $P_2$ from this cycle to cleavage by phosphoinositide specific phospholipase C (PI-PLC). This results in the loss of the hydrophilic headgroup to generate the second messengers, inositol (1,4,5) trisphosphate ($IP_3$) and *sn* -1,2-diacylglycerol (*sn* -1,2-DAG). $IP_3$ binds to specific receptors on the endoplasmic reticulum to release $Ca^{2+}$ from intracellular stores and thus elevate the levels of cytosolic $Ca^{2+}$. *Sn* -1,2-DAG is a physiological activator of protein kinase C (PKC), a $Ca^{2+}$/phospholipid-dependent protein kinase which exerts its effects by the phosphorylation of proteins on serine and threonine residues (Nishizuka, 1984). This enzyme is considered to be involved in the regulation of many cell processes including exocytosis, growth and differentiation (Nishizuka *et al.*,

1989). The second messenger functions of $IP_3$ and *sn* -1,2 DAG are attenuated by their removal via metabolic pathways which result in their salvage and consequent recycling to PtdIns.

$IP_3$ is removed by the concerted interaction of a series of phosphatases and kinases which result in the generation of inositol. There are 2 potential removal pathways. In the first, $IP_3$ is dephosphorylated by a 5-phosphatase (Ahktar and Abdel-Latif, 1980) to yield Ins (1,4) $P_2$ followed by sequential dephosphorylation steps to form inositol. The second pathway involves phosphorylation by a specific inositol 1,4,5 trisphosphate kinase to form inositol 1,3,4,5 tetrakisphosphate (Hawkins *et al.*, 1986;Irvine *et al.*, 1986). This can be dephosphorylated by a 5-phosphatase to form Ins 1,3,4 $P_3$ which is sequentially dephosphorylated to yield inositol. $Li^+$ blocks the dephosphorylation of inositol monophosphate to inositol which results in the accumulation of inositol monophosphate and an inhibition of resynthesis of phosphatidylinositol by preventing the formation of free inositol. This forms the basis of the $Li^+$ assay for agonist stimulated inositol phospholipid breakdown (Berridge and Irvine, 1984).

*Sn* -1,2-DAG may be removed either by its phosphorylation by DAG kinase to form phosphatidic acid or cleavage by DAG lipase to generate arachidonic acid and monoacylglycerol. The DAG kinase pathway is, however, considered to be the quantitatively more important *in vivo* (Bishop *et al.*, 1986). Phosphatidic acid is then primed by combining with CTP to form CDP-diacylglycerol which reacts with inositol to form PtdIns.

Agonist stimulated inositol phospholipid breakdown has generally been linked to the entry of $Ca^{2+}$ into cells in response to occupation of certain receptors by hormone or neurotransmitter agonists. The entry of extracellular $Ca^{2+}$ is required to maintain the elevated cytosolic $Ca^{2+}$ levels which result from the action of $IP_3$ on intracellular stores. This may be achieved by activation of voltage operated (Lee and Tsien, 1983) or receptor operated (Macdougall *et al.*, 1988) $Ca^{2+}$ channels, or by inhibition of a plasma membrane $Ca^{2+}$ pump (Reinhart *et al.*, 1984). Extracellular $Ca^{2+}$ entry into

myoblasts occurs via a voltage dependent $Ca^{2+}$ channel. This may result from phosphorylation by protein kinase C since such a mechanism has been proposed for D600 sensitive $Ca^{2+}$ channels of myotubes (Navarro, 1987) and Aplysia neurones (De Reimer *et al.*, 1985).

The increase in the levels of *sn* -1,2-DAG could also be important for fusion per se, since it is a known fusogen in model systems; it is an erythrocyte fusogen when added exogenously, although its activity was weak (Ahkong *et al.*, 1973) and has been shown to accumulate during erthrocyte vesiculation (Allan *et al.*, 1975). This fusogen, in common with phosphatidylethanolamine (Sessions and Horwitz, 1973) and the ganglioside GD1a (Whatley *et al.*, 1976) forms the hexagonal II ($H_{II}$) structure, a non-lamellar structure that is more fluid than a bilayer (Cullis *et al.*, 1983). This is proposed to be the preferred state for membrane lipids during cell fusion (Verkleij *et al.*, 1984). Fusion may therefore occur via formation of the $H_{II}$ configuration leading to increased membrane fluidity and fusion. The role of *sn* -1,2-DAG in myoblast fusion, both as a potential fusogen and activator of PKC, is further investigated in Chapter 3.

## 1.4. Developmental regulation of differentiation.

Fusion represents a defined switching point in myoblast differentiation,with the formation of multinucleated myotubes being temporally associated with increased muscle specific protein synthesis . It is likely that these events are under developmental control. There may therefore be some form of some form of diffusible signal stimulating the onset of fusion, the production of which is developmentally regulated.

Prostaglandin $E_1$ ($PGE_1$) has been proposed as a candidate, based on the observations that it promoted precocious fusion and that fusion was inhibited in the presence of indomethacin or aspirin, which act on the cyclo-oxygenase enzyme to prevent prostaglandin synthesis (Zalin and Leaver, 1975). In addition, the prostaglandin antagonist PY1 inhibited fusion suggesting that $PGE_1$ effects on fusion were receptor-mediated (David and Higginbotham, 1981). Zalin and Montague (1974)

had previously detected a transient 10-fold increase in the intracellular levels of cyclic adenosine 5' monophosphate (cyclic AMP) and thus $PGE_1$ was proposed to stimulate fusion by elevating cyclic AMP levels via interaction with its plasma membrane receptor (Zalin and Leaver, 1975). David and Higgenbotham (1981) subsequently proposed that this led to an influx of extracellular $Ca^{2+}$ via activation of a $Ca^{2+}$ channel, which occured as a consequence of its phosphorylation by a cyclic AMP-dependent protein kinase. This conclusion has however been challenged by Schutzle *et al.*, (1984) who were unable to detect this rise in cyclic AMP levels prior to fusion. They demonstrated instead that its levels were elevated following fusion and therefore suggest that this increase is a consequence, rather than a cause of fusion. Furthermore, cyclic AMP has been demonstrated, in some studies, to inhibit fusion (Aui *et al.*, 1973; Wahrmann *et al.*, 1973; Moriyama and Murayama, 1977).

$PGE_1$ has also been proposed to stimulate fusion by two alternative mechanisms. In the first, $PGE_1$ causes an increase in chloride permeability leading to a depolarisation of the plasma membrane which causes the entry of extracellular $Ca^{2+}$ through a voltage dependent $Ca^{2+}$ channel. Acetylcholine has been proposed to stimulate fusion in a similar manner by increasing membrane permeability to $Na^+$ and $K^+$ ions (Entwistle *et al.*, 1988). The second mechanism for $PGE_1$ stimulation of fusion involves receptor-mediated inositol phospholipid breakdown (Farzeneh *et al.*, 1989). In addition to its role in the fusion event itself, the interaction of a prostaglandin with its receptor has been suggested to mediate specific myoblast-myoblast adhesion (Hausman *et al.*, 1986), as discussed in section 1.3.1.

A peak of [$^3$H] $PGE_1$ binding to chick embryo skeletal muscle cultures occurs at the time of fusion (Hausman and Velleman, 1981). Specific binding of [$^3$H] $PGE_1$ was demonstrated since it could be displaced by non-radiolabelled $PGE_1$. The specificity of the receptor for the various prostaglandin species, the identity and source of the native prostaglandin are however unknown. Prostaglandins of the 1 and 2 series are derived from dihomo-γ–linoleic acid and arachidonic acid respectively, which are generally obtained from phospholipids (Vogt *et al.*, 1978). Dihomo-γ–linoleic acid is

not present in myoblasts so the prostaglandin involved in myoblast fusion is therefore unlikely to be $PGE_1$. Arachidonic acid is present at the 2 position of the glycerol backbone of phospholipids and can be generated by ~~either~~ phospholipase $A_2$ cleavage. It can also be generated by DAG lipase cleavage of DAG species that possess arachidonic acid as the fatty acyl chain at the carbon 2 position. Since prostaglandins are derived from membrane lipids, it may be argued that some form of extracellular signal is required to stimulate their production.

The inhibition of fusion obtained using indomethacin is not complete. It delays the time course of fusion onset but, under conditions where indomethacin is continuously present, the final extent of fusion obtained is unaffected. The same result is also obtained if nicotinic acetylcholine receptors are blocked using α-bungarotoxin (Entwistle *et al.*, 1988). This would suggest that there is alternative pathway for fusion stimulation.

Myoblast fusion has been proposed to be a receptor mediated event coupled to inositol phospholipid breakdown, which can be initiated *in vitro* by a fusion stimulating factor(s) present in chick embryo extract (Wakelam and Pette, 1984a,b). Chick embryo extract has been demonstrated to be essential for the fusion of chick myoblasts and probably contains an agonist to stimulate fusion, since in the absence of embryo extract, even in the presence of extracellular $Ca^{2+}$, myoblasts are unable to fuse and no inositol phospholipid breakdown is observed. Further support for the involvement of inositol phospholipid breakdown in myoblast fusion comes from the demonstration that $Sr^{2+}$, at fusion stimulating concentrations, also stimulated the breakdown of these lipids. In addition, when myoblasts are prevented from fusing by treatment with sodium butyrate, no stimulation of inositol phospholipid breakdown was observed (Wakelam and Pette, 1982). Furthermore, inositol phospholipid breakdown was not obtained when fusion-incompetent myoblast (cultured for 25 hours in a medium $Ca^{2+}$ depleted culture) were exposed to fusion stimulating conditions. This would suggest that the ability to respond to fusion stimulation is a feature of fusion-competent cells which is acquired during differentiation.

## 1.5. Aims

The aim of this project has been to try to define the biochemical events involved in the regulation of skeletal myoblast differentiation. This has been examined using primary cultures of chick embryonic myoblasts obtained from the breast muscles of 12-day-old chick embryos. Myoblast fusion represents a key event in this process of terminal differentiation, with the fusion of post-mitotic myoblasts to form multinucleated myotubes being temporally associated with the synthesis of muscle specific proteins required for mature muscle function. It is receptor mediated event apparently coupled to inositol phospholipid breakdown (Wakelam and Pette 1984a,b). PI-PLC cleavage of PtdIns 4,5 $P_2$ results in the generation of the second messengers, $IP_3$ and *sn* -1,2-DAG. The generation of *sn* -1,2-DAG in fusion-competent myoblasts upon fusion stimulation was determined and its role in fusion investigated. It is potentially involved in the fusion process because it is a known fusogen and a physiological activator of PKC. The role of PKC in fusion was examined by use of compounds to modulate its activity.

The transfer of information from many agonist activated cell surface receptor to effector enzymes or ion channels is mediated by a family of guanine nucleotide binding proteins (G-proteins). The G-proteins present in myoblasts at various stages in differentiation were characterised and their possible role in fusion investigated.

Myoblast fusion is accompanied by the stimulated synthesis of neurotransmitter receptors e.g. the β–adrenergic and nicotinic acetylcholine receptors. Given the role of G-proteins in signal tranduction, it is possible that there are changes in their relative amounts and subtypes during differentiation. This was determined by examining membranes prepared from myoblasts at various stages in differentiation.

The differentiation of myoblasts is developmentally regulated and is considered to be under neuronal control. Extracts prepared form chick embryonic spinal cord are able to support the fusion of myoblasts in primary culture which suggests that it contains fusion stimulating factor(s). This extract was thus fractionated by gel filtration chromatography in order to further characterise these factor(s).

# Chapter 2
# Methods

## 2.1 Primary culture of myoblasts.

### 2.1.1 Preparation of myoblasts.

Primary cultures of embryonic myoblasts were prepared by enzymic disaggregation of 12 day old chick embryonic breast muscle using the method of Wakelam (1983). The method is summarised in figure 2.1.1. All procedures were carried out under sterile conditions using a Class II laminar flow cabinet. The breast muscle was obtained by dissection from 12-day-old chick embryos. It was then examined using light microscopy and tweezers were used to gently tease away and thus remove any connective tissue present. The muscle was digested with dispase (neutral protease from *Bacillus polymyxa*). This was performed at 37°C for 30 minutes using a sterile filtered solution of 2mg/ml dispase in Earles salts (pH 7.4). The resulting suspension of tissue was triturated to ensure its dispersal into single cells which were collected by centrifugation at 1000g for 5 minutes in a MSE bench top centrifuge. The cell pellet was washed by resuspending into 50ml '$Ca^{2+}$-free' Dulbecco modified Eagle's medium (DMEM),containing $Ca^{2+}$ at a concentration of approximately 0.5mM and supplemented with 10%w/v dialysed horse serum which inhibits any remaining dispase activity. The cells were collected by centrifugation at 1000g for 5 minutes and then resuspended in $Ca^{2+}$-free DMEM and preplated for 10 minutes in 60mm tissue culture coated plastic dishes in order to selectively remove fibroblasts. During this time the fibroblasts attach to the plastic dish whereas the myoblasts do not so that on removal of the medium from the dish, the myoblasts are present in suspension leaving the fibroblasts attached to the dish. The two cell types are thus separated. The myoblasts were then cultured for 50 hours, until fusion-competence, in '$Ca^{2+}$-free' DMEM supplemented with 10% v/v dialysed heat inactivated horse serum and 2% v/v dialysed chick embryo extract. In later experiments 20μg/ml conalbumin replaced chick embryo extract as described by Neff and Horwitz (1982). Cells were cultured as monolayers using either 35mm tissue culture dishes or multiwell plates (6 or 24 wells per plate) coated with 0.3% w/v gelatin in phosphate buffered saline (PBS) or collagen.. Alternatively, the myoblasts were plated into 60mm tissue culture dishes

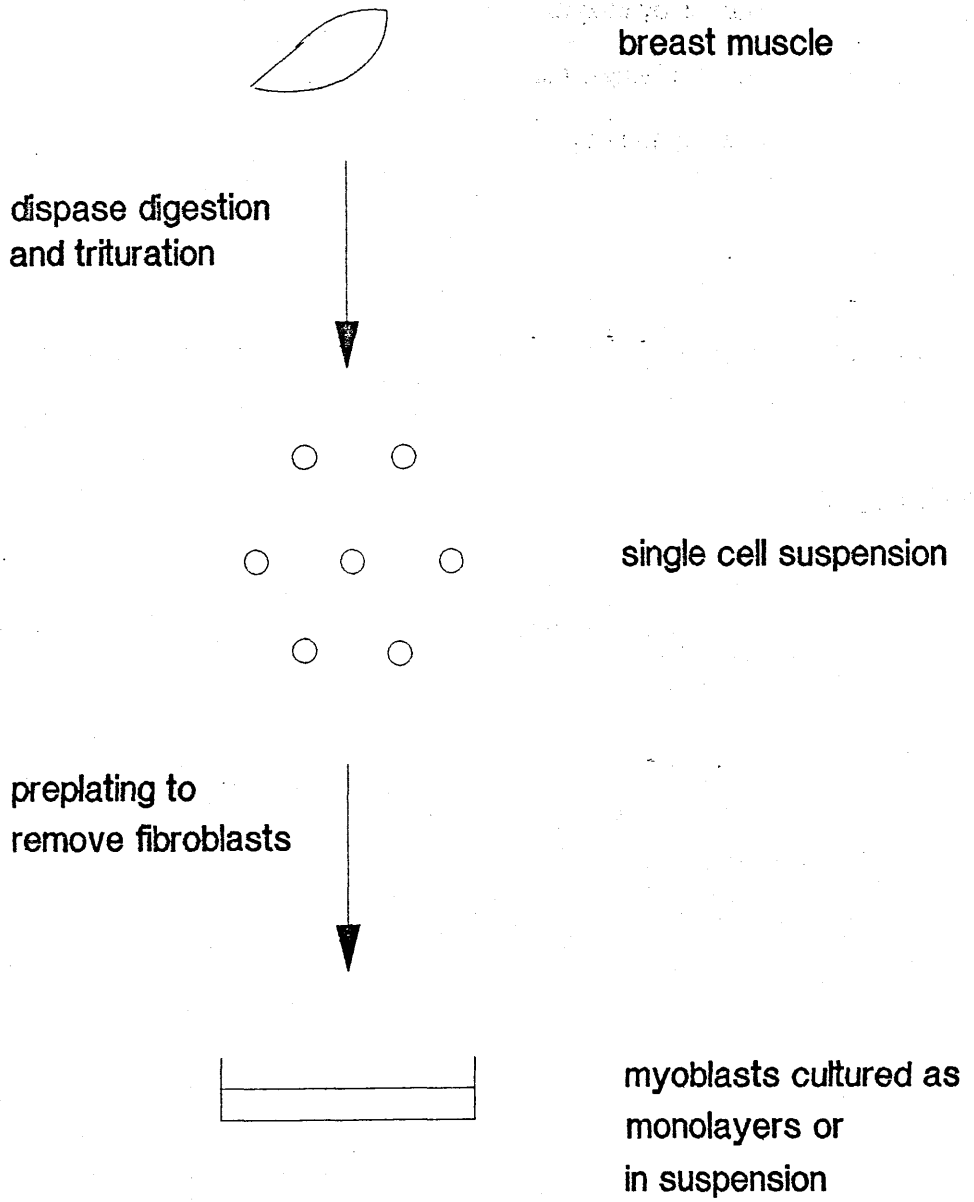
**Figure 2.1.1.**

**The preparation of primary cultures of myoblasts.**

Skeletal myoblasts were prepared from the breast muscle of 12-day-old chick embryos by enzymic disaggregation and trituration to generate a population of single cells which was enriched for myoblasts by the technique of pre-plating to remove fibroblasts.

Figure 2.1.1.

Schematic representation of the preparation of primary cultures of myoblasts from chick embryonic breast muscle



coated with 1.5% w/v gelatin in PBS to which they become loosely attached and may be easily harvested by rinsing using repeated pipetting.

### 2.1.2. Preparation of supplements.

#### 2.1.2.1. Heat inactivation of horse serum.

The horse serum was heat inactivated in order to destroy complement. This was achieved by placing the serum in a water bath at 56°C for 1.5 hours. The heat inactivated serum was then dispensed into 50ml aliquots and stored frozen at -20°C until use.

#### 2.1.2.2. Preparation of $Ca^{2+}$-free supplements.

Essentially $Ca^{2+}$-free horse serum was prepared by extensive dialysis against Earles' salts. The dialysis tubing was prepared by boiling in 20mM EDTA for 20 minutes followed by thorough washing in distilled deionised water. The tubing was either used immediately or stored in 20% (v/v) ethanol at 4°C. A known volume of chick embryo extract or heat inactivated horse serum was aliquoted into dialysis tubing and dialysed against 2 changes of 100x volume dialysis medium of 1:19 dilution of a 20x strength Earles' salts solution in distilled deionised water adjusted to pH 7.4 with 1M sodium hydroxide. The dialysed supplement was sterilised by filtration using a 0.2μm filter and stored at -20°C until use.

### 2.1.3. Fusion assay.

#### 2.1.3.1. Sample preparation.

**(i) Determining the effect of possible inhibitors of myoblast fusion.**

Myoblasts were cultured in '$Ca^{2+}$ free' DMEM/ 20μg/ml conalbumin or 2% v/v dialysed chick embryo extract/ 10% v/v dialysed heat inactivated horse serum until fusion-competence. The cells were cultured in 60mm tissue culture dishes coated with 1.5% w/v gelatin in PBS to which they become loosely attached. At fusion-competence the medium was aspirated and the cells carefully washed twice with'$Ca^{2+}$-

free' DMEM/ 20µg/ml conalbumin to remove non-attached dead cells. The cells were harvested from the dish by repeated pipetting and collected by centrifugation at 1000g for 5 minutes in a MSE Centaur 2 bench top centrifuge. The resultant cell pellet was washed with '$Ca^{2+}$-free' DMEM/ 20µg/ml conalbumin and centrifuged as before. The cells were then resuspended into '$Ca^{2+}$ free' DMEM/ 20µg/ml conalbumin to the required volume, plated into 6 well plates coated with 0.3% w/v gelatin in PBS and incubated at 37°C for 1 hour. The samples were then pretreated with the test compound prior to addition of the fusion stimulating factors (10% heat inactivated horse serum/ 5% chick embryo extract). Myoblasts treated with no addition, and if necessary, the vehicle for addition of the test compound, provided the control samples. Fusion was assessed 20 hours later as described below in sections 2.1.3.2. and 2.1.3.3.

**(ii) Determination of fusion stimulating ability.**

Myoblasts were cultured in '$Ca^{2+}$-free' DMEM/ 2% v/v dialysed chick embryo extract/ 10 % heat inactivated dialysed horse serum until fusion competence. At this time, the cells were removed from the dish by repeated pipetting and collected by centrifugation at 1000g for 5 minutes in a MSE Centaur 2 bench top centrifuge. The resultant cell pellet was washed in' $Ca^{2+}$-free' DMEM and centrifuged as before. This process was repeated. The myoblasts were resuspended to the required volume and plated at a cell density of 15,000 cells/ well into collagen coated 24 well plates in 1ml DMEM. The cells were left to stand for 1 hour in a 37°C incubator. In this time they become attached to the collagen substrate. A 75µl aliquot of the samples to be tested was added to each well. Fusion was assessed 20 hours later as described below in sections 2.1.3.2. and 2.1.3.3.

### 2.1.3.2. Acridine orange staining.

The monolayers of cells were washed four times with 1ml PBS and allowed to air dry. Cells were fixed and dehydrated in 1ml absolute ethanol for 10 minutes. At this time, the ethanol was aspirated and the cells allowed to air dry before rehydration with 1ml 1% (v/v) acetic acid in distilled water for 10 minutes also prevents fading of the fluorescence on visualisation. The samples were then rinsed three times with distilled water which was followed by staining with 1ml acridine orange (0.01% (w/v) in phosphate buffer pH 7.0) for 3 minutes, and destaining with 1ml phosphate buffer pH 7 for 1minute. The stain was differentiated with 1.1% w/v calcium chloride for approximately 30 seconds. This results in yellow-green nuclei and red-orange cytoplasmic staining.

### 2.1.3.3. Determination of percentage fused cells.

The percentage of fused cells in each sample was calculated by taking the average of

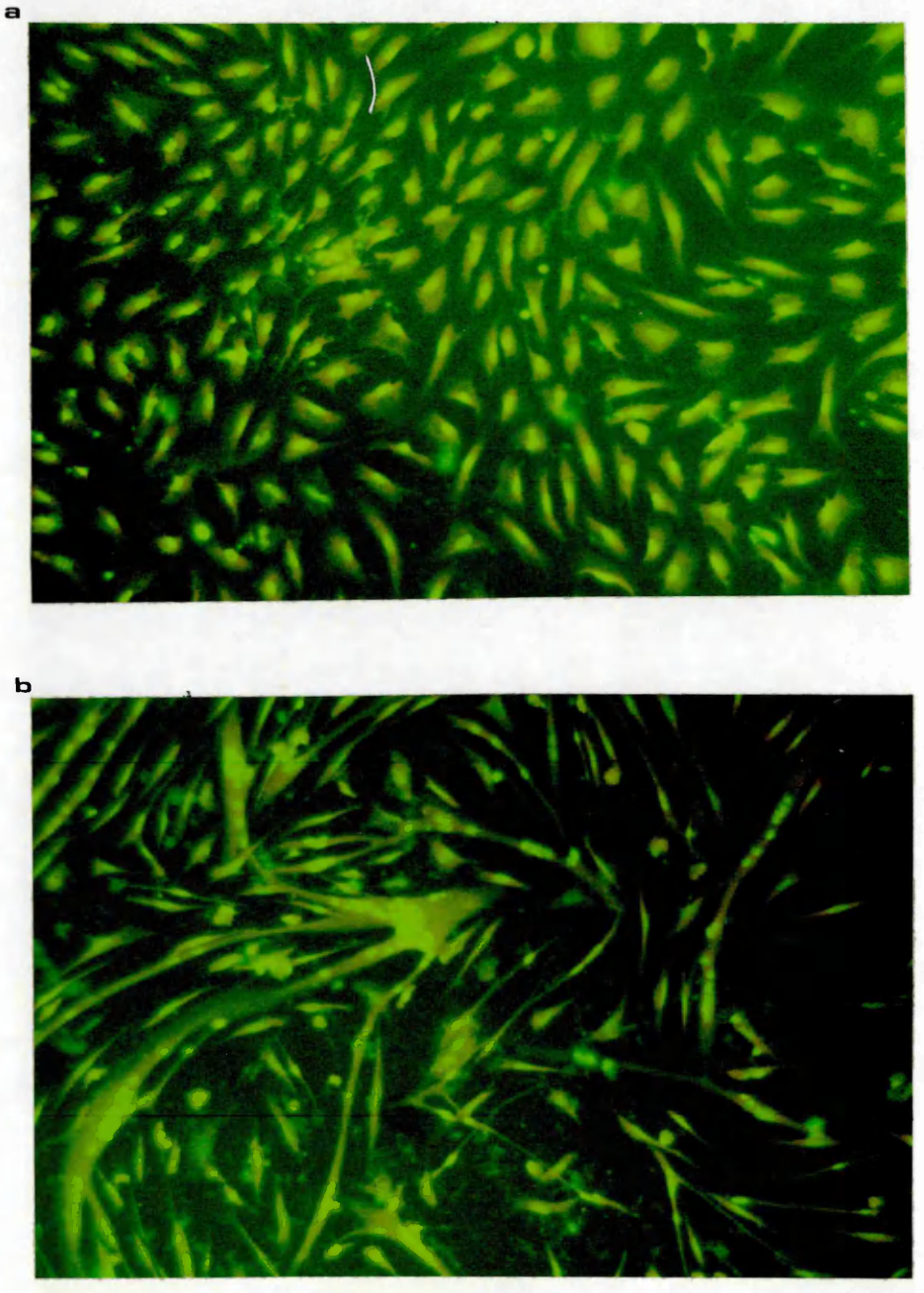
$$\frac{\text{The number of nuclei in cells with 3 or more nuclei} \times 100}{\text{Total number of nuclei}}$$

as determined for 5 fields of view, with 200 - 400 nuclei per field in triplicate samples stained with acridine orange. Cells with 2 nuclei may be the result of either the fusion of 2 mononucleated cells or in the process of mitotic cell division. They are therefore excluded from the determination in order to avoid overestimating the extent of fusion. Samples of non-fused and fused myoblasts are shown in figure 2.1.3.3.a) and b) respectively. It should be noted that the percentage fusion in the 'non-fused' sample is never zero, due to the presence of a few preformed myotubes at the time of plating.

**Figure. 2.1.3.3.**

**Acridine orange staining of myoblast samples.**

Samples of (a) 'non-fused' and (b) 'fused' myoblast were stained with acridine orange. The 'non-fused' sample is composed mainly of mono- and binucleated cells whilst multinucleated myotubes are predominant in the 'fused' sample.



## 2.2. Quantitation of *sn*-1,2-diacylglycerol

Diacylglycerol levels in crude lipid extracts of myoblasts were measured using the method of Preiss *et al.*, (1986).

### 2.2.1. Sample preparation.

Fusion competent myoblasts were harvested from 1.5 w/v gelatin coated 60mm tissue culture dishes as described in 2.1.1. The cells were resuspended in HBG to the required volume and dispensed in 200µl aliquots into plastic scintillation vial inserts and left at 37°C for 30 minutes. 300,000 cells per sample were used. Cells were stimulated with fusion mix (10% horse serum/ 5% chick embryo extract) and after the appropriate time the reaction was terminated by addition of 0.94ml chloroform:methanol (1:2). Samples were capped and vortex mixed and left to extract at room temperature for 20 minutes. 0.31ml water and 0.31ml chloroform were added to each sample. The tubes were capped and vortex mixed prior to centrifugation at 2000g for 5 minutes in a MSE bench top centrifuge to separate the aqueous and organic phases.

### 2.2.2. Assay for *sn*-1,2-diacylglycerol.

A 600µl aliquot of the lower organic phase was transferred to a plastic 2ml Sarstedt tube and dried down under vacuum in a Univap centrifugal evaporator. Mixed micelles were prepared by solubilising an aliquot of the dried crude lipid in 20µl in 7.5% octyl-β D glucopyranoside and 5mM cardiolipin in 1mM di-ethylene-triamine-penta-acetic-acid (DETAPAC) by sonication in a Decon FS 100 bath sonicator. The following additions were then made to the solubilized lipid/octylglucoside samples: 10µl E.coli membranes containing *sn* -1,2-diacylglycerol kinase (diluted 1:4 in 10mM Imidazole/HCl 1mM DETAPAC pH 6.6), 10µl 100mM freshly prepared 100mM dithiothreitol and 50µl incubation buffer (100mM Imidazole/HCl, 100mM NaCl, 25mM $MgCl_2$ and 2mM EDTA, pH 6.6). The reaction was started by addition of 10µl 5mM ATP containing 1.25 µCi [$\gamma$-$^{32}$P] ATP in 100mM Imidazole/HCl, 1mM DETAPAC pH 6.6. All solutions were prepared in double distilled water. Following

vortex mixing the samples were incubated at 30°C for 30 minutes. The reaction was terminated by addition of 450µl chloroform:methanol (1:2) and 20µl 1% (w/v) perchloric acid. Samples were left to extract at room temperature for 15 minutes before addition of 150µl chloroform and 1ml 1% perchloric acid to split the phases. Following capping and vortex mixing the samples were centrifuged at 12,000 rpm for 2 minutes in a Hettisch microfuge. The upper aqueous phase was removed and a further 1ml 1% perchloric acid added to wash the lower chloroform phase. The tubes were capped vortex mixed and centrifuged at 12,000rpm for 2minutes as before. The upper aqueous phase was removed. The lower phase was dried under vacuum in a Univap centrifugal evaporator. The products of the reaction were separated by t.l.c using Silica Gel 60 F254 (Merck) 20cm x 10cm plates preactivated in acetone. Plates were air dried and used immediately. Samples were dissolved in 20µl chloroform:methanol (95:5) and 10µl were spotted over 1cm of the origin. Plates were developed with chloroform:methanol:acetic acid (65:15:5, v/v), air dried and subjected to autoradiography. The band co-migrating with *sn* -1-stearoyl-2-arachidonyl-phosphatidic acid ($R_F$ 0.41) was scraped from the plate and its radioactivity determined by scintillation counting in 6ml Ecoscint.

### 2.2.3. Assay for total lipid phosphorus

Total lipid phosphorus was determined using the free phosphate assay method of Bartlett (1959). A 100µl aliquot of the chloroform phase from cell samples was evaporated to dryness using a Univap centrifugal evaporator. The samples was digested in 600µl 72% w/v perchloric acid at 260°C for 2 hours to release free phosphate. After cooling, 1.6ml distilled water was added to each sample together with 0.5ml (143mM $Na_2S_2O_5$, 15.9mM $Na_2SO_3$ and 0.1% (w/v) 1-amino-2-napthol-4-sulphonic acid). Samples were vortex mixed before addition of 1ml 2.5% w/v ammonium molybdate, followed by vortex mixing and addition of a further 1.3ml distilled water. Samples were heated at 90°C for 30 minutes prior to determining the absorbance at 830nm using an LKB Ultrospec 2 spectrophotometer.

## 2.3. [$^3$H] Phorbol 12,13 dibutyrate binding

Binding of [$^3$H] phorbol 12,13 dibutyrate (PDBu) was determined using monolayers of intact myoblasts cultured in 6 well tissue culture plates. Following aspiration of the culture medium, the myoblasts were washed three times with 1ml PBS. Cells were then incubated with 500µl assay buffer (1mg/ml bovine serum albumin (fraction V) in PBS) containing 10nM [$^3$H] PDBu in the presence or absence of an excess of unlabelled PDBu (10mM)in order to determine non-specific binding and total binding respectively. After incubation for the required length of time, the cells were washed three times with ice cold assay buffer and solubilised in 500µl 0.1M sodium hydroxide. The radioactivity of 500µl cell lysate was determined by scintillation counting in 4ml Ecoscint. Protein was determined by the method of Lowry *et al.,* (1951) as described in section 2.12.

## 2.4. [$^3$H] thymidine incorporation

These experiments were performed to provide a measure of cell proliferation according to the method of Adams (1969). Freshly prepared myoblasts were plated into 24 well plates at an initial density of 25,000 cells per well. The cells were cultured in DMEM/ 20µg/ml conalbumin/ 10% heat inactivated horse serum. 1µCi/ml [$^3$H] thymidine was added with the appropriate additives 24 hours after initial plating. The medium was aspirated 24 hours later and the cell monolayers washed three times with 500µl phosphate buffered saline followed by three washes with 500µl 5% w/v ice cold trichloroacetic acid. The acid insoluble material which contained [$^3$H] thymidine incorporated into DNA was then solubilised in 600µl 0.3M sodium hydroxide. A 500µl aliquot of this was added to 100µl 1.5M hydrochloric acid in a scintillation insert vial and the associated radioactivity in the sample determined by scintillation counting in 4ml Ecoscint.

## 2.5. Fluorescence microscopy and associated techniques.

### 2.5.1. Leitz Microscope and photography.

Fluorescence observations and photomicrographs were obtained with a Leitz Ortholux II fluorescence microscope, a standard camera attachment and Ektachrome 800-1600 ASA professional film.

### 2.5.2. Fluorescence Recovery After Photobleaching.

Monolayer cultures of fusion competent-myoblasts in 0.3% (w/v) gelatin coated 35mm tissue culture dishes were labelled using the fluorescent lipid analogue 5-N-(octadecanoyl) aminofluorescein (AF-C18) which becomes inserted into the plasma membrane. A 5µl aliquot of AF-C18 (2mg/ml in absolute ethanol) was added to 1ml culture medium and the cells were incubated at 37°C for 5 minutes before washing five times with 1ml '$Ca^{2+}$ free' DMEM/ 10mM HEPES/1% w/v lactalbumin hydrolysate. Samples were placed on a heated stage at 37°C and visualised by microscopy using a Leitz (Ortholux) epifluorescence microscope. A layout of the apparatus constructed by Dr. John Gordon and Dr John Kusel, Biochemistry Dept, Glasgow University is shown in figure 2.5.2.1. A highly attenuated argon ion laser beam of wavelength 490 nm was directed into the side of the microscope and focused down through the objective lens onto a small spot of 1µm radius on the surface of the specimen. The fluorescent molecules within this spot were excited, the fluorescence was detected by a photomultiplier unit which converts the number of photons hitting it into an electrical signal which was viewed on an oscilloscope and recorded.

Light of the excitiation wavelength was produced by the attenuated laser beam which allowed the power of the excitation light to be varied. This facility was used to very briefly unattenuate the laser beam for approximately 100 milliseconds which increased the power of the laser by $10^4$ to provide a bleach pulse which caused a proportion of the fluorophores in the spot to be irreversibly bleached. The laser beam returned to low power immediately which was sufficient to excite molecules in that area to fluoresce without bleaching and allowed the fluorescence in the illuminated spot

**Figure 2.5.2.1.**

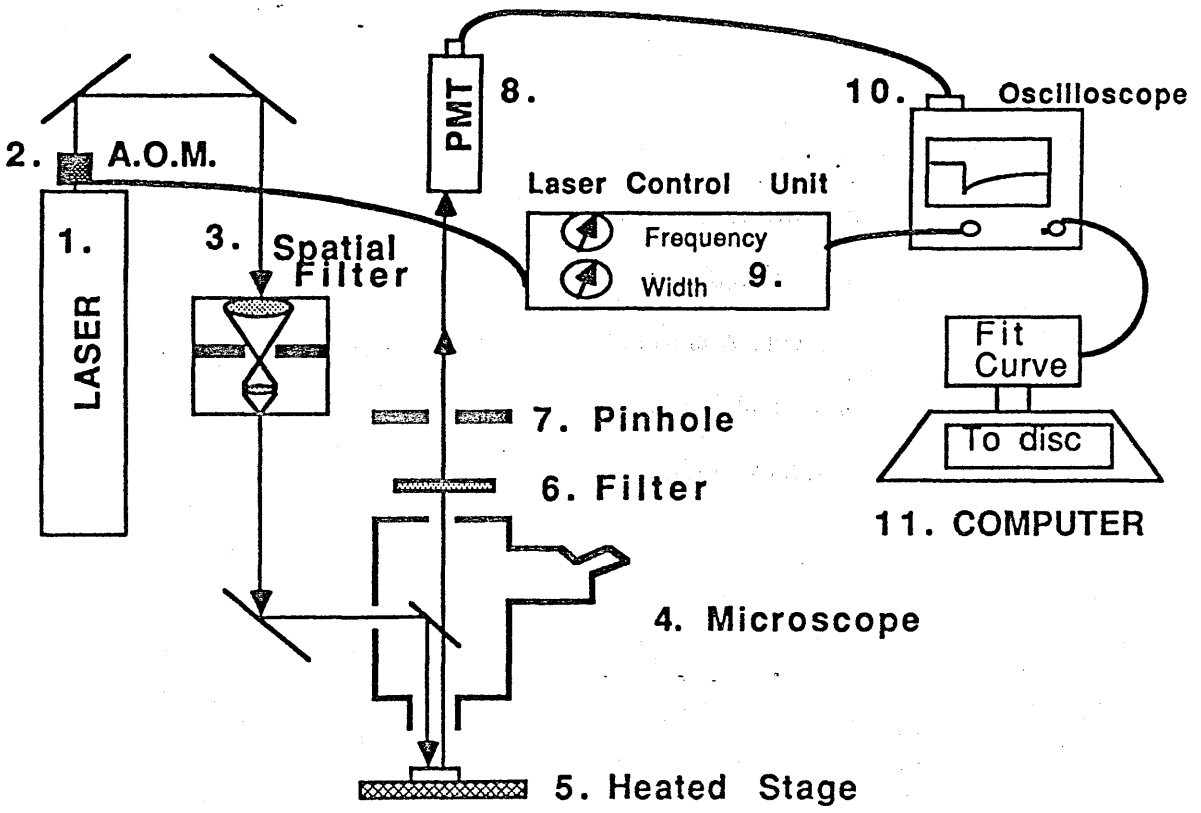
**Apparatus for Fluorescence Recovery After Photobleaching (FRAP).**

Diagramatic representation of the FRAP apparatus constructed by Drs John Gordon and John Kusel of the Biochemistry department , University of Glasgow.

Figure 2.5.2.1.

Fluorescence Recovery after Photobleaching

Layout of apparatus Glasgow 1987




1. LEXEL MODEL 85 Argon ion laser HERTS. U.K.
2. Acousto Optic Modulator 304D COHERENT U.K.
3. Spatial Filter PHOTON CONTROL , CAMBRIDGE U.K.
4 Microscope LEITZ (Ortholux) , LUTON, BEDS. U.K
5. Heated Stage LEITZ.
6. Filters Leitz K530 , k590 .
7. Pinhole 0.5mm Dept Nat. Phil. Workshop Glasgow Univ.
8. PMT Photomultiplier tube Model 9924b THORN EMI U.K.
9. Control Unit Dept. Electronic engineering Glasgow Univ.
10 Oscilloscope DIGITAL STORAGE MODEL 4035 GOULD U.K.
11. Computer Hewlett Packard Model 82927A

to be monitored as a function of time. Recovery of the fluorescence intensity is due to lateral diffusion of unbleached molecules from the surrounding area into the spot. The lateral diffusion coefficient can be calculated using the value obtained for the half time of fluorescence recovery i.e. the time taken for the intensity fluorescence to reach 50% of the final intensity. The fraction of fluorescently labelled molecules mobile in the plane of the membrane can be determined from the percentage recovery relative to the initial fluorescence.

### 2.5.3 Fluorescence Quenching.

The depth of insertion of the lipid probe AF-C18 into the membrane was examined by determining the ability of a non permeant molecule - trypan blue (0.25% w/v) to quench the probe fluorescence as described in Chapter 3.

## 2.6. Determination of total [$^3$H] inositol phosphate generation.

### 2.6.1. Sample preparation.

Myoblasts were labelled for 48 hours prior to fusion-competence with 5μCi /ml [$^3$H] inositol in '$Ca^{2+}$ free' DMEM/ 20μg/ml conalbumin /10% (v/v) heat inactivated dialysed horse serum. The cells were cultured in 60mm tissue culture dishes coated with 1.5% (w/v) gelatin in PBS to which they become loosely attached. After the labelling period the medium was aspirated and the cells carefully washed twice with 3ml Hanks buffered saline to remove the non-attached dead cells. The cells were harvested from the dish by repeated pipetting and collected by centrifugation at 1000g for 5 minutes in a MSE Centaur 2 bench top centrifuge. The resultant cell pellet was washed with Hanks buffered saline and centrifuged as before. The cells were resuspended into Hanks buffered saline containing 1% (w/v) bovine serum albumin (fraction V) and 10mM glucose (HBG) and incubated for 10 minutes at 37°C prior to pelleting by centrifugation and washing in HBG

The cells were resuspended in HBG to the required volume and then left for 30 minutes at 37°C before being aliquoted into plastic scintillation vial inserts containing

the appropriate additions and 10µl 250mM lithium chloride to a final volume of 250 ml per sample. The samples were incubated at 37°C in a shaking water bath for 30 minutes and the reactions terminated by addition of 0.94ml chloroform : methanol (1:2). The vials were capped, vortex mixed and left to extract either for 20 minutes at room temperature or overnight at 4°C.

### 2.6.2. Extraction of [$^3$H] inositol phosphates and [$^3$H] inositol phospholipids.

0.31ml water and 0.31ml chloroform were added to each sample. The tubes were capped and vortex mixed prior to centrifugation at 2000g in a MSE Centaur 2 bench top centrifuge for 5 minutes to separate the aqueous and organic phases.

### 2.6.3. [$^3$H] inositol phosphates measurement.

The accumulation of inositol phosphates, present in the upper aqueous phase, was determined by batch chromatography on Dowex 1x8 formate anion exchange resin. A 0.8ml aliquot of the aqueous phase was transferred to clean tubes and 2.2ml water added to lower the ionic strength. 0.25ml Dowex formate slurry was added to each sample. The tubes were vortex mixed and left for 10 minutes to allow the Dowex to settle out. The water was aspirated and the Dowex washed with a further 3ml water. The Dowex was left to settle out as before and the water was then aspirated. The Dowex was then washed in a similar manner with 2x3ml 60mM ammonium formate/ 5mM sodium tetraborate followed by 3ml water.

Inositol phosphates were eluted from the resin with 0.6ml 1M ammonium formate/ 0.1M formic acid. The tubes were vortex mixed and the Dowex left to settle. A 0.5ml aliquot of the supernatent was removed to a scintillation vial. A further 0.6ml 1M ammonium formate/ 0.1M formic acid was added to the Dowex. The tubes were vortex mixed, the Dowex allowed to settle and 0.6ml supernatent was removed and added to the scintillation vial. This step was repeated once to give a final volume 1.7ml

eluate from each sample. 12 ml Ecoscint or 8ml Optiphase scintillant was added to each vial and the radioactivity in each sample determined by scintillation counting.

### 2.6.4. [$^3$H] Inositol phospholipid measurement.

The lower organic phase contains the [$^3$H] inositol phospholipids. A 250µl aliquot of this phase was dried down in scintillation vials and the radioactivity in each sample determined by scintillation counting in 3ml Ecoscint or 1ml Optiphase.

### 2.6.5. Preparation Dowex 1x8-200 formate.

Dowex 1x8-200 chloride was obtained from the Sigma Chemical Company and converted to the formate form for use as a resin for anion exchange chromatography of inositol phophates.

A known packed volume of Dowex was washed with distilled water and left to settle. The unsettled fines were discarded. This process was repeated twice. The Dowex was transferred to a scintered glass funnel and washed with 20 times its volume of 2M sodium hydroxide, 10 times its volume of distilled water and 5 times its volume of 1M formic acid respectively. Following these treatments the Dowex was washed with 50 times its volume of distilled water until the pH of the slurry was constant at approximately pH 5.5.

## 2.7. Preparation of membrane samples.

### 2.7.1. Cell harvesting.

Fusion-competent myoblasts cultured on 1.5% w/v gelatin in PBS in 60mm tissue culture dishes were harvested. The medium was aspirated from the dishes and the cells carefully washed three times with 3ml PBS to remove dead non-attached cells. Cells were harvested by rinsing from the dish using repeated pipetting, placed into 13.5ml centrifuge tubes and collected by centrifugation at 1000g for 5 minutes in a MSE bench top centrifuge. After aspiration of the supernatent, the cells were washed

by resuspending into PBS followed by centrifugation at 1000g for 5 minutes. The supernatent was again aspirated and the samples stored as cell pastes at -80°C.

### 2.7.2. Membrane preparation.

Cell pastes were disrupted in 10mM Tris/HCl pH 7.5/ 0.1mM EDTA (buffer 1) using a glass-on-teflon homogeniser. The resulting homogenates were centrifuged in a Beckman L5-50B ultracentrifuge in a Ti 50 rotor at 1000g for 10 minutes to remove cellular debris and the resulting supernatent was collected and centrifuged at 40,000g for 10 minutes to generate a membrane pellet. This was washed by resuspending in buffer 1 followed by centrifugation for a further 10 minutes at 40,000g. The pellet was resuspended in 400µl buffer 1 and stored at -80°C until use. Protein was determined by the method of Lowry *et al.*, (1951) using bovine serum albumin (fraction V) as the standard, see section 2.12.

## 2.8. Bacterial-toxin catalysed ADP-ribosylation.

Samples for pertussis-toxin/ cholera-toxin catalysed ADP-ribosylation were prepared as above in section 2.4.2. but omitting the first step of centrifugation at 1000g. The pertussis toxin and cholera toxin were pre-activated by incubating with 50mM freshly prepared dithiothreitol for 1 hour prior to the experiment. At this time, the membrane samples in 20µl Tris/HCl pH 7.5/ 0.1mM EDTA were mixed with 5µl of either thiol-preactivated pertussis or cholera toxin and 20mM thymidine, 0.1mM GTP, 250mM potassium phosphate buffer pH 7.0, 1mM ATP, 20mM arginine hydrochloride and 2µCi [$^{32}$P] $NAD^+$ in a final incubation volume of 50µl. Samples were incubated for 1 hour at 37°C.

Cholera-toxin catalysed ADP-ribosylated membrane samples were collected by the method of sodium deoxycholate/ trichloroacetic acid precipitation. 6.25µl 2% w/v sodium deoxycholate 750µl distilled water and 250µl 24% w/v trichloroacetic acid were added sequentially to the samples followed by centrifugation at 12,000rpm for 15 minutes in a refrigerated Hettisch bench top microfuge. The supernatent was discarded

and the pellet resuspended in 20µl 1M Tris base and 20µl Laemmli buffer. Samples were resolved by SDS-PAGE (10% w/v acrylamide/ 0.27% bisacrylamide) in 16cm x 18cm slab gels run overnight at 60V and 15mA. Autoradiographs were derived from the dried gels using Kodak-O-Mat-S X-Ray film.

Pertussis-toxin catalysed ADP-ribosylated membrane samples were alkylated prior to resolution by SDS-PAGE. The samples were collected by centrifugation at 12,000rpm for 15 minutes in a Hettisch refrigerated bench top microfuge. The supernatent was discarded and the membrane pellet resuspended in 20µl 10mM Tris/HCl pH 7.5/ 0.1mM EDTA. A 10µl aliquot of 50mM freshly prepared dithiothreitol and 5% w/v SDS was added and the samples placed in a boiling water bath for 10 minutes. Following cooling on ice, 25µl 100mM N-ethyl maleimide was added and the samples left to alkylate for 45 minutes. The reaction was terminated by addition of Laemmli buffer prior to resolution by SDS-PAGE (12.5% w/v acrylamide/ 0.06% w/v bisacrylamide) in 20cm x 20cm slab gels run overnight at 120V and 40mA. Autoradiographs were derived from the dried gels using Kodak-O-Mat S X-ray film.

## 2.9. Western Blotting.

Western immunoblotting was performed using a panel of anti-peptide anti-G-protein antisera raised against synthetic peptides corresponding to amino acid sequences unique to the various G-proteins. Each of the antisera was raised in a New Zealand White Rabbit using the synthetic peptide of choice, coupled to Keyhole Limpet Haemocyanin, as the antigen. These were provided by Dr. Graeme Milligan.

### 2.9.1. Membrane preparation.

Membranes were prepared from myoblasts cultured as monolayers in 60mm tissue culture dishes in $Ca^{2+}$ free DMEM/ 10% dialysed heat inactivated horse serum/ 20µg/ml conalbumin for 24 hours 50 hours and 98 hours. These cells are proliferating, fusion-competent and non-fused respectively. The cells harvested at 98 hours are non fused controls to compare with myotubes formed by fusion of fusion-competent

myoblasts cultured in medium containing 1.4mM $Ca^{2+}$ for 48 hours. At the time of harvesting the medium was aspirated and the cells washed three times with 3ml PBS to remove dead, non-attached cells. Cells were then scraped into 2ml PBS and collected into 13.5ml plastic centrifuge tubes. The dish was rinsed with a further 1ml PBS which was added to the centrifuge tube. The samples were centrifuged at 1000g in a MSE bench top centrifuge for 5 minutes to pellet the cells and the supernatent was aspirated. Cells were washed by resuspending in 10 ml PBS followed by centrifugation at 1000g for 5 minutes and removal of the supernatent by aspiration. Harvested cells were stored as cell pastes at -80°C. Membrane samples were prepared as described in section 2.7.2.

### 2.9.2. Immunoblotting.

Samples for immunoblotting using the anti-peptide anti-G-protein antiserum CS1 were collected by the method of sodium deoxycholate/ trichloroacetic acid precipitation. This was achieved by sequential addition of aliquots of 2% w/v sodium deoxycholate (6.25µl) distilled water (750µl) and 24% w/v trichloroacetic acid (250µl) to the membrane sample. Samples were then centrifuged at 12,000rpm for 15 minutes in a Hettisch refrigerated bench top microfuge. The supernatent was discarded and the pellet resuspended in 20µl 1M Tris base and 20µl Laemmli buffer. The samples were resolved by SDS-PAGE (10% (w/v) acrylamide, 0.27% w/v bisacrylamide) in 16 cm x 18cm slab gels run overnight at 60V and 15 mA. Samples for immunoblotting using the anti-peptide antisera SG1 I3B ON1 were first alkylated as this has been shown to enhance the resolution of mammalian pertussis toxin substrates recognised by these antisera. Membrane protein was resuspended in 20µl 10mM Tris/HCl pH 7.5/ 0.1mM EDTA. A 10µl aliquot of 50mM dithiothreitol/ 5% (w/v) SDS was added and the samples placed in a boiling water bath for 10 minutes. Following cooling on ice, 25µl 100mM N-ethyl maleimide was added, and the samples left to alkylate at room temperature for 45 minutes. The reaction was terminated by addition of 20µl Laemmli

buffer prior to resolution by SDS-PAGE (12.5% w/v acrylamide, 0.06% bisacrylamide w/v) in 20cm x 20cm slab gels run overnight at 120V 40mA.

Proteins were transferred from the slab gels to nitrocellulose (0.45mm) using a LKB 2005 Transphor electrophoretic transfer unit for 1.5 hours at 2A. The blotting buffer was 2M glycine 0.25M Tris and 20% v/v methanol. The nitrocellulose sheets were treated for 6 hours at 37°C with a blocking solution of 3% w/v skimmed milk (Marvel) or 3% w/v gelatin in PBS. Sheets were transferred to the primary antiserum (1 in 200) dilution in 1% Marvel in PBS/ 0.2% (v/v) NP40 or 1% gelatin under equivalent conditions. and incubated overnight at 37°C. The primary antiserum was removed and the blot thoroughly washed in PBS/ 0.2% NP40. The secondary antiserum of donkey anti-rabbit IgG coupled to horseradish peroxidase (1 in 200 dilution) was added and left at 37°C for 4 hours. After removal of the secondary antiserum the blot was thoroughly washed with PBS/ 0.2% NP40 followed by PBS for 10 minutes each. The antibody complex was detected using orthodiansidine hydrochloride (0.025mg/ml in PBS) as the substrate for horseradish peroxidase.

## 2.10. Assay of GTPase activity.

Samples of membrane protein (10µg in 20µl 10mM Tris/HCl pH 7.5/ 0.1mM EDTA) were added to a ice cold reaction mix of 10mM creatine phosphate, 1mM ATP, 0.1mM App(NH)p, 1mM ouabain, 100mM NaCl, 5mM $MgCl_2$, 2mM freshly prepared dithiothreitol, 0.1mM EDTA, 20mM Tris pH 7.5, 0.5mM GTP with 5 units creatine kinase and approximately 50,000 cpm [$\gamma$–$^{32}$P] GTP per assay tube, in a final incubation volume of 100µl. The samples were incubated in the absence and presence of agonist. to determine the basal and agonist stimulated GTPase activity respectively. Non-specific GTPase activity was determined in the presence of 1mM GTP. Sample incubation was carried out at 37°C for 20 minutes. At this time, the reaction was terminated by placing the samples on ice. An aliquot of 0.9ml activated charcoal (5% w/v in 20mM orthophosphoric acid) was added to each sample to remove non-hydrolysed [$\gamma$–$^{32}$P] GTP. The tubes were then capped and vortex mixed prior to

centrifugation in a microfuge for 5 minutes. A 0.5ml aliquot of the supernatent was removed into a scintillation insert vial and the associated radioactivity determined by Cerenkov counting.

## 2.11. Electroporation.

Myoblasts were labelled for 48 hours prior to fusion competence with 5μCi/ml [$^3$H] inositol in '$Ca^{2+}$ free' DMEM/ 10% w/v heat inactivated dialysed horse serum/ 20μg/ml conalbumin. Susequent harvesting and washing of the cells was performed as described in section 2.7.1.with the modification of permeabilisation buffer (120mM KCl, 0.1mM EGTA, 6mM $MgCl_2$, 20mM Na Hepes, 2mM $KH_2PO_4$ 1mg/ml bovine serum albumin (fraction V) and 2.5mM ATP) replacing Hanks buffered saline. Cells were resuspended in 1.6ml permeabilisation buffer and electropermeabilised using discharge pulses of 1600V/cm. The cells were then diluted to the required volume with permeabilisation buffer and dispensed into plastic scintillation insert vials containing the appropriate additions and10μl 250mM LiCl to a final volume of 250μl. The samples were incubated in a shaking water bath at 37°C for 30 minutes and the reactions terminated by addition of 0.94ml chloroform : methanol (1:2). The vials were capped, vortex mixed and left to extract for 20 minutes at room temperature or overnight at 4°C. Samples were subsequently processed as described in sections 2.6.2. to 2.6.4.

## 2.12. Protein determination

The method is essentially that of Lowry *et al.*, (1951). A standard curve for the assay was constructed using 0, 3μg, 5μg, 7.5μg, 10μg, 15μg 20μg and 30μg bovine serum albumin (fraction V). Aliquots of the unknown sample were assayed in triplicate. 2% w/v sodium carbonate in 0.1M sodium hydroxide, 1% w/v copper (II) sulphate and 2% w/v sodium potassium tartrate (100:1:1) were freshly mixed together and 1ml added to each sample. Samples were vortex mixed and left at room temperature for 10 minutes. A 100μl aliquot of Folin-Ciocalteau reagent (1:2 dilution in distilled water) was added to each sample. Samples were left at room temperature

for 30 minutes to allow the coloured complex to fully develop. The absorbance of the samples at 750nm was determined spectrophotometrically using a LKB Ultrospec 2.

## 2.13. Sephadex G100 gel filtration chromatography.

### 2.13.1. Column packing.

A column of 90cm in height and 1.6cm in diameter was poured using a 75% v/v settled gel that was a fairly thick slurry. Sephadex was supplied as a dry powder with a bed volume of 15 - 20ml/g dry powder. 15g dry powder were swollen in excess (500ml) 100mM acetic acid - the eluting buffer - to give an approximate bed volume of 225 - 300ml. The gel was swollen using a boiling water bath overnight which also served to deaerate the buffer. Adaptors which are adjustable column end pieces were used to support the bed surface and protect the bed from any insoluble particles in the sample. The column was run at 4°C with a downward arrangement of flow. After pouring and settling of the column, three column volumes of 100mM acetic acid were passed through the column, in order to stabilise the bed and equilibrate it with the eluent buffer.

### 2.13.2. Checking column homogeneity.

The homogeneity of the column was checked by running through a sample of a coloured substrate. 0.5ml Blue Dextran at a concentration of 2mg/ml in 100mM acetic acid was used. The quality of the packing was checked by watching the progress of the band of this substance through the column bed. Skewing of the band represents a fault in the column but this was not seen thus indicating that there was homogeneity of packing. The elution of Blue Dextran from the column was followed by measuring the absorbance at 280nm. The flow rate was 0.3 ml/min and 1.5ml fractions were collected.

### 2.13.3. Determination of the void volume.

Sephadex G100 has a fractionation range of 4 - 150kDa. Molecules with molecular weights above the upper limit of this range, which is the exclusion limit, are totally excluded from the gel matrix and elute at the void volume. The elution volume Ve was determined for Blue Dextran (2000 kDa molecular weight) and represents the void volume Vo of the column.

### 2.13.4. Determination of total column volume.

The total column volume was determined by running the potassium dichromate of molecular weight 294 through the column. Its molecular weight is less than 4kDa, the lower limit of the molecular weight fractionation range, and it therefore eluted at the bed volume or total volume of the column. 0.5ml 0.05M potassium dichromate was loaded onto the column and its elution monitored by visual inspection since it has a bright orange colour. The centre of the eluent peak was determined and the total column volume calculated.

### 2.13.5. Molecular weight calibration.

Sephadex G100 has a molecular weight fractionation range between 4-150kDa. The column was calibrated by determining the elution volumes, Ve, for a series of protein standards. The calibration curve was obtained by plotting log10 molecular weight against Ve/Vo and is shown in fig. 2.13.5.1. The standard proteins used were horse heart cytochrome C, bovine carbonic anhydrase, bovine serum albumin, yeast alcohol dehydrogenase and sweet potato β amylase with molecular weights 12.4, 29, 66, 150 and 200kDa respectively. The standards bovine carbonic anhydrase and yeast alcohol dehydrogenase were mixed together and run down the column as were horse heart cytochrome C and sweet potato β amylase. 0.5ml each sample was loaded onto the column and 1.5ml fractions collected. Elution profiles were obtained by monitoring absorbance at 280nm of the eluent and the elution volumes determined.

### 2.13.6. Gel filtration chromatography of extracts.

Extracts of chick embryonic brain and spinal cord were prepared from 12 day old chick embryos. Following dissection from the embryo, the tissues were homogenised in 50% w/v PBS followed by centrifugation at 100,000gfor 2 hours. The supernatent containing the soluble fraction of the brain and spinal cord homogenates was collected and frozen. Samples were prepared for gel filtration chromatography by lyophilisation of 2ml sample followed by dissolving in 1ml eluent buffer 100 mM acetic acid. The sample was run through the column at a flow rate 0.3ml/minute and 1.5ml fractions collected. The elution of the sample from the column was determined by monitoring the absorbance at 280nm. Each fraction to be tested for fusion stimulating ability was lyophilised. The factor(s) present were dissolved in 0.5ml 1mM acetic acid.

### 2.14. Reverse phase HPLC analysis.

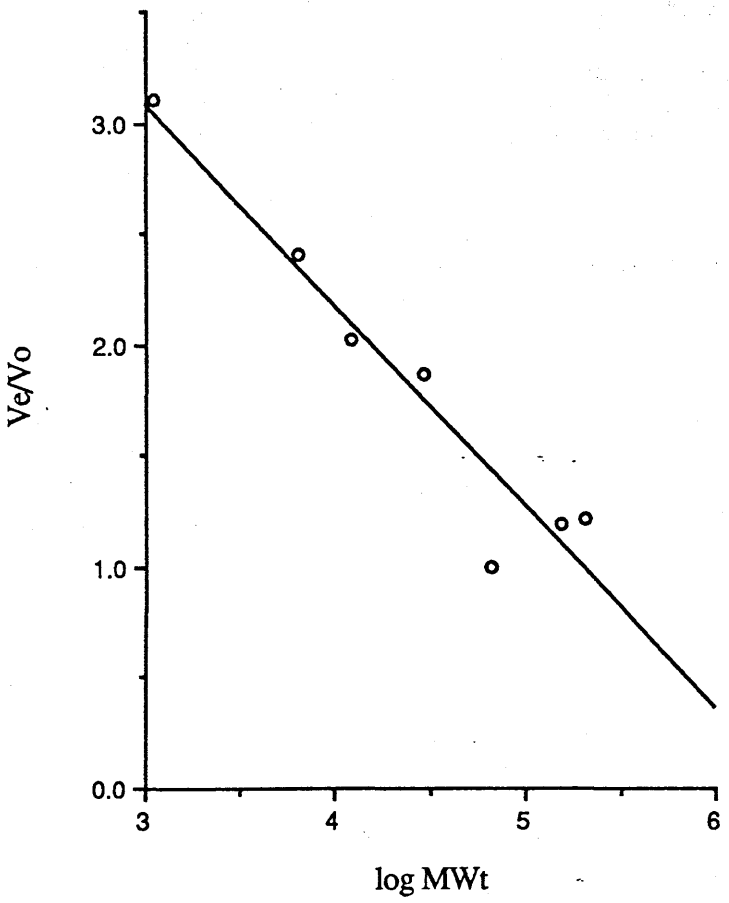
A Waters μ bondapack C18 reverse phase column (4.6mm x 250mm 10μm particle size) was used with the buffers 0.1% trifluoroacetic acid in distilled deionised water (buffer A) and 0.1% trifluoroacetic acid in Propan-2-ol (buffer B). A linear gradient of buffer A:buffer Bfrom (90:10) to (10:90) over 80 minutes was used with a flow rate of 1ml/min. Samples were loaded using a Rheodyne injector (100μl sample loop). The HPLC apparatus consisted of a LKB 2152 controller, 2 LKB 2150 pumps a LKB Uvicord SD UV monitor and a LKB Superrac collection rack. Elution of peptides was determined by monitoring the absorbance of the eluent at 215nm.

**Figure 2.13.5.1.**

**Calibration curve for Sephadex G100 column.**

The elution volume for blue dextran (Vo) and those (Ve) for the standard proteins horse heart cytochrome C, bovine carbonic anhydrase, bovine serum albumin, yeast alcohol dehydrogenase and sweet potato β amylase were determined using 10mM ammonium acetate (pH 4.0 with acetic acid)as the eluent.

Figure 2.13.5.1.
Sephadex G100 calibration curve



# Chapter 3
# The role of *sn*-1,2-diacylglycerol in myoblast fusion

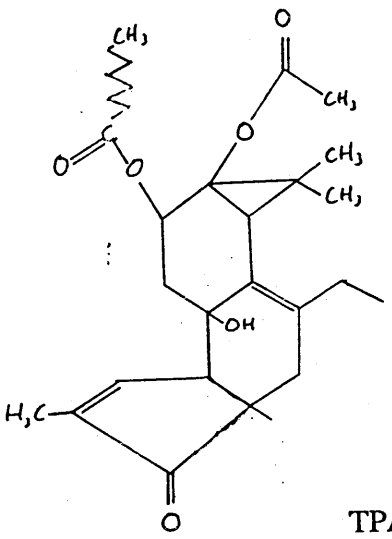
## Introduction

The fusion of myoblasts requires that there are transient changes in membrane structure together with cytoskeletal rearrangements (Kalderon and Gilula, 1979; Fulton *et al.*, 1981). It is $Ca^{2+}$-dependent receptor-mediated event that is apparently coupled to inositol phospholipid breakdown (Wakelam and Pette, 1984a,b). The cleavage of phosphatidylinositol 4,5 bisphosphate (PtdIns 4,5 $P_2$) by phospholipase C results in the generation of the second messengers, inositol trisphosphate ($IP_3$) and *sn* -1,2 diacylglycerol (*sn* -1,2-DAG) (Berridge *et al.*, 1984). The generation of *sn* -1,2-DAG in the membranes of fusion-competent myoblasts may be a key event in the fusion process because (i) *sn* -1,2-DAG and phosphatidic acid, which is the product of its phosphorylation by *sn* -1,2-DAG kinase act as fusogens in model membrane systems (Ahkong *et al.*, 1973; Sundler and Papajadopoulos, 1981) and (ii) it is a physiological activator of protein kinase C (PKC), a $Ca^{2+}$/phospholipid dependent serine threonine protein kinase whose activation is considered to play a key role in many cell processes, including growth and differentiation (Nishizuka, 1984, 1986). The changes in its levels upon fusion stimulation of fusion-competent myoblasts were thus determined using an assay specific for *sn* -1,2-DAG.

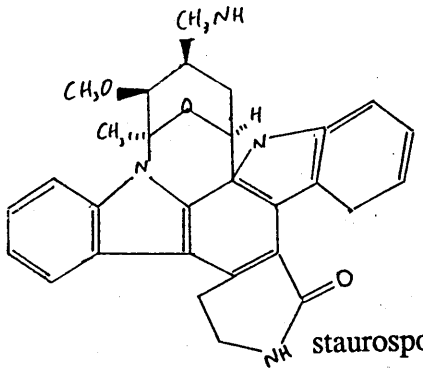
PKC is an allosteric enzyme possessing a regulatory domain which, in the resting state, keeps the catalytic domain inactive. Activation occurs when $Ca^{2+}$, phospholipid and *sn* -1,2-DAG bind to the regulatory domain and cause a conformational change in the enzyme which exposes its active site in the catalytic domain. The role of PKC in myoblast fusion was examined using compounds which modulate its activity. Phorbol esters, of which 12-0-tetradecanoyl-13-acetate (TPA) is an example, activate the enzyme directly by substituting for *sn* -1,2-DAG (Castagna *et al.*, 1982). Long term treatment of cells with TPA has been demonstrated to result in the down-regulation of PKC (Rodriguez-Pena and Rozengurt, 1984) by increasing its rate of degradation (Young *et al.*, 1987). Myoblasts were treated with 100nM TPA, from 24 hours in culture to fusion-competence, in order to determine the effect of PKC down-regulation on fusion. The changes in PKC levels, resulting from this treatment,

Figure 3.1.

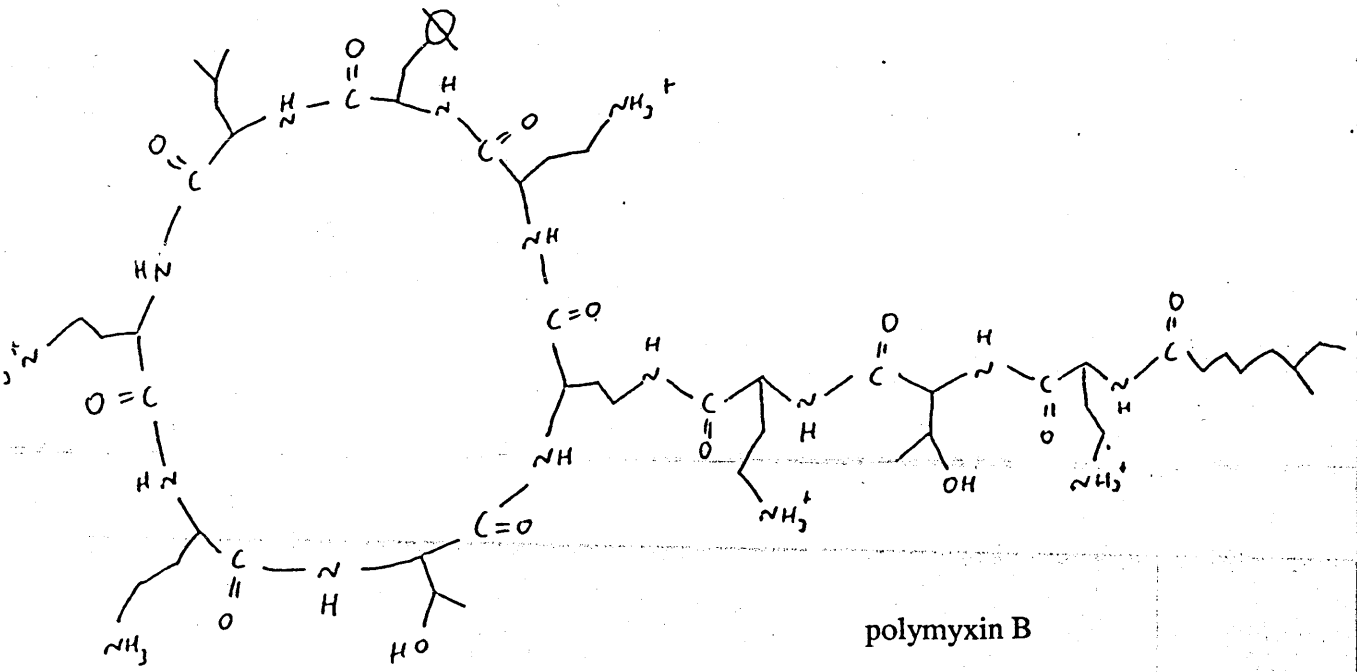
Structural formulae of TPA, staurosporine and polymyxin B



TPA



staurosporine



polymyxin B

were examined by determining specific binding of the radiolabelled phorbol ester, [$^3$H] phorbol 12, 13 dibutyrate (PDBu).

Polymyxin B and staurosporine are putative inhibitors of PKC (Kuo *et al.*, 1983; Tamaoki *et al.*, 1986). They exert their effects on PKC by different mechanisms: polymyxin B inhibits PKC by binding to the regulatory domain (Kuo *et al.*, 1983) whereas staurosporine interacts with the catalytic domain (Huang, 1989). Their effects on myoblast fusion were determined. The structural formulae of these putative PKC inhibitors, together with that of TPA, are shown in fig. 3.1.

Experiments were then performed to further investigate the effects of these PKC modulators on fusion. A possible model of myoblast fusion is discussed to explain the data presented.

## Results

### 3.1. Generation of *sn*-1,2-diacylglycerol

The levels of *sn* -1,2-DAG in crude neutral lipid extracts prepared from fusion-competent myoblasts were were measured using an assay based on the enzymatic conversion of *sn* -1,2-DAG to phosphatidic acid by *sn* -1,2-DAG kinase. Lipid extracts were dispersed into mixed detergent micelles and incubated in the presence of [$\gamma$-$^{32}$P] ATP, together with membranes from an overproducing strain of *E.coli*, which contain *sn* -1,2-DAG kinase, as approximately 15% of the membrane protein. The products of the reaction were separated by t.l.c. and the radioactivity associated with the band co-migrating with 1-stearoyl-2-arachidonyl-*sn* -glycerophosphate determined. This species of *sn* -1,2-DAG was used since most PtdIns 4,5 $P_2$, which is assumed to be the major precursor of *sn* -1,2-DAG, has this fatty acid composition. Figs. 3.1.1.a) and b) show representative time courses of *sn* -1,2-DAG production in fusion-competent myoblasts stimulated with 'fusion mix' (1.4mM $Ca^{2+}$, 10% (v/v) heat inactivated horse serum and 5% (v/v) chick embryo extract). An increase in *sn* -1,2-DAG, in response to the agonist was apparent by 5 seconds, which was the earliest time point measured.

**Figure 3.1.1.a) and b)**

**Time course of *sn*-1,2-DAG production in fusion mix stimulated myoblasts a) 0 - 600 seconds b) 0 - 30 minutes.**

Fusion-competent myoblasts were washed and resuspended in HBG prior to incubation for the times indicated, with fusion mix (1.4mM $Ca^{2+}$, 10% (v/v) heat-inactivated horse serum, and 5% (v/v) chick embryo extract) (open circles) or HBG (closed circles). The results are mean ± S.D. from single experiments (n=3) typical of 3.

Figure 3.1.1.a)

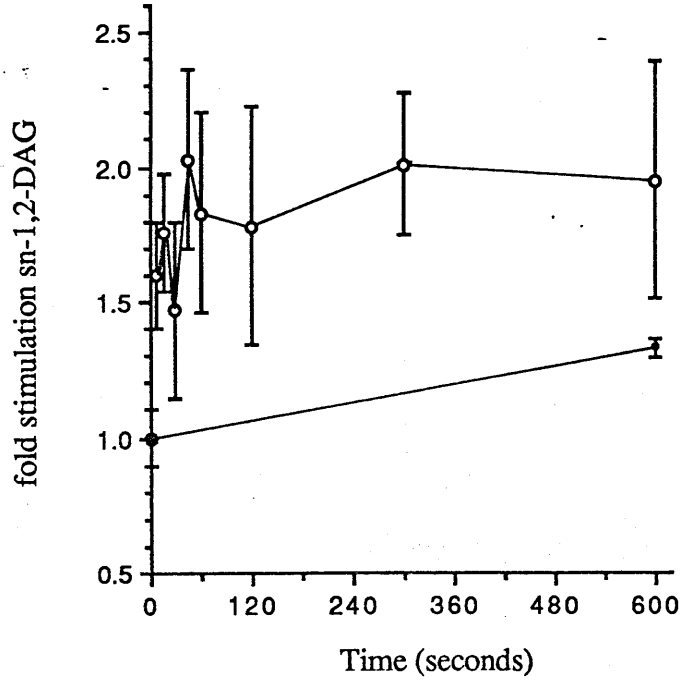
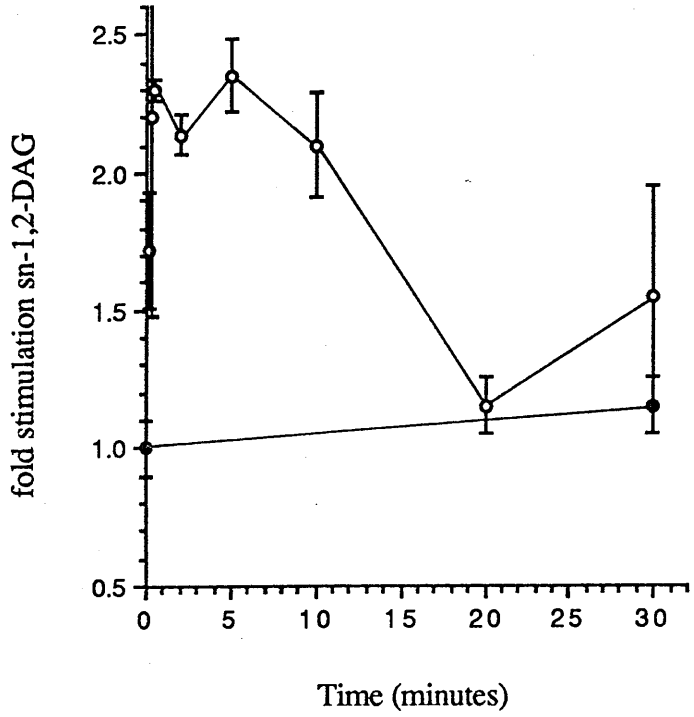



Fig. 3.1.1.b)



Levels of *sn* -1,2-DAG levels remained elevated at subsequent time points and returned to control i.e. basal unstimulated levels by 20 minutes.

Assuming 100% conversion of DAG to phosphatidic acid, the basal level of *sn* -1,2-DAG in fusion-competent myoblasts is 0·09 ± 0·017 nmol DAG/ 100nmole phospholipid i.e. mol% (mean ± S.E.M. of 3 separate experiments). This rises by 2-fold on stimulation with fusion mix to 0·175 ± 0·028 mol % (mean of 3 separate experiments ± S.E.M.). Phospholipid phosphorus was determined using the method of Bartlett (1957).

### 3.2 Examination of the effect of long term treatment of myoblasts with the phorbol ester TPA.

Long term treatment of cells with TPA has been demonstrated to result in down-regulation of PKC in many cell types (Nishizuka, 1989). The possible involvement of PKC in myoblast fusion was therefore examined, by determining the effect of long term treatment of myoblasts with TPA from 24 hours after initial plating to fusion-competence. TPA was kept as a stock solution of 5mg/ml in DMSO and diluted into absolute ethanol as the vehicle for addition to cells. The vehicle concentration did not exceed 5µl/ml.

#### 3.2.1. Effect of long term treatment with TPA on myoblast fusion.

The effect of long term treatment of myoblasts with TPA was examined by treating myoblasts with TPA from 24 hours after initial plating in '$Ca^{2+}$-free' medium to fusion-competence. At this time, myoblasts were harvested and fusion initiated by plating into fresh medium containing 'fusion mix' (1.4mM $Ca^{2+}$ together with 10% (v/v) heat inactivated horse serum and 5% (v/v) chick embryo extract). Fusion was assessed 20 hours later. The results obtained are shown in fig. 3.2.1.1. and the data are expressed in terms of fusion index. The percentage fusion in the 'fusion mix' sample was arbitrarily assigned a fusion index of 1. These cells were cultured in the presence of fusion mix at fusion-competence and no addition was made at 24 hours

after initial plating. This sample represents the amount of fusion obtained in the absence of test additons. Control, non-fused cells were cultured in parallel in '$Ca^{2+}$-free' medium for the duration of the experiment. Their fusion index was 0·09 ± 0·01 (mean of 3 separate experiments ± S.E.M.) due to the presence of pre-formed myotubes at the time of plating.

The effect of long term treatment of myoblasts with TPA was examined by incubation of the cells in the presence of 100nM TPA from 24 hours after initial plating in '$Ca^{2+}$-free' medium to fusion-competence. Fusion was initiated by harvesting and plating these cells into fresh medium containing fusion mix. Fusion was determined 20 hours later and expressed relative to that of cells not pretreated with TPA (fusion index = 1, see above).

Long term TPA treatment resulted in an inhibition of fusion. The extent of fusion in TPA pretreated cells was not significantly different from that of 'non-fused' control cells. The vehicle for addition of TPA to myoblasts or the PKC-inactive structural analogue of TPA, β-phorbol, were without effect on fusion. This suggests that TPA-induced inhibition of myoblast fusion is due to PKC activation or PKC down-regulation.

The dose-dependence of this inhibition by TPA was determined using a range of concentrations of TPA ($5 \times 10^{-7}$M - $10^{-10}$M). The results are shown in fig. 3.2.1.2. and the data are expressed in terms of fusion index. TPA inhibited fusion in a dose-dependent manner with an IC 50 value of 8·3 ± 1·3nM (mean of 3 separate experiments± S.E.M.). Maximal inhibition of fusion occurred with 100nM TPA, which inhibited fusion to levels obtained for myoblasts cultured in '$Ca^{2+}$-free' medium for the duration of the experiment. The $IC_{50}$ value for the inhibition of fusion by TPA, is of the same order of magnitude, as the half maximal concentration of TPA required for activation of PKC from rat brain (Castagna *et al.*, 1982). These results are thus consistent with the activation of PKC.

**Figure 3.2.1.1.**

**The effect of long term TPA treatment on myoblast fusion.**

Myoblasts were plated into '$Ca^{2+}$-free' medium and various addition were made 24 hours later. Fusion was initiated, after 50 hours in culture, by plating the cells into medium containing fusion mix. Control, 'non-fused cells' were cultured in '$Ca^{2+}$-free' medium for the duration of the experiment. The results are mean ± S.D. (n=3) from a single experiment typical of 3.

Figure 3.2.1.1.

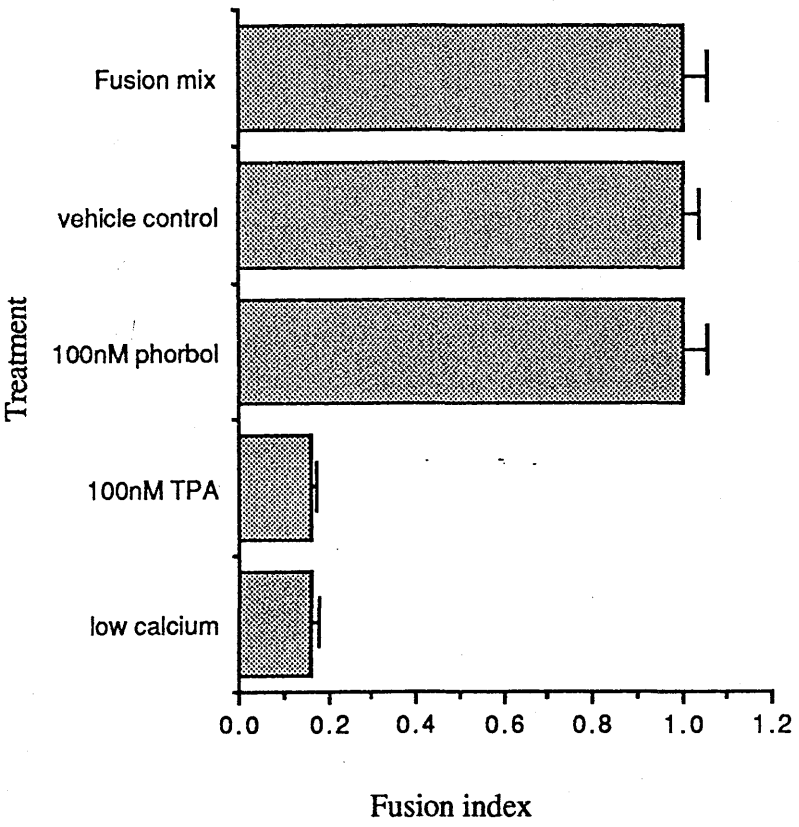

Fusion mix
vehicle control
100nM phorbol
100nM TPA
low calcium
Treatment
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Fusion index

**Figure 3.2.1.2.**

**The dose-dependence of TPA inhibition of myoblast fusion.**

Myoblasts were cultured in '$Ca^{2+}$-free' medium and increasing concentrations of TPA were added 24 hours later. Fusion was initiated, after 50 hours in culture, by plating the cells into medium containing fusion mix. Control, non-fused cells were cultured in'$Ca^{2+}$-free' medium for the duration of the experiment. The results are mean ± S.D. (n=3) from a single experiment typical of 3.

Figure 3.2.1.2.

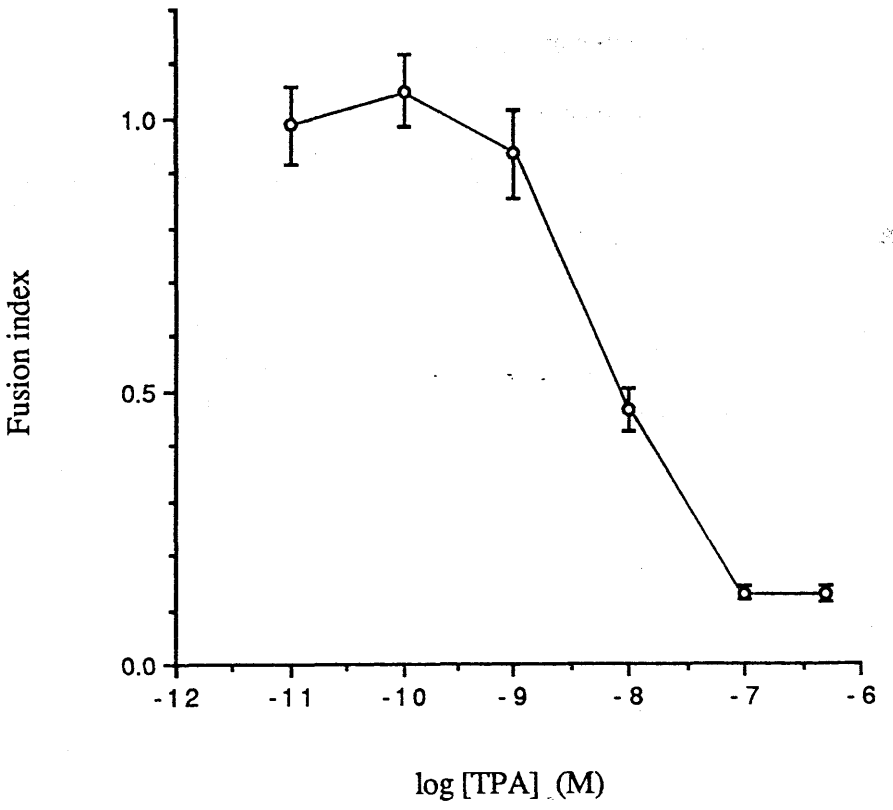
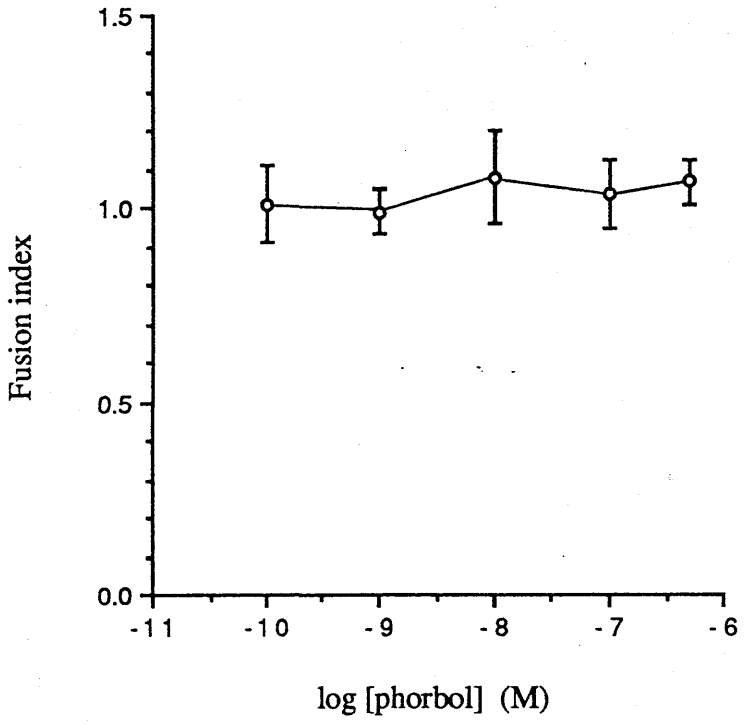


**Figure 3.2.1.3.**

**The effect of β-phorbol on myoblast fusion.**

Myoblasts were cultured in '$Ca^{2+}$-free' medium and increasing concentrations of β-phorbol were added 24 hours later. Fusion was initiated, after 50 hours in culture, by plating the cells into medium containing fusion mix. Control, non-fused cells were cultured in'$Ca^{2+}$-free' medium for the duration of the experiment. The results are mean ± S.D. (n=3) from a single experiment typical of 3.

Figure 3.2.1.3.



In order to confirm this, the effects of TPA were compared to those obtained using the PKC inactive β-phorbol. The results are shown in fig. 3.2.1.3. and the data are expressed in terms of fusion index. It may be seen that β-phorbol was without effect on myoblast fusion at concentrations up to 500nM. The vehicle control was also non-inhibitory for myoblast fusion. The inhibition of fusion by TPA was therefore specific to TPA.

### 3.2.2 [$^3$H] PDBu binding.

In order to investigate whether long term treatment of myoblasts with TPA resulted in the down-regulation of PKC, PKC levels were assessed by determining specific binding of the radiolabelled phorbol ester, [$^3$H] phorbol 12,13-dibutyrate ([$^3$H] PDBu). Samples were incubated in PBS containing 1mg/ml BSA and 10nM [$^3$H PDBu] in the presence and absence of an excess (10μM) of unlabelled PDBu, to determine non-specific and total binding respectively. Specific binding was determined by subtracting the amount of non-specific binding from the amount of total binding. The non-specific binding was 40·8 ± 2·1% of the total binding. Fig. 3.2.2.1 shows the time course of change in [$^3$H PDBu] binding to fusion-competent myoblasts at 37°C. The data are expressed as d.p.m.bound per sample. Specific binding occured rapidly and reached equilibrium by 5 minutes. Subsequent experiments were performed using a 10 minute incubation with [$^3$H] PDBu.

Fig. 3.2.2.2. shows the effect of long term treatment of myoblasts with TPA on specific [$^3$H PDBu] binding. Myoblasts were treated from 24 hours after initial plating in '$Ca^{2+}$ free' DMEM until fusion-competence with TPA, β-phorbol, the vehicle (DMSO/ ethanol) or no addition . The results are shown in fig. 3.2.2.3. and the data are expressed as percentage of specific [$^3$H PDBu] binding obtained relative to the control sample to which no addition was made. TPA treatment resulted in a 45·7 ± 4% (mean of 3 separate experiments ± S.E.M.) reduction in binding relative to the control.

**Figure 3.2.2.1.**

**Time course of specific binding of [$^3$H] PDBu to fusion-competent myoblasts.**

Myoblasts were cultured until fusion-competence in '$Ca^{2+}$-free' medium. At this time, samples were washed with PBS containing 1mg/ml BSA and incubated with 10nM [$^3$H] PDBu, in the presence and absence of 10μM PDBu, for the times indicated. Results are mean ± S.D. (n=3) from a single experiment typical of 3.

Figure 3.2.2.1.

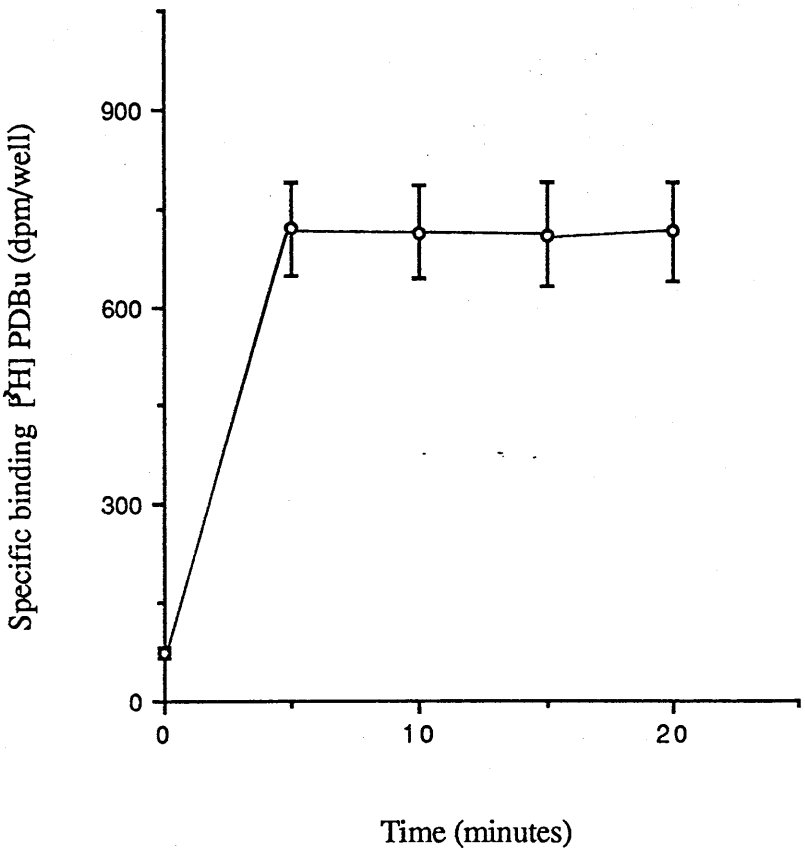
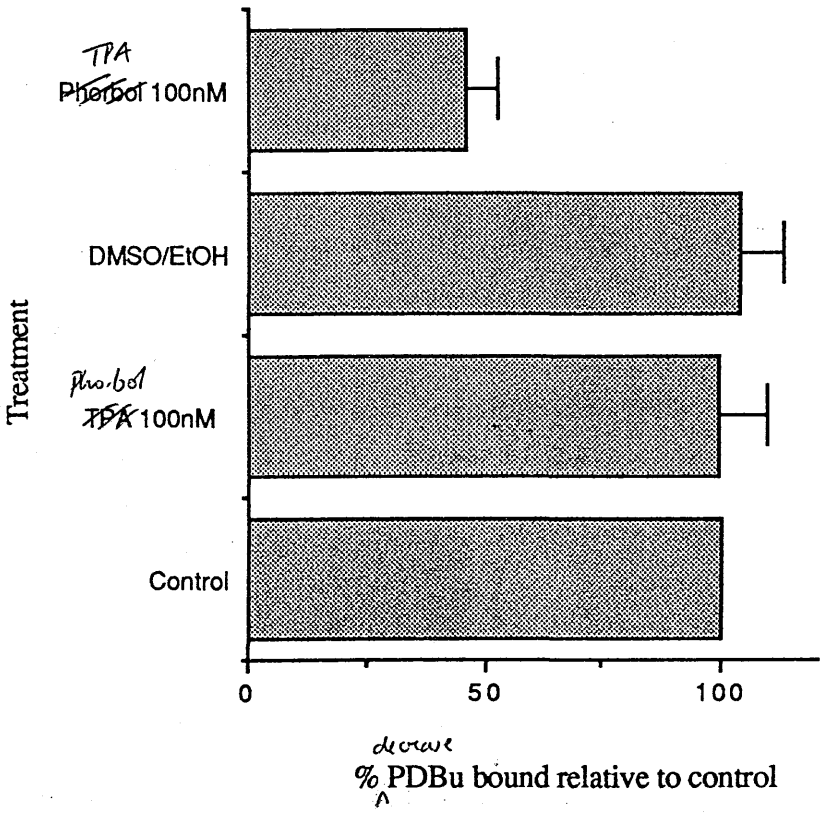

Specific binding [³H] PDBu (dpm/well)
900
600
300
0
0
10
20
Time (minutes)

**Figure 3.2.2.2.**

**Effect of long term treatment of myoblasts with TPA on specific binding of [$^3$H] PDBu.**

Myoblasts were plated into '$Ca^{2+}$-free' medium and various additions were made 24 hours later. After 50 hours in culture, the myoblasts were washed and the specific binding of [$^3$H] PDBu determined. The results are mean ± S.D. (n=3) from a single experiment typical of 3.

Figure 3.2.2.2.


TPA
Phorbol 100nM
DMSO/EtOH
Phorbol
TPA 100nM
Control
Treatment
0
50
100
% PDBu bound relative to control

Treatment with 100nM β-phorbol or the vehicle was without effect on the specific binding of [$^3$H]PDBu, a result which is consistent with TPA exerting its effect by binding to PKC.

The results from section 3.2 indicate that TPA inhibition of fusion resulted in the inhibition of myoblast fusion to levels obtained for the myoblasts cultured in '$Ca^{2+}$-free' medium for the duration of the experiment. In addition, this treatment resulted in a reduction in the amount of PKC present, as demonstrated by a 45·7% decrease in the specific binding of [$^3$H] PDBu. This suggests that PKC activation is involved in the process of myoblast fusion.

The possible role of PKC in myoblast fusion was further investigated by determining the effects of treatment with the putative PKC inhibitors, polymyxin B and staurosporine.

## 3.3 Effect of treatment with PKC inhibitors on myoblast fusion.

### 3.3.1 Effect of polymyxin B treatment on myoblast fusion.

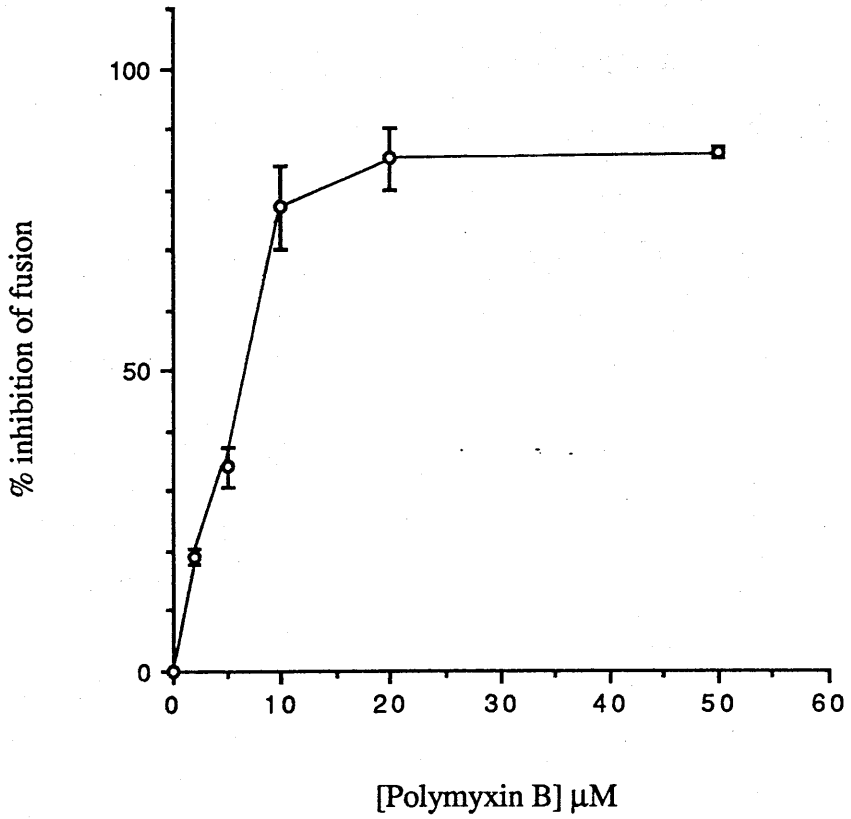
Polymyxin B is an amphipathic peptide antibiotic which interacts with $Ca^{2+}$ and acidic phospholipids and inhibits PKC by binding to its regulatory domain (Kuo *et al.*, 1983). The effect of long term polymyxin B treatment on myoblast fusion was determined using myoblasts cultured medium containing 1.4mM $Ca^{2+}$. Polymyxin B, at concentrations of 2 to 50μM was added to myoblasts 24 hours after initial plating. This method of culture is commonly used to determine the effect of various compounds on myoblast fusion (see Bischoff, 1978). The percentage of fused cells present in each sample was assessed 46 hours later. The results are shown in Fig. 3.3.1.1. and the data expressed as % inhibition relative to the 'fused' control sample, to which no addition of polymyxin B was made. Polymyxin B treatment resulted in a dose-dependent inhibition of myoblast fusion, with maximal inhibition resulting in a reduction of fusion, to the level observed for 'non-fused' myoblasts, which were cultured in '$Ca^{2+}$-free' medium for the duration of the experiment.

**Figure 3.3.1.1.**

**Dose-dependence of the effect of treatment with polymyxin B on myoblast fusion.**

Myoblasts were cultured in medium containing 1.4mM $Ca^{2+}$. Increasing concentrations of polymyxin B were added 24 hours after initial plating. Results are mean ± S.D. (n=3) from a single experiment typical of 3.

Figure 3.3.1.1.



The $IC_{50}$ value for polymyxin B inhibition of fusion was 7·2M ± 3·2μM (mean of 3 separate experiments ± S.E.M.).

### 3.3.2 Effect of staurosporine treatment on myoblast fusion.

Staurosporine is an inhibitor of PKC (Tamaoki *et al.*, 1986) which prevents the interaction of ATP and the protein substrate with the catalytic domain. It possesses a rigid aromatic indole carbazole system, which suggests, that it may act at the site where the adenine of ATP binds. However, the potency of staurosporine in cells appears to be incompatible with competitive inhibition at the ATP site (Nakamishi *et al.*, 1988). Staurosporine, therefore, appears to exert its effects by interacting directly with the catalytic domain of PKC (Huang, 1989).

The effect of long term staurosporine treatment on myoblast fusion was determined for a range of concentrations from 3 $x10^{-7}$ to $10^{-10}$M. Myoblasts were cultured in medium containing 1.4mM $Ca^{2+}$ and staurosporine was added from 24 hours after initial plating. Fusion was assessed 46 hours later and the results are shown in fig. 3.3.2.1. The data are expressed as the % inhibition of fusion obtained relative to the control, 'fused' sample, grown in the absence of staurosporine. The addition of staurosporine to cultures at 24 hours after initial plating resulted in a dose-dependent inhibition of myoblast fusion. Maximal inhibition was obtained using 100nM staurosporine which reduced fusion to the level observed for 'non-fused' myoblasts, which were cultured in '$Ca^{2+}$-free' medium for the duration of the experiment. The $IC_{50}$ of this fusion inhibition was 7·1 ± 1·4nM (mean of 3 separate experiments ± S.E.M.).

Long term treatment of myoblasts in culture with the putative PKC inhibitors, staurosporine and polymyxin B, resulted in the inhibition of fusion (section 3.3). In these experiments, monolayers of myoblasts were cultured in medium containing 1.4mM $Ca^{2+}$ and the additions of staurosporine and polymyxin B were made 24 hours after initial plating.

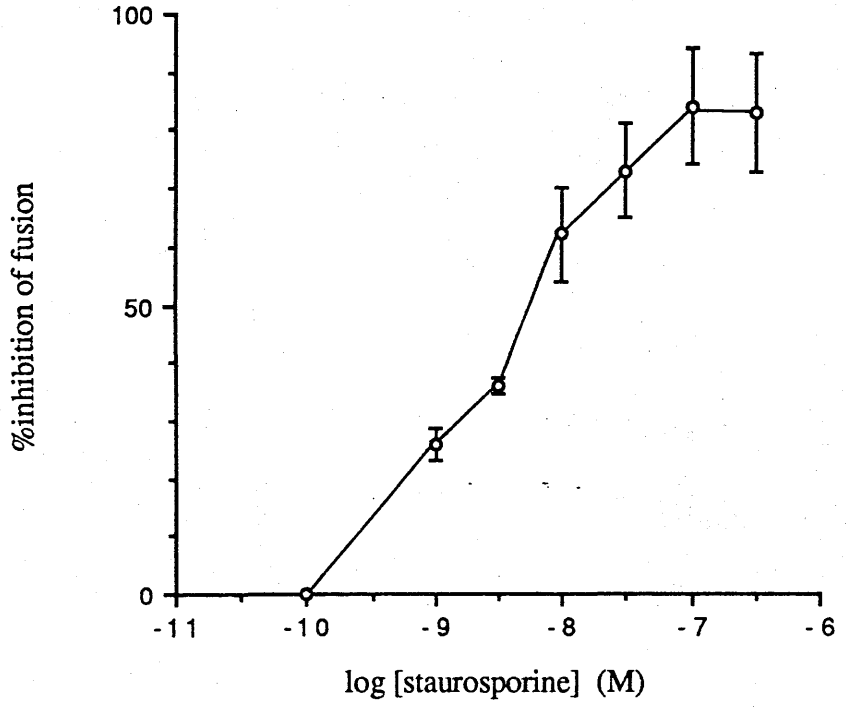
**Figure 3.3.2.1.**

**Dose-dependence of the effect of treatment with staurosporine on myoblast fusion.**

Myoblasts were cultured in medium containing 1.4mM $Ca^{2+}$.

Increasing concentrations of staurosporine were added 24 hours after initial plating. Results are mean ± S.D. (n=3) from a single experiment typical of 3.

Figure 3.3.2.1.



At this time, there is extensive alignment of the myoblasts, a process which precedes fusion. The inhibition of fusion observed with staurosporine and polymyxin B is therefore probably due to inhibition of the fusion event itself. In this culture system, fusion proceeds asynchronously (Wakelam and Pette, 1982), which hinders the mechanistic analysis of the events underlying the process of fusion.

Fusion may be made synchronous using the low $Ca^{2+}$ culture system (section 2.2.), in which myoblasts cultured in a medium containing a low concentration of $Ca^{2+}$ become fusion-competent by 50 hours after initial plating. They are however prevented from fusing because the $Ca^{2+}$ concentration is not permissive for fusion. Addition of $Ca^{2+}$ to these cells results in rapid synchronous fusion. This culture system has the potential to yield information on the mechanism(s) of the event of myoblast fusion. The effects of short term (10 or 15 minute) pre-incubation of fusion-competent myoblasts with PKC modulators were therefore determined using this culture method, in order to further investigate the role of PKC activation in myoblast fusion.

## 3.4 Investigation of the effects of short term pre-incubation with PKC modulators on myoblast fusion.

Myoblasts were cultured in '$Ca^{2+}$-free' medium until fusion-competence. Following harvesting, cells were plated into '$Ca^{2+}$-free' DMEM supplemented with 20μg/ml conalbumin to maintain cell viability. After 1 hour at 37°C, cells were pre-incubated for 10 or 15 minutes, in the presence and absence of the PKC modulators. Fusion was then initiated by the addition of fusion mix. A range of concentrations of polymyxin B (2 - 50μM), staurosporine ($3 \times 10^{-7}$M - $10^{-10}$M) and TPA ($5 \times 10^{-7}$M - $10^{-10}$M) were used. Fusion was assessed 20 hours later and the results obtained are shown in figs 3.4.1., 3.4.2. and 3.4.3. for polymyxin B, staurosporine and TPA respectively. The data are expressed as % inhibition relative to the control 'fused' sample to which no addition was made prior to the addition of fusion mix.

**Figure 3.4.1.**

**Dose-dependence of the effect of short term treatment with polymyxin B on myoblast fusion.**

Myoblasts were plated into '$Ca^{2+}$-free' medium and cultured until fusion-competence. The cells were pretreated for 15 minutes with increasing concentrations of polymyxin B prior to the initiation of fusion by the addition of fusion mix. Results are mean ± S.D. (n=3) from a single experiment typical of 3.

Figure 3.4.1.

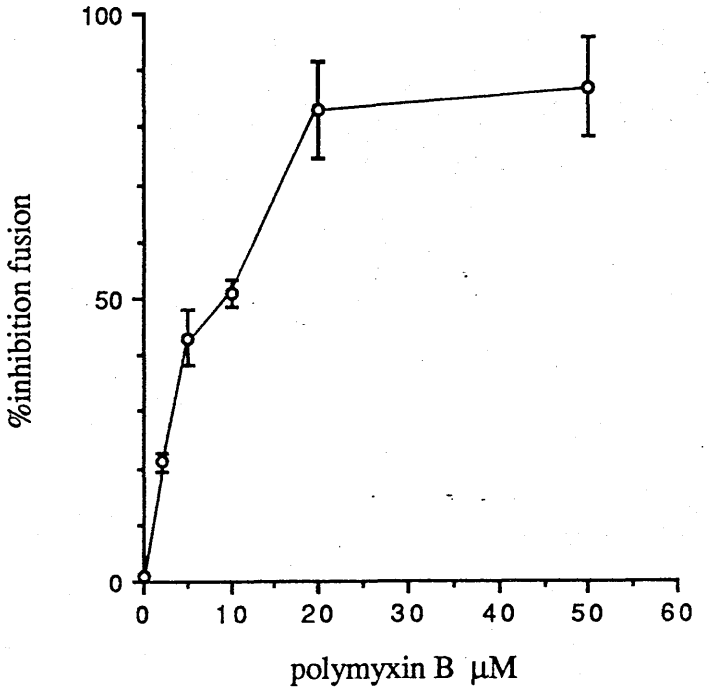


**Figure 3.4.2.**

**Dose-dependence of the effect of short term treatment with staurosporine on myoblast fusion.**

Myoblasts were cultured until fusion-competence in '$Ca^{2+}$-free' medium. The cells were pretreated for 10 minutes with increasing concentrations of staurosporine prior to the addition of fusion mix. Results are mean ± S.D. (n=3) from a single experiment typical of 3.

Figure 3.4.2.

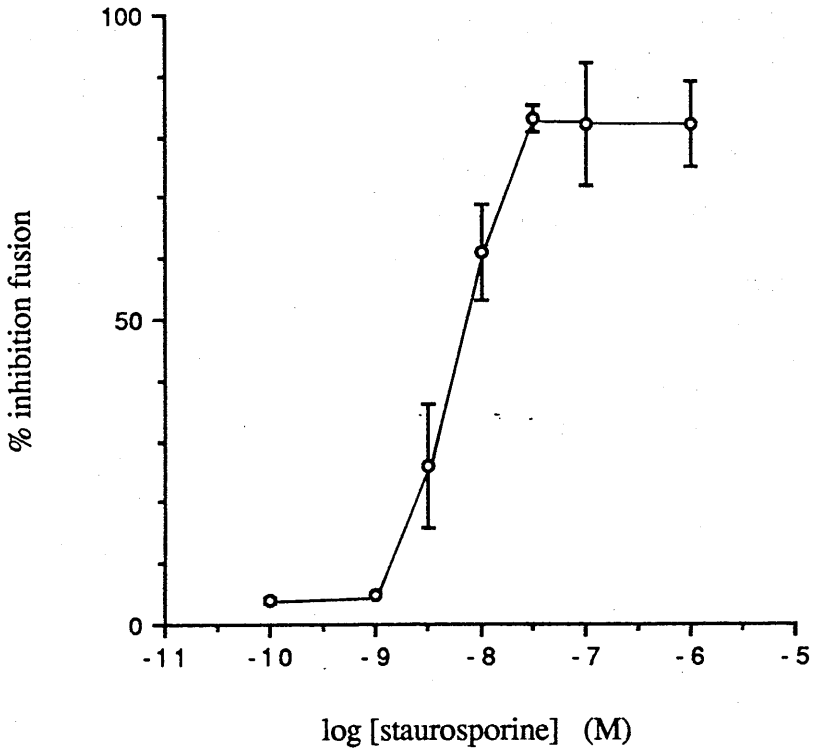


**Figure 3.4.3.**

**Dose-dependence of the effect of short term treatment with TPA on myoblast fusion.**

Myoblasts were cultured until fusion-competence in '$Ca^{2+}$-free' medium. The cells were pretreated for 10 minutes with increasing concentrations of TPA prior to the initiation of fusion by the addition of fusion mix. Results are mean ± S.D. (n=3) from a single experiment typical of 3.

Figure 3.4.3.

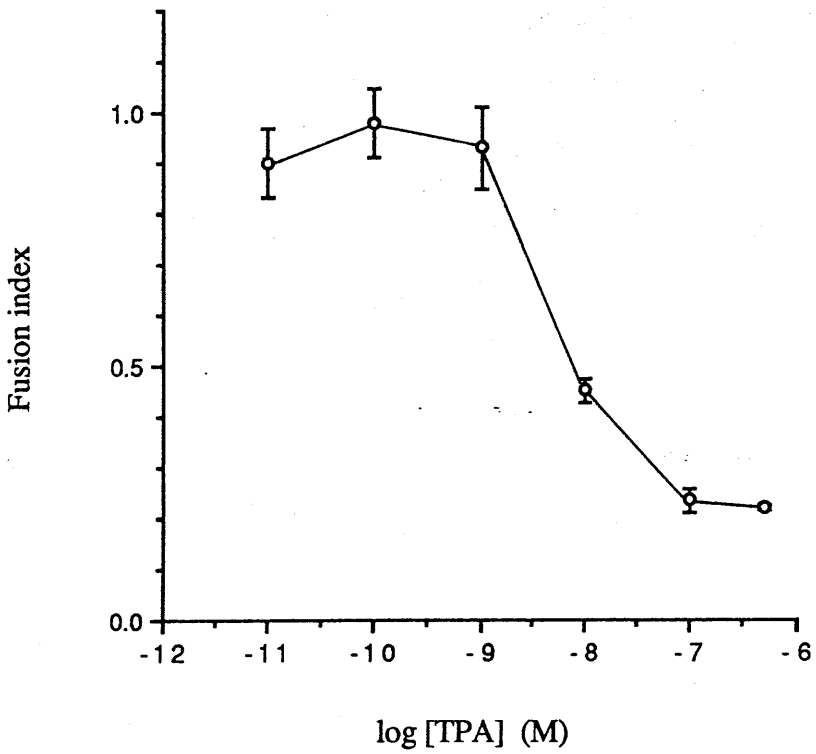
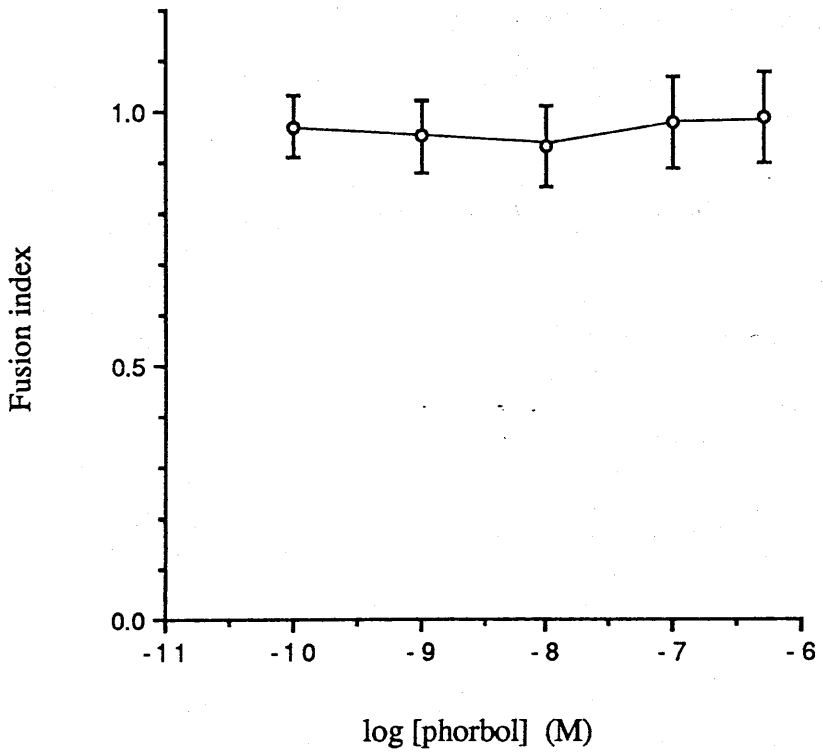


**Figure 3.4.4.**

**Dose-dependence of the effect of short term treatment with β-phorbol on myoblast fusion.**

Myoblasts were cultured until fusion-competence in '$Ca^{2+}$-free' medium. The cells were pretreated for 10 minutes with increasing concentrations of β-phorbol prior to initiation of fusion by the addition of fusion mix. Results are mean ± S.D. (n=3) from a single experiment typical of 3.

Figure 3.4.4.



Pre-incubation of fusion-competent myoblasts with polymyxin B resulted in a dose-dependent inhibition of fusion. Maximal inhibition caused a reduction in fusion to levels obtained for the 'non-fused' samples which were cultured in $Ca^{2+}$-free medium. Polymyxin B inhibited myoblast fusion with an $IC_{50}$ value of 9·3 ± 1·7μM (mean of 3 separate experiments ± S.E.M.). This was the same order of magnitude in the previous experiments using polymyxin B (fig. 3.3.1.2.).

Pre-incubation of fusion-competent myoblasts with staurosporine resulted in a dose-dependent inhibition of myoblast fusion. Maximal inhibition was obtained with 100nM staurosporine which resulted in an inhibition of fusion obtained for 'non-fused'samples. The $IC_{50}$ value for inhibtion was 13·5 ± 2·8nM (mean of 3 separate experiments ± S.E.M.). This was of the same order of magnitude as that observed in the previous experiments using staurosporine (fig. 3.3.2.2.)

The pre-incubation of fusion-competent myoblasts with TPA resulted in an inhibition of fusion (Fig. 3.4.3.). This inhibition was dose-dependent, with maximal inhibition being obtained with 100nM TPA, which reduced fusion to that of 'non-fused' samples. The vehicle for TPA addition was without effect on fusion (data not shown). The $IC_{50}$ value for TPA inhibition was 10 ± 1·2nM (mean of 3 separate experiments ± S.E.M.). This is consistent with activation of PKC (Castagna *et al.*, 1982).

In order to confirm that TPA was exerting its effect via activation of PKC, β-phorbol, a structural analogue of TPA that cannot activate PKC, was tested for its ability to inhibit fusion. The results obtained are shown in fig. 3.4.4. and the data are expressed in terms of fusion index, which was calculated relative to the control fused sample to which no addition of β-phorbol was made. β-phorbol was without effect on fusion at concentrations of up to 500nM. This was in contrast to TPA which caused maximal inhibition of fusion at a concentration of 100nM.

The results presented thus far demonstrate that treatment of myoblasts with TPA, polymyxin B and staurosporine resulted in fusion inhibition. These compounds have different effects on PKC activity; polymyxin B and staurosporine are putative

PKC inhibitors whilst TPA activates the enzyme. It thus appears that, under the experimental condition used, inhibition and activation of PKC both prevent myoblast fusion.

These results present a paradox and, in an attempt to resolve this, experiments were performed to try to establish the mechanism of fusion inhibition by these compounds. These included studies on the effect of TPA on (i) membrane fluidity; (ii) myoblast proliferation and (iii) fusion mix stimulated total [$^3$H] inositol phosphate generation, and are described in the following section

## 3.5. Investigation of the mechanism of fusion inhibition by PKC modulators.

### 3.5.1. Investigation of the effect of TPA on membrane fluidity.

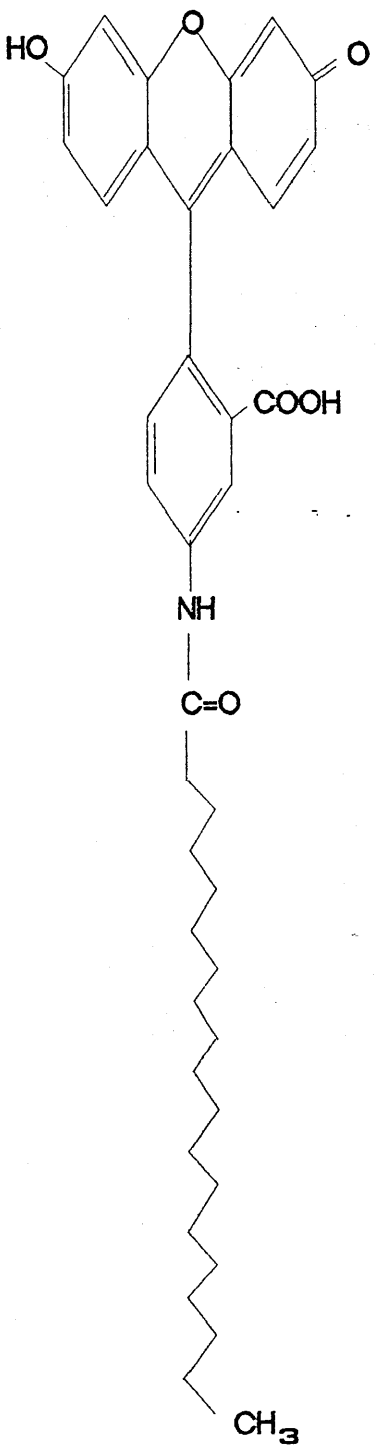
TPA is a hydrophobic molecule, which a high concentrations (>200nM), may act in a non-specific manner to perturb the membrane bilayer (Castagna *et al.*, 1979; Weinstein *et al.*, 1980). Such perturbation of the plasma membrane could result in the inhibition of fusion by interfering with the controlled transient changes in membrane structure which are required for fusion. The lipids of the bilayer are constantly in lateral motion and non-specific membrane perturbation by TPA could affect their lateral mobility. The effect of TPA on the lateral mobility of membrane molecules in fusion-competent myoblasts was therefore examined by the technique of Fluorescence Recovery After Photobleaching (FRAP) using the fluorescent lipid probe 5-N-(octadecanoyl) aminofluorescein (AF C-18), the structural formula of which is shown in fig. 3.5.1.1.. Fusion-competent myoblasts were labelled with AF C-18 which became plasma membrane inserted. An argon ion laser beam set at the excitation wavelength, 490nm, was focused onto a small area (approx 1μm radius) of the cell surface. The fluorescent molecules in this region were then destroyed or 'bleached' by an intense pulse of light from the laser. The subsequent recovery of fluorescence intensity in this area of the cell surface was monitored, as a function of time, using a light level that was low enough to prevent further bleaching.

**Figure 3.5.1.1.**

The structural formula of aminofluorescein C-18 (AF C-18).

Figure 3.5.1.1.

Structural formula of 5-N-(octadecanoyl) aminofluorescein (AF C-18)



This recovery in fluorescence occured due to the exit of bleached molecules from the region and the entry of unbleached molecules from the surrounding area. The rate and extent of recovery depend on the rate of lateral movement of AF C-18 and the fraction of these fluorescent molecules that are mobile in the plane of the membrane. The lateral mobility of AF C-18 may be expressed in terms of a lateral diffusion coefficient ($D_L$). The average distance s (in centimetres) traversed in 2 dimensions of time t (in seconds) depends on $D_L$ ($cm^2$/second) according to the equation:

$$s = (4D_Lt)^{1/2}$$

$D_L$ may be calculated using the value obtained for the half time of fluorescence recovery i.e. the time taken for the intensity of fluorescence to reach 50% of the final intensity. A typical trace obtained is shown in fig. 3.5.1.2. The effect of 10 minute TPA treatment on the $D_L$ value and % recovery of fluorescence of AF C-18 were determined and the results obtained are summarised in Table 3.5.1. below.

**Table 3.5.1.**

**Effect of TPA on the lateral diffusion coefficient ($D_L$) and % recovery of fluorescence of the fluorescent lipid probe AF C-18 in the plasma membranes of fusion competent myoblasts.**

Fusion-competent myoblasts were labelled with the fluorescent lipid probe, AF C-18. The lateral diffusion coefficient ($D_L$) and % recovery of fluorescence were measured in myoblasts prior to and following a 10 minute preincubation with 100nM TPA. Results are mean ± S.D. (n=10) from a single experiment typical of 3.

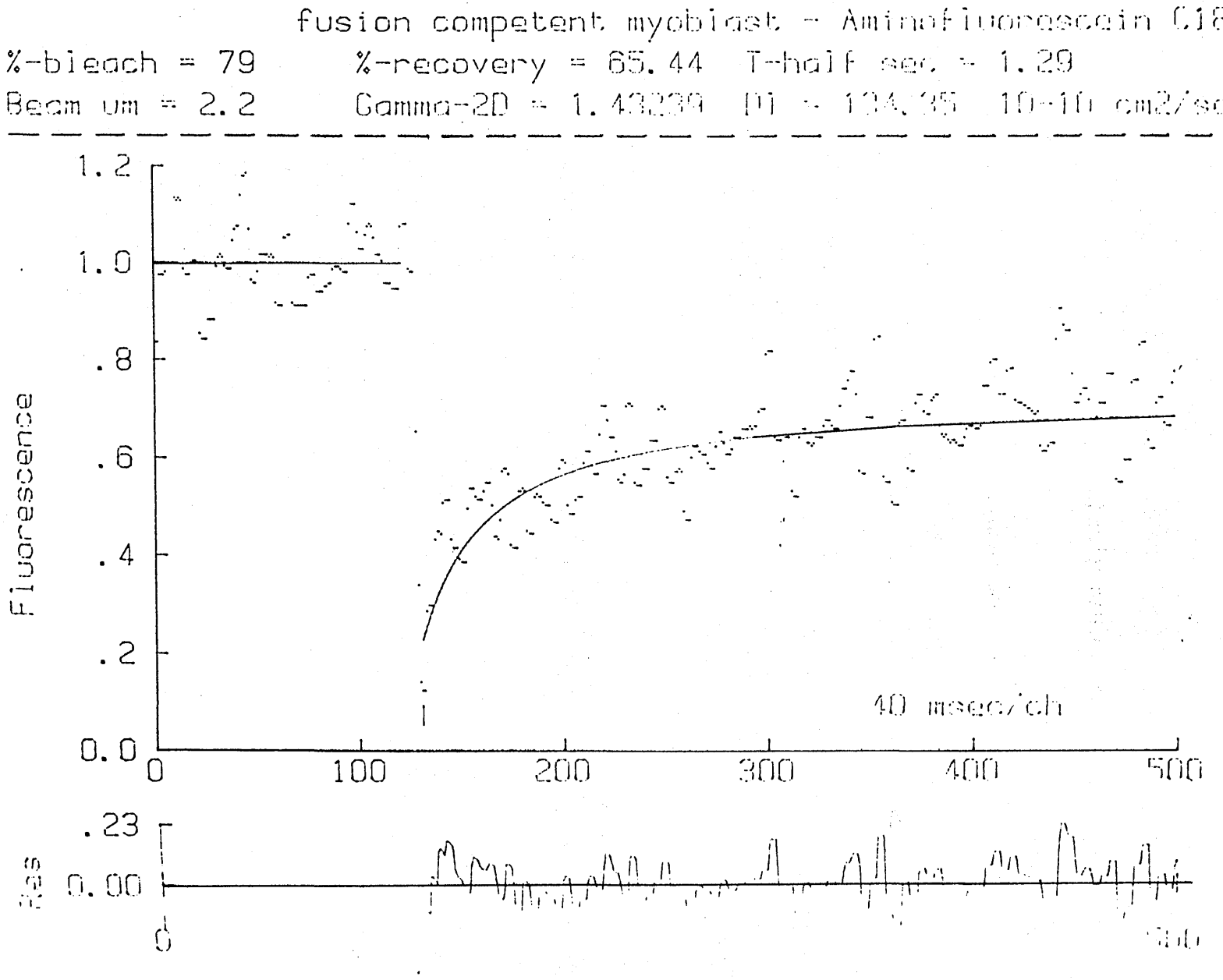
| Treatment | $D_L$ x $10^{-10}$ $cm^2$/sec | % recovery fluorescence |
|---|---|---|
| Control | 8·3 ±3·4 | 58·1 ± 7 |
| 100nM TPA | 10·4 ± 2.5 | 58·7 ± 5·9 |

**Figure 3.5.1.2.**

**Typical FRAP curve obtained from fusion-competent myoblasts labelled with the fluorescent lipid probe AF C-18.**

Fusion-competent myblasts were labelled with AF C-18. An argon ion laser beam, of wavelength 490nm, was focused onto an area of the myoblast plasma membrane. The laser was briefly unattenuated to provide the bleach pulse. The recovery of fluorescence was monitored as a function of time.

**Figure 3.5.1.2.**


Filename : afr4
fusion competent myoblast - Aminofluorescein C18
%-bleach = 79
%-recovery = 65.44
T-half sec. = 1.29
Beam um = 2.2
Gamma-2D = 1.43239
D1 = 134.35 10-10 cm2/sec
Fluorescence
1.2
1.0
.8
.6
.4
.2
0.0
0
100
200
300
400
500
40 msec/ch
.23
0.00
res
0

TPA treatment of fusion-competent myoblasts did not affect these parameters, relative to those obtained for the control non-TPA treated samples. It did not therefore perturb the myoblast membrane as assessed by FRAP using AF C-18.

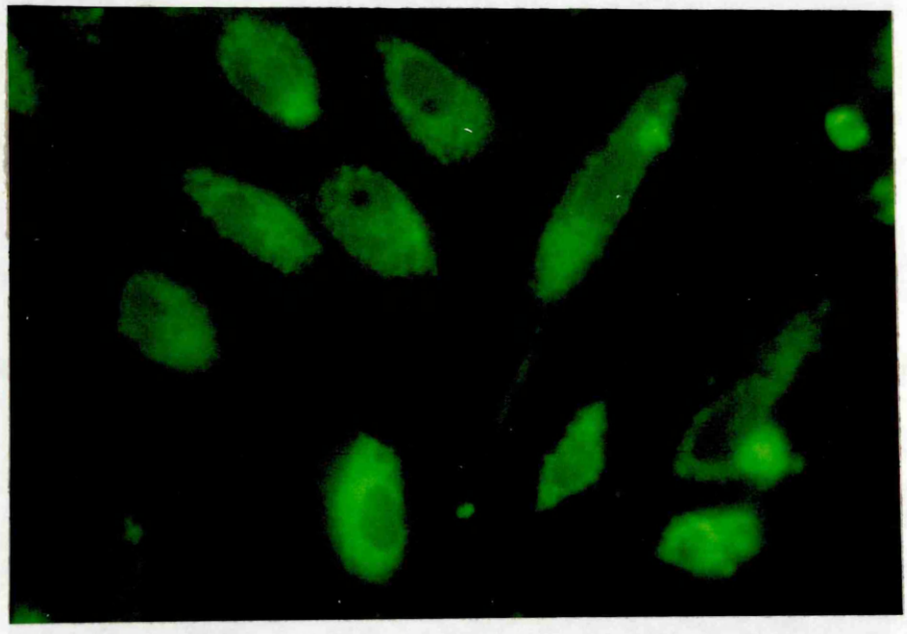
Use of AF C-18 labelling as a monitor of the plasma membrane bilayer clearly implies that the probe itself locates within one of the two leaflets of the bilayer. Its location in the plasma membrane was confirmed by the technique of fluorescence quenching using trypan blue. Trypan blue quenches the fluorescence of AF C-18 due to the phenomenon of Resonance Energy Transfer (RET). AF C-18 labelled fusion-competent myoblasts were examined before and after the addition of 0.25% (v/v) trypan blue using a fluorescence microscope equipped with a camera attachment. The results obtained are shown photographically in fig. 3.5.1.3. Addition of trypan blue to AF C-18 labelled myoblasts resulted in a quenching of the fluorescence which confirms the membrane location of the fluorescent probe (Gordon, 1989).

**Figure 3.5.1.3. a) and b)**

**Effect of trypan blue treatment on myoblasts labelled with AF C-18.**

Fusion competent myoblasts were labelled with 10µg/ml AF C-18 for 5 minutes and examined by fluorescence microscopy before (a) and after (b) the addition of 0.25% (w/v) trypan blue.

a



b

### 3.5.2. Effect of TPA on [methyl-$^3$H] thymidine incorporation.

Fusion-competent myoblasts are post-mitotic cells and withdrawal from the cell cycle is a prerequisite for fusion. A possible mechanism for TPA inhibition of fusion thus exists, where TPA stimulates myoblasts to proliferate, thus maintaining them in the cell cycle and consequently preventing their fusion. The incorporation of [methyl-$^3$H] thymidine incorporation into acid insoluble material was used to determine DNA synthesis (Adams, 1969), to provide a measure of cell proliferation. The effect of TPA on [methyl-$^3$H] thymidine incorporation was examined by adding TPA and 1μCi/ml [methyl-$^3$H] thymidine, to cultures of myoblasts, 24 hours after plating into medium containing 1.4mM $Ca^{2+}$. At this time in culture the myoblasts are predominantly proliferative (Wakelam, 1985). The results are shown in fig. 3.5.2.1. with the data expressed as d.p.m. [methyl-$^3$H] thymidine incorporated per sample. TPA inhibited [methyl-$^3$H] thymidine incorporation into myoblasts in a dose-dependent manner. The vehicle for TPA addition was without effect and therefore the observed inhibition was not due to a component of the vehicle. Maximal inhibition of 31.1 ± 2.5% was obtained using 100nM TPA. The $IC_{50}$ value for inhibition was 6·6 ± 2·9nM (mean of 3 separate experiments ± S.E.M.). PKC activation was observed to inhibit [methyl-$^3$H] thymidine incorporation and, thus, myoblast proliferation. The proposed mechanism of TPA inducing myoblast proliferation and, thereby, inhibiting their fusion is, therefore, unlikely to occur.

In order to confirm this, the experiment was repeated using β-phorbol, a PKC inactive phorbol ester. The results are shown in fig. 3.5.2.2. and the data are expressed as d.p.m. [methyl-$^3$H] thymidine incorporated per sample. β-phorbol was ineffective at concentrations up to 500nM which suggests that the effects of TPA are PKC specific.

**Figure 3.5.2.1.**

**Dose-dependence of the effect of TPA on [methyl-$^3$H] thymidine incorporation.**

Myoblasts were cultured in medium containing 1.4mM $Ca^{2+}$. A range of concentrations of TPA were added, together with 1μCi/sample [methyl-$^3$H] thymidine at 24 hours after initial plating. The radioactivity associated with acid-insoluble material was determined 24 hours later. Results are mean ± S.D. (n=3) of a single experiment typical of 3.

Figure 3.5.2.1.

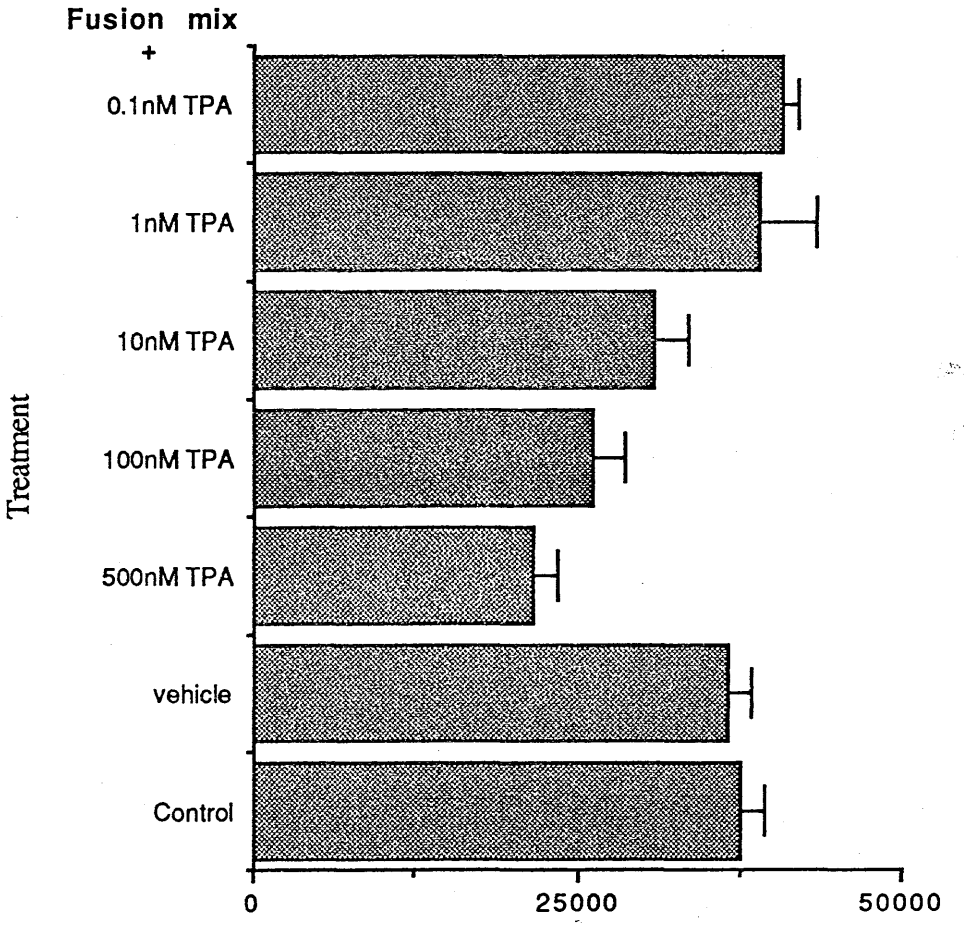
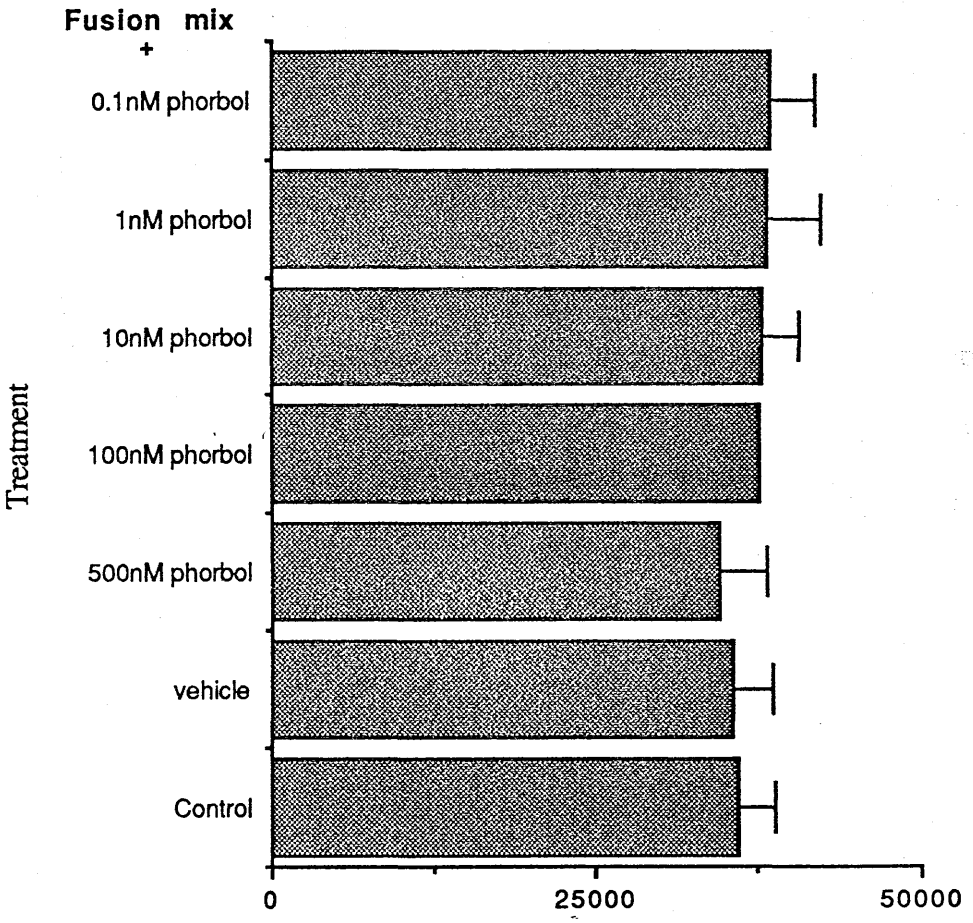

Fusion mix
+
0.1nM TPA
1nM TPA
10nM TPA
100nM TPA
500nM TPA
vehicle
Control
Treatment
0
25000
50000
[methyl-³H] thymidine incorporated (dpm/sample)

**Figure 3.5.2.2.**

**Effect of β-phorbol on [methyl-$^3$H] thymidine incorporation.**

Myoblasts were cultured in medium containing 1.4mM $Ca^{2+}$. A range of concentrations of β-phorbol were added, together with 1μCi/sample [methyl-$^3$H] thymidine at 24 hours after initial plating. The radioactivity associated with acid-insoluble material was determined 24 hours later. Results are mean ± S.D. (n=3) of a single experiment typical of 3.

Figure 3.5.2.2.


Fusion mix
+
0.1nM phorbol
1nM phorbol
10nM phorbol
100nM phorbol
500nM phorbol
vehicle
Control
Treatment
0
25000
50000
[methyl-3H] thymidine incorporated (dpm/sample)

### 3.5.3. Effect TPA treatment on total [$^3$H] inositol phosphate generation.

PKC activation has been proposed to be involved in the negative feedback regulation of inositol phospholipid hydrolysis (Watson and Lapetina, 1985). For example, it has been demonstrated that short term pretreatment of cells with TPA, to activate PKC, results in the attenuation of agonist-stimulated inositol phospholipid breakdown in a number of systems (Leeb-Lundberg *et al.*, 1985; Rittenhouse and Sasson, 1985; Watson and Lapetina, 1985 and Brown *et al.*, 1987). The effect of TPA on total [$^3$H] inositol phosphate generation was therefore examined as a possible mechanism for its inhibition of myoblast fusion.

Fusion-competent myoblasts, labelled to a constant specific activity with [$^3$H] inositol, were treated with TPA for 10 minutes prior to addition of fusion mix. Total [$^3$H] inositol phosphate generation was determined using the $Li^+$ assay as previously described (section 2.6 ). 500nM β-phorbol and the vehicle for the addition of TPA/β-phorbol to myoblasts were also tested. Fig. 3.5.3.1 shows a dose-response curve of TPA effects on total [$^3$H] inositol phosphate generation and the data are expressed as fold stimulation over the control.

Maximal inhibition was obtained with 100nM TPA, which inhibited total [$^3$H] inositol phosphate generation by 38·9 ± 4·4% (mean from 3 separate experiments ± S.E.M.). This inhibition was dose-dependent with an $IC_{50}$ of 4·1 ± 0·7nM (mean from 3 separate experiments ± S.E.M.), which is consistent with activation of PKC (Castagna *et al.*, 1982). This observation was supported by the lack of inhibition obtained using the non-PKC-activating TPA analogue, β-phorbol, and the vehicle for TPA/β-phorbol addition to myoblasts.

Earlier experiments (section 3.2), showed that long term treatment of myoblasts with TPA resulted in the inhibiton of myoblast fusion. In order to determine whether this was due to the inhibition of inositol phospholipid breakdown in response to the fusion stimulator, fusion mix, the effect of long term treatment on fusion mix-stimulated total [$^3$H] inositol phosphate generation was examined. Myoblasts were treated with either no addition, 100nM TPA, 100nM β-phorbol or the vehicle for

TPA/β-phorbol addition, from 24 hours after initial plating to fusion-competence. Total [$^3$H] inositol phosphate generation in response to fusion mix was determined using the $Li^+$ assay as before. The results are shown in fig. 3.5.3.2. and the data are expressed as fold stimulation of total [$^3$H] inositol phosphate generation over the control (no addition at 24 hours after initial plating). Long term treatment of myoblasts with 100nM TPA resulted in a 22·5 ± 2·5% inhibition of total [$^3$H] inositol phosphate generation in response to fusion mix (mean of 3 separate experiments ± S.E.M.). This effect was specific to TPA since β-phorbol and the vehicle for addition were without effect.

Long term treatment of myoblasts with TPA also results in down-regulation of PKC (sections 3.2.1. and 3.2.2). Under these conditions it might be expected that fusion-mix stimulated [$^3$H] inositol phosphate accumulation might be enhanced, as is observed in some other systems e.g. Swiss 3T3 fibroblasts (Brown *et al.*, 1987). However, Swiss 3T3 cells are unique in that prolonged TPA pretreatment results in the complete down-regulation of PKC (Rodriguez-Pena and Rozengurt, 1984). In contrast, myoblast PKC was only partially down-regulated by 45·7 ± 4% (see section 5.2.2). Thus, under these conditions, the remaining PKC will be activated by TPA and, thereby, result in the inhibition of fusion-mix stimulated [$^3$H] inositol phosphate accumulation in myoblasts.

The pre-incubation of fusion-competent myoblasts with 100nM TPA resulted in the reduction in total [$^3$H] inositol phosphate generation in response to fusion mix and in the inhibition of myoblast fusion to control, 'non-fused' levels. PKC-induced negative regulation of agonist stimulated inositol phospholipid hydrolysis may thus underlie the mechanism of fusion inhibition by TPA.

**Figure 3.5.3.1.**

**The effect of short term TPA or β-phorbol pre-incubation on agonist-stimulated total [$^3$H] inositol phosphate generation.**

Fusion-competent myoblasts that were labelled with [$^3$H] inositol were collected by centrifugation and washed. The cells were resuspended in HBG containing 10mM LiCl and pretreated with 100nM TPA or 100nM β-phorbol for 10 minutes prior to incubation in the presence and absence of fusion mix for 30 minutes. Total [$^3$H] inositol phoshates were batch eluted from Dowex-formate anion exchange resin. Results are mean ± S.D. (n=3) from a single experiment typical of 3.

Figure 3.5.3.1.

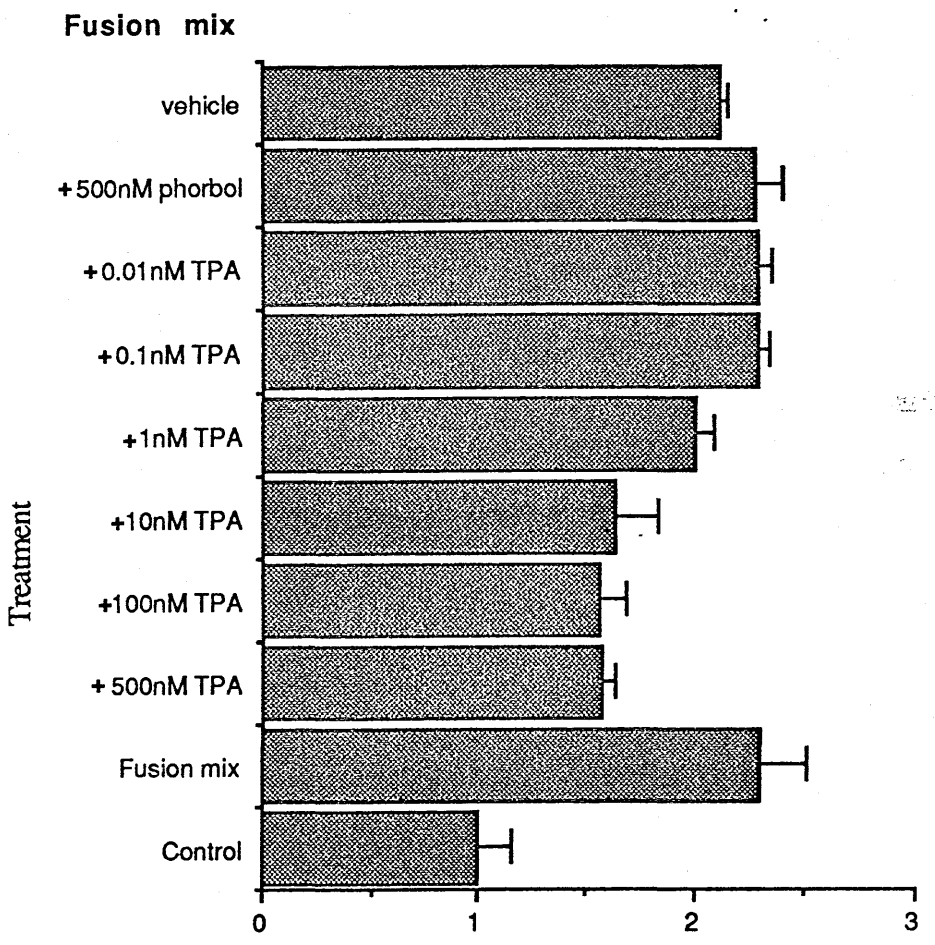
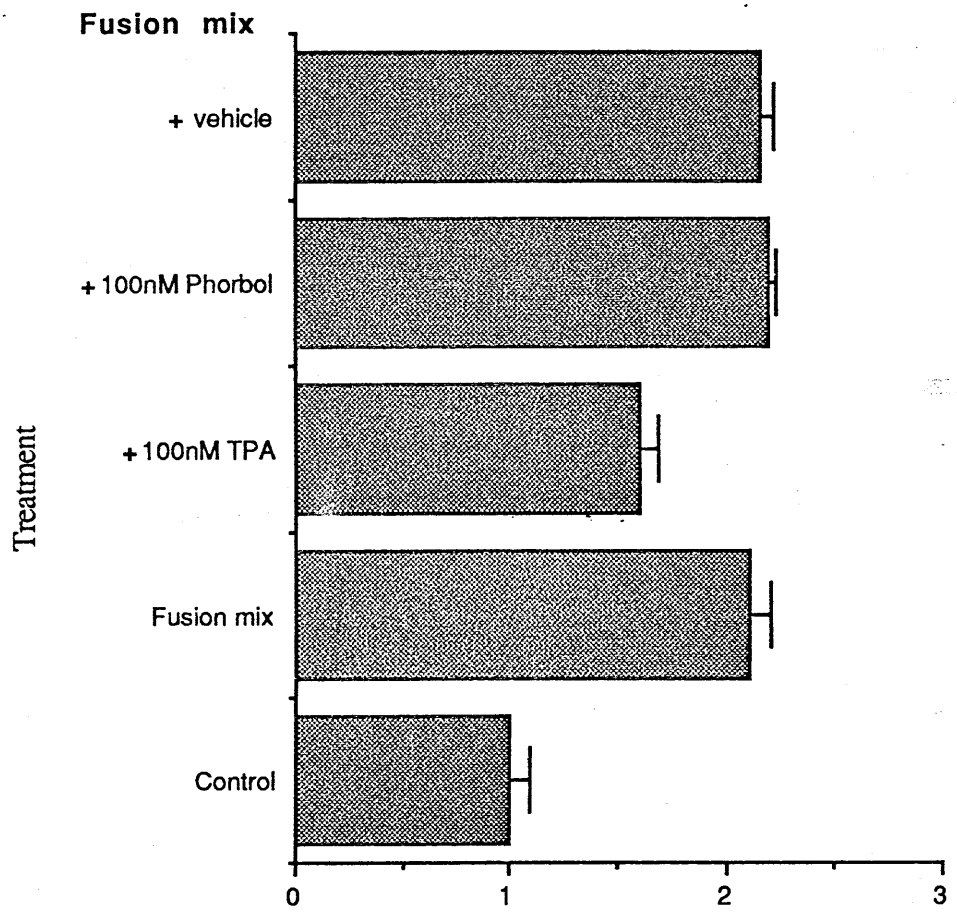

Fusion mix
vehicle
+500nM phorbol
+0.01nM TPA
+0.1nM TPA
+1nM TPA
+10nM TPA
+100nM TPA
+500nM TPA
Fusion mix
Control
Treatment
0
1
2
3
fold stimulation of total inositol phosphate generation

**Figure 3.5.3.2.**

**Effect of long term TPA or β-phorbol treatment on agonist-stimulated total [$^3$H] inositol phosphate generation.**

Myoblasts were plated into '$Ca^{2+}$-free' medium and labelled with [$^3$H] inositol. 100nM TPA or 100nM β-phorbol were added 24 hours after initial plating. After 50 hours in culture, fusion-competent myoblasts were washed and resuspended into HBG containing 10mM LiCl and incubated in the presence and absence of fusion mix for 30 minutes. Total [$^3$H] inositol phosphates were batch eluted from Dowex-formate anion exchange resin. Results are mean ± S.D. (n=3) from a single experiment typicalof 3.

Figure 3.5.3.2.


Fusion mix
+ vehicle
+ 100nM Phorbol
+ 100nM TPA
Fusion mix
Control
Treatment
0
1
2
3
fold stimulation of total inositol phosphate generation

### 3.6. Effect of short term pre-incubation with PKC inhibitors on total [$^3$H] inositol phosphate generation.

Polymyxin B and staurosporine were also inhibitory to fusion (sections 3.3 and 3.4). Their effects on upon fusion mix stimulated of total [$^3$H] inositol phosphate generation were therefore investigated, as a possible mechanism for their inhibition of fusion.

Fusion-competent myoblasts were pre-incubated for 10 or 15 minutes in the presence and absence of polymyxin B (50μM) and staurosporine (100nM), at maximal concentrations for fusion inhibition, prior to the addition of fusion mix. Total [$^3$H] inositol phosphate generation was determined using the $Li^+$ assay as previously described (section 2.6). The results obtained are shown in Tables 3.6.1. and 3.6.2. below.

**Table 3.6.1.**

**Effect of short term pre-incubation with polymyxin B on agonist-stimulated total [$^3$H] inositol phosphate generation in fusion-competent myoblasts.**

Fusion-competent myoblasts that were labelled with [$^3$H] inositol were washed and resuspended into HBG containing 10mM LiCl and pretreated with polymyxin B (50μg/ml) for 15 minutes. The cells were then incubated in the presence and absence of fusion mix for 30 minutes. Total [$^3$H] inositol phosphates were batch eluted from Dowex-formate anion exchange resin. Results are means ± S.D. (n=3) from a single experiment typical of 2.

| Treatment | Total [$^3$H] inositol phosphates (d.p.m.) |
|---|---|
| Control | 1401 ± 168 |
| fusion mix | 2697 ± 211 |
| fusion mix + 50μg/ml polymyxin B | 2518 ± 30 |

**Table 3.6.2.**

**Effect of short term pre-incubation with staurosporine on agonist-stimulated total [$^3$H] inositol phosphate generation in fusion-competent myoblasts.**

Fusion-competent myoblasts that were labelled with [$^3$H] inositol were washed and resuspended into HBG containing 10mM LiCl and pretreated with staurosporine (100nM) for 10 minutes. The cells were then incubated in the presence and absence of fusion mix for 30 minutes. Total [$^3$H] inositol phosphates were batch eluted from Dowex-formate anion exchange resin. Results are means ± S.D. (n=3) from a single experiment typical of 2.

| Treatment | Total [$^3$H] inositol phosphates (d.p.m.) |
|---|---|
| Control | 2426 ± 71 |
| fusion mix | 3490 ± 111 |
| fusion mix + vehicle for addition of staurosporine | 3453 ± 519 |
| fusion mix + 100nM staurosporine | 3324 ± 165 |

## Discussion.

### 3.7. Possible roles for *sn*-1,2-DAG in myoblast fusion.

Myotube formation occurs when the plasma membranes of two or more myoblasts fuse to become continuous and allow intermixing of their membrane components and cytosolic contents. This requires the close apposition of membranes followed by a local merging of the bilayers at the point of contact. The fusion of myoblasts apparently occurs via receptor-mediated inositol phospholipid breakdown (Wakelam and Pette, 1984a,b). The hydrolysis of PtdIns 4,5 $P_2$ results in the loss of the hydrophilic headgroup and the consequent generation of *sn* -1,2-DAG in the plasma membrane. As discussed in the introduction, this lipid may be involved in the events that underlie the fusion process since it has fusogenic properties and is a physiological activator of PKC. The generation of DAG in fusion-competent myoblasts upon fusion stimulation has previously been demonstrated using [U-$^{14}$C] glycerol labelled cells. The lipids of these cells were extracted and separated by t.l.c. after fusion stimulation for 8 minutes. [U-$^{14}$C] glycerol labelled DAG was found to be increased relative to the control untreated cells. The t.l.c. method used in this experiment was however unable to resolve the *sn* -1,2-DAG from the *sn* -2,3-enantiomer. The activation of PKC by *sn* -1,2-DAG is stereospecific for this isomer; the *sn* -2,3-DAG and *sn* -1,3-DAG are PKC inactive (Boni and Rando,1985). Thus an assay specific for *sn* -1,2-DAG was used to determine the changes in its levels upon fusion stimulation. This method is based on the phosphorylation of *sn* -1,2-DAG by *E.coli sn* -1,2-DAG kinase in the presence of [$\gamma$-$^{32}$P] ATP (Preiss *et al.*, 1986). It also has the advantage over [U-$^{14}$C] glycerol labelling in that it precludes the assumption that [U-$^{14}$C] glycerol is distributed equally throughout the membrane phospholipid pools. From fig. 3.1.1a) and b), there was approximately a 2-fold increase in *sn* -1,2-DAG upon fusion stimulation with fusion mix. This increase was apparent at five seconds following stimulation, which was the earliest time point measured. Its levels remained elevated at subsequent time points and returned to control, i.e. basal unstimulated levels by 20 minutes.

DAG is a neutral hydrophobic molecule which tends to destabilise membrane bilayers by causing increased spreading of phospholipid headgroups (Das and Rand, 1986). This leads, potentially, to the formation of a non-bilayer, inverted hexagonal II ($H_{II}$) structure and has been demonstrated to occur in model membrane systems (Epand, 1985). Such structures have been proposed as possible fusion intermediates (Cullis and de Kruijff, 1979).

In addition to its fusogenic properties, *sn* -1,2-DAG is a physiological activator of PKC, an enzyme which is considered to be involved in the regulation many cell processes, for example, exocytosis, growth and differentiation (Nishizuka, 1986). It is a $Ca^{2+}$/phospholipid dependent serine threonine protein kinase that is active when inserted into the membrane, which provides the correct lipid environment for activation. The membrane destabilising effect of *sn* -1,2-DAG has been suggested to allow PKC to penetrate the plasma membrane (Epand and Lester, 1990). The hydrophobic domain of *sn* -1,2-DAG ensures that the PKC molecule is inserted into the membrane with the correct orientation to allow binding of phosphatidylserine, $Ca^{2+}$ and *sn* -1,2-DAG at the regulatory domain (Hannun *et al.*, 1986). As a result, the PKC complex adopts a specific conformation which 'unmasks' the active site in the catalytic domain (Brumfeld and Lester, 1990).

These two roles for *sn* -1,2-DAG are further discussed in the following sections.

## 3.8 *sn*-1,2-DAG as a fusogen.

The fusion of myoblasts involves transient changes in the plasma membrane structure. The lipids of the apposed bilayers mix during fusion, which implies that there is a temporary loss of the bilayer configuration at the fusion site. This loss of the lipid bilayer conformation at the point of contact would destabilise the bilayer, to allow mixing of the apposed individual membrane lipids and thus the establishment of membrane continuity. Non-bilayer structures have been proposed to play a role in the fusion of membranes (Cullis and de Kruijff, 1979) based on the observation that

phospholipids in the membranes of these ghost cells undergo a conformational change from the lamellar bilayer ($L_\alpha$) to non-bilayer hexagonal II ($H_{II}$) structure in the presence of the fusogenic lipid, oleic acid, as determined using $^{31}P$ Nuclear Magnetic Resonance spectroscopy. The lamellar and $H_{II}$ phases possess characteristic $^{31}P$ NMR spectra which result from the degree of diffusional freedom of the phospholipid molecule in the lipid aggregate (Cullis and de Kruijff 1976). In the $H_{II}$ phase, lipids are organised in hexagonally arranged cylinders, in which the polar head groups of the lipid molecules surround a narrow aqueous channel. Evidence to support the hypothesis that lipids in the $H_{II}$ phase play a critical role in membrane fusion comes from experiments using model membrane systems. For example, vesicles composed of equimolar amounts of bovine heart cardiolipin and egg phosphatidylcholine, lipids which prefentially adopt the $H_{II}$ configuration, fuse on addition of $Ca^{2+}$ (Wilschut *et al.*, 1982).

Physico-chemical forces exist between membranes which result in their short range repulsion. These present a barrier to close membrane contact and fusion and therefore must be overcome for fusion to occur. They consist mainly of electrostatic and hydration forces (Parsegian *et al.*, 1984) with electrostatic repulsion being predominant at distances of greater than 2-3nm. This is due to the negatively charged polar headgroups of the phospholipids which stabilise the membrane. At less than 2-3nm, the hydration force is dominant and it results from the water solvation of the phospholipid headgroups (Parsegian *et al.*, 1979). Dehydration, i.e. the removal of water from the membrane surfaces is thus considered to be the primary requirement for intermembrane apposition and fusion (Parsegian *et al.*, 1984). Myoblasts fuse at discrete areas of the plasma membrane, so the need to remove water from the outer membrane surface is localised to the fusion site. The $H_{II}$ structure is externally dehydrated and thus fusion via an inverted $H_{II}$ lipid orientation would be compatible with this requirement to remove water from the membrane surfaces at the fusion site. Such a non-bilayer structure is also consistent with local point fusion, in that it allows

intermixing of the membrane lipids in their region of contact. In addition, it permits fusion that is non-leaky i.e. the permeability of the cell would be maintained.

The formation of the $H_{II}$ phase is an inter-lamellar event, occuring only between closely opposed bilayers (Siegel, 1982). The concept of an intrinsic radius of curvature ($R_o$), has been adopted to describe the radius of the channel formed by the close packing of the lipid groups in the hexagonal phase. Lipids that form stable lamellar phases possess near-infinite values of $R_o$, while lipids that form the $H_{II}$ phase possess small values of $R_o$. A small value of $R_o$ thus favours the formation of the $H_{II}$ phase. Conditions which favour the $L_\alpha$ to $H_{II}$ phase transition result in a reduction in the phase transition temperature ($T_H$). A decrease in $T_H$ and consequent stabilisation of the $H_{II}$ phase may be achieved by factors which decrease $R_o$. DAG reduces $T_H$ by this mechanism. Siegel *et al.*, (1989) demonstrated, by the use of X ray diffraction that 2 mol% 1-oleoyl-2-arachidonyl glycerol, dipalmitoylglycerol and dioleoylglycerol were each sufficient to reduce the $T_H$ of phospholipid lattices of monomethylated dioleoyl-phosphatidylethanolamine (DOPE-Me) by 20°C. The proximity to $L_\alpha/H_{II}$ transitions has been shown to affect fusion rates (Ellens *et al.*, 1986,1989) and many biomembrane systems are close to this phase boundary under physiological conditions (Siegel, 1986b). The transient production of diacylglycerol may be sufficient to bring these lipids to the temperature interval for phase transition. In support of this, the $L_\alpha$ to $H_{II}$ transition has been demonstrated to occur upon the incorporation of DAG into phosphatidylcholine or phosphatidylethanolamine vesicles (Das and Rand, 1984). The DAG content of the membrane will thus regulate the stability of the lipid bilayer. Transient, controlled alteration in the levels of DAG present in the plasma membrane therefore provides a potential mechanism for the metabolic regulation of membrane stability.

The orientation of diacylglycerol in the $L_\alpha$ and $H_{II}$ phase in phospholipid systems has been investigated by Siegel *et al.*, (1989) using acyl chain-perdeuterated 1,2 dipalmitoylglycerol (DPG) in lattices of dioleoylphosphatidylcholine (DOPC) and DOPE-Me. $^2H$ and $^{31}P$ NMR spectroscopy were used to determine the orientation of

DPG. DPG behaves like a phospholipid in both phases with the acyl chains roughly parallel to those of DOPE-Me and the glycerol moiety oriented at the lipid/water interface. These observations support the model for myoblast fusion (Wakelam, 1987) which is outlined in fig. 3.7.1. The production of *sn* -1,2-DAG in the inner leaflet of the plasma membrane upon agonist stimulation of fusion-competent myoblasts, results in the $L_\alpha$ to $H_{II}$ phase transition. This occurs because the loss of the hydrophilic headgroup of PtdIns 4,5 $P_2$ causes compensatory phospholipid spreading. Membrane curvature is generated due to stretching of the outer leaflets of the two apposed bilayers. When the phospholipid headgroups become spread so far from each other that they are no longer able to protect the hydrophobic interior from contact with water, the bilayer destabilises and the lipids repack to form an $H_{II}$ phase, which generates an inverted micellar fusion intermediate. As a result, the lipids of the membranes of the two cells are free to mix at the point of contact. This structure is inherently unstable andwill break down, leading either to fusion or reformation of two independent cells. There are approximately 50 molecules from each monolayer present in this intermediate (Siegel 1984). A single molecule of DAG is thus equivalent to 2 mol%, the value required for the $L_\alpha$ to $H_{II}$ formation in egg PE (Das and Rand, 1986).

The stimulation of fusion-competent myoblasts with fusion mix results in a 2-fold increase in the levels of *sn* -1,2-DAG to $0{\cdot}175 \pm 0{\cdot}028$ mol% in the membrane (see section 3.2.1). Fusion between myoblasts only occurs at discrete sites of the membrane, thus, if *sn* -1,2-DAG production is confined to these sites, the effective local concentration of *sn* -1,2-DAG generated, may approach that required to effect the $L_\alpha$ to $H_{II}$ phase transition.

Evidence for localised changes in membrane structure on fusion comes from studies on the changes in membrane fluidity when myoblasts fuse. For example, Weidekamm *et al.*, (1976) demonstrated that addition of $Ca^{2+}$ to fusion -competent myoblasts resulted in an increase in membrane fluidity as measured by changes in the fluorescence polarisation of a membrane probe, A23 187.

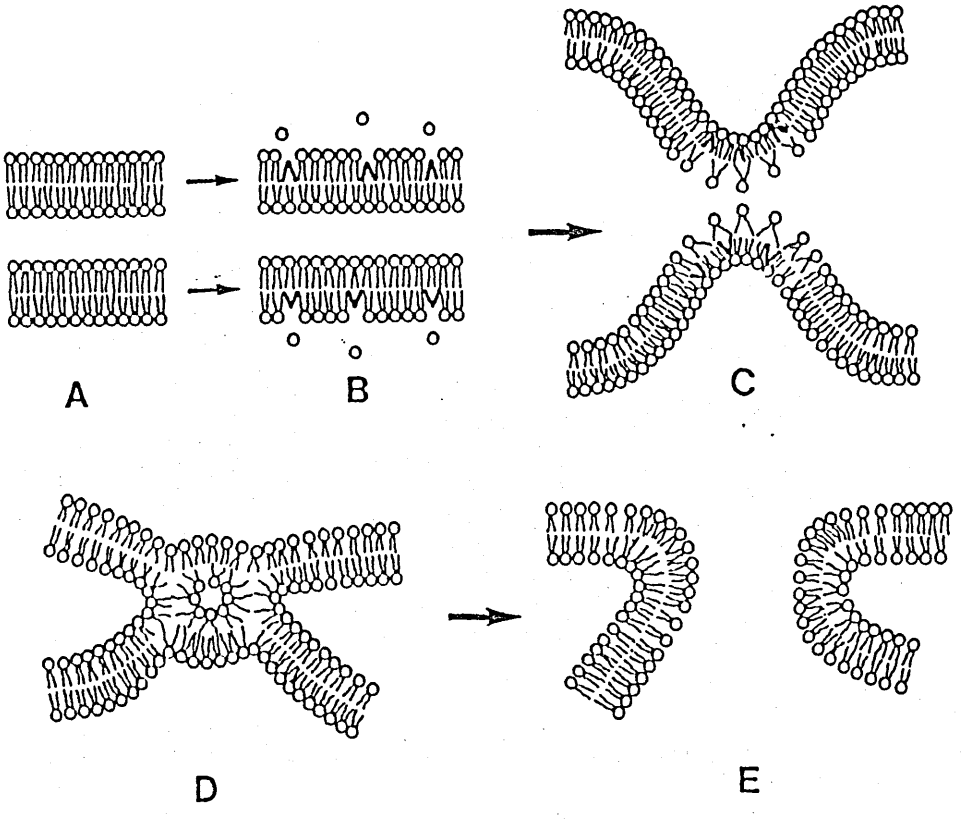



**Figure 3.7.1. A possible model for the fusion of myoblasts.**

**A.** The plasma membranes of fusion-competent myoblasts are closely apposed but unable to fuse due to intermembrane repulsive forces.

**B.** Agonist stimulated Ptd Ins 4,5 $P_2$ breakdown results in the loss of its hydrophilic head group and consequent generation of *sn* -1,2-DAG in the plasma membrane.

**C.** The phospholipid head groups separate and spread to compensate for the loss of the hydrophilic head group of Ptd Ins 4,5 $P_2$. Membrane curvature is generated due to stretching of the outer leaflets of the bilayers.

**D.** Eventually the spreading polar head groups are insufficient to protect the hydrophobic interior from contact with water. This leads to destabilisation of the bilayer which results in the formation of the $H_{II}$ structure and generation of an inverted micellar fusion intermediate.

**E.** The $H_{II}$ phase is inherently unstable and breaks down, resulting in either fusion or the reformation of separate cells.

Studies using the technique of resonance energy transfer have shown that when cells fuse, the greatest changes in fluidity occur in areas of contact between fusing cells (Herman and Fernandez, 1982). Localised transient membrane destabilisation may occur by the production of *sn* -1,2-DAG in the plasma membrane at the sites of fusion. In this way, the susceptibility of these specific membrane regions to form the inverted micellar fusion intermediate may be controlled by *sn* -1,2-DAG. The levels of *sn* -1,2-DAG return to basal by 20 minutes after stimulation thus providing a mechanism for restabilisation of the membrane following fusion which is essential for cell viability.

Myoblast fusion does not occur instantaneously. Using the criterion of membrane continuity between of living cells using phase contrast microscopy, it has been demonstrated to require at least 10-30 minutes (Holtzer *et al.*, 1958; Bischoff, 1978). The kinetics of fusion have also been studied by using two complementary assays to measure myoblast fusion in suspension culture (Neff *et al.*, 1984). The first is the assay of Knudsen and Horwitz (1977). This defines fusion as the formation of cell aggregates that are resistant to dispersion by trypsin and EDTA. The second assay measured membrane continuity which occurs on fusion, by determining the transfer of a fluorescent lipid from labeled to unlabeled cells on fusion. The addition of $Ca^{2+}$ to fusion-competent myoblasts in '$Ca^{2+}$-free'medium resulted in membrane continuity within 20 - 30 minutes. This has a similar time course to that observed for *sn* -1,2-DAG generation in the membranes of fusion-competent myoblasts (Fig. 3.1.1.*b*.).

## 3.8. Evidence for protein kinase C involvement in fusion.

PKC is a $Ca^{2+}$/phospholipid-dependent enzyme whose activation is considered to play a role in cell growth and differentiation (Nishizuka, 1986). Its activity is regulated by the binding of the activator, *sn* -1,2-DAG which reduces the $Ca^{2+}$ concentration required for activation, so that the enzyme is stimulated at physiological resting $Ca^{2+}$ concentrations (Yamanishi *et al.*, 1979).

Phorbol esters, of which TPA is an example, bind to the regulatory domain of PKC at the *sn* -1,2-DAG binding site and cause its sustained activation (Castagna *et al.*, 1982). These molecules resemble *sn* -1,2-DAG in structure since they possess ester bonds at positions 12 and 13. This allows them to mimic, and thus substitute for *sn* -1,2-DAG. TPA can be used to directly activate PKC in intact cells, where it effectively by-passes the requirement for agonist-stimulated generation of *sn* -1,2-DAG. It thus provides a biochemical tool for examining the involvement of PKC in myoblast fusion.

Long term treatment of myoblasts with 100nM TPA resulted in the inhibition of fusion to levels obtainedfor myoblasts cultured in '$Ca^{2+}$-free' medium (Fig. 3.2.1.1.). Previous studies have also demonstrated that TPA inhibits myoblast fusion (Cohen *et al.*, 1977; Sulakhe *et al.*, 1985). The results in fig. 3.2.1.1. are consistent with these observations since TPA was present at high concentrations and for prolonged exposure periods; Sulakhe and co-workers studied the effect of addition of 100nM to myoblast from 8 hours after initial plating until 96 hours in culure.

The inhibition of fusion resulting from long term TPA treatment was dose-dependent with an $IC_{50}$ value of 8·3 ± 1·3nM (Fig. 3.2.1.2.). This is the same order of magnitude as the half maximal concentration required for activation of rat brain PKC (Castagna *et al.*, 1982). The structural analogue of TPA, β-phorbol, which is unable to activate PKC, was without effect at concentrations up to 500nM (Fig. 3.2.1.3.). The vehicle for addition was also without effect. These results are consistent with the activation of PKC by TPA.

Long term treatment of TPA has been demonstrated to result in the down-regulation of both phorbol ester binding sites (Collins and Rozengurt, 1982) and PKC activity (Rodriguez-Pena and Rozengurt, 1984). This is due to an increase in the rate of proteolytic degradation of PKC so that there is a reduction in the steady-state levels of the enzyme (Young *et al.*, 1987). This results in a decrease in PKC activity and phorbol ester binding sites, which correlates with PKC activity (Stabel *et al.*, 1987), since PKC is the phorbol ester 'receptor'.

~~The effect of long term TPA treatment of myoblasts on PKC levels was therefore assessed by determining changes in the specific binding of the radiolabelled phorbol ester, [$^3$H] phorbol 12, 13 dibutyrate (PDBu). Long term treatment of~~

The effect of long term TPA treatment of myoblasts on PKC levels was therefore assessed by determining changes in the specific binding of the radiolabelled phorbol ester, [$^3$H] phorbol 12, 13 dibutyrate (PDBu). Long term treatment of myoblasts from 24 hours after initial plating in $Ca^{2+}$ free medium to fusion-competence results in a 45·7 ± 7% reduction in the levels of [$^3$H PDBu] binding relative to the control (Fig. 3.2.2.2.). This is interpreted as reflecting a loss of the TPA sensitive isoform of PKC. This down-regulation was specific for TPA since the PKC inactive β-phorbol and the vehicle for phorbol addition were without effect. The data obtained using TPA suggest a role for PKC in fusion.

PKC involvement was further investigated by examining the effects of two putative PKC inhibitors on fusion. Those tested were polymyxin B and staurosporine. PKC inhibitors have been used extensively to define its physiological functions. They can be classified into two groups, depending upon their site of action, at either the regulatory or catalytic domain of the enzyme. Polymyxin B interacts with $Ca^{2+}$ and acidic phospholipids and inhibits PKC by binding at the regulatory domain. Staurosporine probably interacts with the catalytic domain to exert its effect. The addition of these compounds to myoblasts cultured in medium containing 1.4mM $Ca^{2+}$, resulted in the inhibition of fusion, to control levels, obtained for myoblasts cultured in '$Ca^{2+}$-free' medium (Figs. 3.3.1.1. and 3.3.2.1). This inhibition was dose-dependent with $IC_{50}$ values for fusion inhibition of 7·2 ± 3·2μM and 7·1 ± 2·4nM, respectively. These inhibitors were added at 24 hours after initial plating. At this time, myoblasts are extensively aligned in culture, in preparation for fusion (Wakelam, 1985). Thus the inhibition of fusion obtained with these compounds is probably due to inhibition of the fusion event itself. Further information on their mode(s) of fusion inhibition is difficult to obtain using this culture system, since fusion occurs asynchronously, which hinders the mechanistic analysis of fusion.

The mechanism of fusion inhibition by the PKC modulators TPA, polymyxin B and staurosporine may be more clearly defined using the low $Ca^{2+}$ culture system, in which rapid synchronous fusion occurs in the presence of extracellular $Ca^{2+}$. Their

effects on fusion were therefore determined using this system. Myoblasts were cultured in '$Ca^{2+}$-free' medium until fusion-competence and then preincubated for 10 or 15 minutes in the presence and absence of the PKC modulators prior to the addition of fusion mix. Fusion was assessed 20 hours later.

Treatment with polymyxin B, staurosporine resulted in a dose-dependent inhibition of fusion (figs 3.4.1., 3.4.2. and 3.4.3. respectively). Maximal inhibition caused a reduction in fusion to levels obtained for cells cultured in '$Ca^{2+}$-free' medium. The $IC_{50}$ values for inhibition by polymyxin B, staurosporine and TPA were 9·3 ± 1·7μM, 13·5 ± 2·8nM and 10 ± 1·2nM respectively. These are of the same order of magnitude as the $IC_{50}$ values obtained for the inhibition of fusion by long term treatment with these PKC modulators. β-phorbol was without effect at concentrations up to 500nM (Fig. 3.2.1.2.. and 3.4.4.). The results obtained using two different culture systems are thus consistent with the same target site of action for these compounds.

The results obtained with the protein kinase C inhibitors should be interpreted with caution since neither of these inhibitors is completely selective for PKC. The entry of $Ca^{2+}$ which occurs just prior to fusion (David *et al.*, 1981) will also potentially affect events regulated by calmodulin (CaM) the intracellular $Ca^{2+}$ receptor protein, calmodulin (CaM) (Means, 1982). Staurosporine has been reported to inhibit myosin light chain kinase (MLCK) which is a $Ca^{2+}$/CaM dependent enzyme (Watson *et al.*, 1988). Polymyxin B also inhibits this enzyme by competing for CaM (Kuo *et al.*, 1983). They do, however, exhibit some selectivity for PKC over MLCK *in vitro*, with $IC_{50}$ values of 2μM (PKC) and 105μM (MLCK) for polymyxin B (Kuo *et al.*, 1983) and 3nM (PKC) and 100nM (MLCK) for staurosporine (Watson *et al.*, 1988).

Staurosporine also inhibits protein kinase A (PKA), ($IC_{50}$ value: 8nM (Ruegg and Burgess, 1988), which is activated by cyclic adenosine 5' monophosphate (cyclic AMP). Cyclic AMP has been proposed as the signal which initiates the onset of fusion (Zalin and Montague, 1975). This was based on the observation that a transient rise in cyclic AMP precedes fusion (Zalin and Montague, 1974) and that prostaglandin $E_1$ and

isoproterenol induce precocious fusion by elevating levels of cyclic AMP (Zalin and Montague, 1975). These findings have however been challenged by Schutzle and co-workers, who were unable to detect this rise in cyclic AMP prior to fusion. They demonstrated instead that its levels are elevated following fusion, and suggest that this increase is a consequence, rather than a cause of fusion (Schutzle *et al.*, 1984). Another line of evidence against cyclic AMP involvement in fusion comes from the demonstration that that chronic exposure of myoblasts to TPA, which prevents fusion, results in the activation of β–adrenergic receptors and consequently cyclic AMP levels are increased (Sulakhe *et al.*, 1985). PKA activation is therefore unlikely to play a role in fusion, unless TPA prevents cyclic AMP mediated fusion by blocking some event in the fusion process which ocurs subsequent to activation of this enzyme.

If the data obtained using staurosporine and polymyxin B are taken in conjunction with for TPA; the results presented suggest a role for PKC activation in myoblast fusion. Supporting evidence for PKC involvement in fusion comes from the observation that changes in PKC activity and its subcellular distribution occur during chick embryonic myoblast differentiation (Adamo *et al.*, 1989). This enzyme is active in the membrane inserted 'particulate' form (May *et al.*, 1985) and soluble PKC thus represents a potential source of enzyme activity for recruitment to the plasma membrane upon agonist stimulation of the cell. At the time of fusion, PKC is predominantly particulate whereas, following fusion, there is a decrease in particulate and an increase in cytosolic activity (Adamo *et al.*, 1985). This probably reflects detachment of PKC from the membrane and its recovery in the cytosolic fraction. These results suggest that PKC is active when myoblasts fuse to form myotubes. The subsequent decline in particulate and rise in cytosolic PKC activity probably corresponds to myotube maturation following muscle specific proteins synthesis. This would be consistent with data indicating that PKC is predominantly cytosolic in cultured mouse myotubes (Cossu *et al.*, 1986).

## 3.9. Mechanism of the inhibition of fusion by short term preincubation with TPA.

The data presented in this chapter demonstrate that TPA treatment of myoblasts resulted in the inhibition of fusion, to levels seen in the control, 'non-fused' samples. The percentage of fusion is never zero, due to the presence of preformed myotubes at the time of plating, and is approximately 5-10%. The inhibition of fusion by TPA is dose-dependent and is consistent with activation of PKC. This is confirmed by the inability of β-phorbol, a structural analogue of TPA, which is unable to activate PKC, to inhibit fusion. This lack of inhibition with β-phorbol also implies that the effect of TPA on fusion is probably not due to its hydrophobic properties resulting in non-specific perturbation of the membrane. This was further demonstrated by use of the fluorescent lipid probe, AF C-18, a molecule which is considered to behave like a phospholipid since it possesses an acyl chain which has 18 carbon atoms. It is believed to insert alongside to endogenous phospholipids of the myoblast membrane. The fluorescence observed after AF C-18 labelling was not due to fluorescent micelles adhering to the membrane surface, since the fluorescent label, aminofluorescein, self-quenches when it is present in micelles. The location of AF C-18 in the plasma membrane was confirmed by quenching of its fluorescence by trypan blue (fig. 3.5.1.3.). This is due to resonance energy transfer which occurs due to the overlap between the emission spectrum of AF C-18 and the absorption spectrum of trypan blue.

The effect of TPA on membrane fluidity was investigated by Fluorescence Recovery After Photobleaching (FRAP) using the fluorescent lipid probe AF-C18. The lateral diffusion coefficient ($D_L$) and percentage recovery of fluorescence was determined in the presence and absence of TPA (Table 3.5.1.). TPA was found to have no effect on either the rate of diffusion of the probe in the membrane or its percentage mobility. These observations suggest that TPA inhibition of fusion is mediated through PKC activation rather than by non-specific perturbation of the plasma membrane bilayer structure.

Activation of PKC is considered to be involved in the process of cell proliferation (Rodriguez-Pena and Rozengurt, 1984). Myoblasts must withdraw from the cell cycle prior to fusion. The effect of TPA on myoblast proliferation was therefore examined as a possible mechanism for its inhibition of fusion. The incorporation of [methyl-$^3$H] thymidine into acid-insoluble material was used to determine DNA synthesis (Adams, 1969) as a measure of proliferation. TPA inhibited the incorporation of [methyl-$^3$H] thymidine into myoblasts (fig.3.5.2.1), by a maximum of 31·1 ± 2·4%. This inhibition was dose-dependent with an $IC_{50}$ value of 6·6 ± 2·9nM which is consistent with activation of PKC (Castagna *et al.*, 1982) and is supported by the lack of inhibition obtained in the presence of β-phorbol (fig. 3.5.2.2.). The maintenance of myoblasts in the cell-cycle to prevent them from fusing, does not therefore provide an explanation for TPA inhibition of fusion. In fact, TPA inhibits myoblast proliferation by a mechanism which apparently involves activation of PKC.

The activation of PKC by *sn* -1,2-DAG is a transient event due to its removal from the plasma membrane by phosphorylation or hydrolysis. TPA activation is however long lived and prolonged exposure of cells to this compound results in the PKC down-regulation (Collins and Rozengurt, 1982). This is due to an increase in the rate of degradation of the enzyme leading to reduced steady-state levels (Young *et al.*, 1987). The inhibition of proliferation observed with TPA is, therefore, probably due to a reduction in the amount of PKC present in myoblasts which occurs following long term treatment TPA, as demonstrated by a decrease in the specific binding of [$^3$H] PDBu (Fig. 3.2.2.2.)

Short term treatment of cells with TPA prior to challenge with agonist has been demonstrated to inhibit inositol phospholipid breakdown (Rittenhouse and Sassan, 1985; Brown *et al.*, 1987). The effect of TPA on the generation of total [$^3$H] inositol phosphates was therefore examined as a possible mechanism for its inhibition of myoblast fusion. Ten minute pretreatment of fusion-competent myoblasts with TPA resulted in a 38·9 ± 4·4% maximal inhibition of stimulated inositol phospholipid

breakdown, in response to fusion mix as determinedby measuring total inositol phosphate generation using the $Li^+$ assay (fig 3.5.3.1). The $IC_{50}$ value for this inhibition is 4·1 ± 0·7nM which is consistent with activation of PKC (Castagna *et al.*, 1982), and is supported by the lack of effect of the PKC inactive β-phorbol at a concentration of 500nM and the vehicle for TPA/β-phorbol addition. These results suggest that the inhibition of PtdIns 4,5 $P_2$ hydrolysis is mediated by activation of PKC.

There are several possible sites at which TPA could act to exert this effect via PKC activation. It may inhibit binding of the agonist to its receptor, as has been demonstrated for TPA inhibition of total inositol phosphate generation in hepatocytes, in response to α1-adrenergic stimulation by noradrenaline (Corvera *et al.*, 1986). Alternatively, TPA may uncouple agonist stimulated PtdIns 4,5 $P_2$ breakdown by phosphorylation of either PLC itself (Geny *et al.*, 1989) or the putative G-protein coupling it to the receptor. Phosphorylation of this G-protein could potentially interfere with the receptor-G-protein and/or G-protein-PLC interaction(s). Such effects of TPA treatment on G-protein function have been demonstrated to occur (Plevin *et al.*, 1990; Smith *et al.*, 1987).

For example, it has been shown that pre-incubation of electropermeabilised Swiss 3T3 fibroblasts with TPA resulted in the inhibition of bombesin stimulated total [$^3$H] inositol phosphate generation and the potentiation of this response by GTPγS. The stimulation obtained with GTPγS alone was however unaffected. It was therefore suggested that, in these cells, TPA impairs the coupling of the bombesin receptor to the G-protein (Plevin *et al.*, 1990). A different mechanism for the TPA inhibition of agonist stimulated PtdIns 4,5 $P_2$ breakdown has been proposed to occur in human neutrophils (Smith *et al.*, 1987). In these cells TPA inhibits GTPγS stimulated total inositol phosphate generation and therefore probably impairs the interaction of the G-protein with PLC.

Long term treatment of myoblasts with TPA, which inhibits fusion, also results in an inhibition of total [$^3$H] inositol phosphate generation in response to the fusion

stimulator, fusion mix. Such long term treatment resulted in a 22·5 ± 2·5% inhibition of fusion mix stimulated total [$^3$H] inositol phosphate generation. This effect is specific for TPA since it was not obtained in the presence of β-phorbol or the vehicle for TPA/β-phorbol addition to myoblasts.

It therefore appears that fusion mix stimulated myoblast fusion and total [$^3$H] inositol phosphate generation show different sensitivities to inhibition by TPA. Long or short term treatment of myoblasts with TPA results in the complete inhibition of fusion to control 'non-fused' levels (figs. 3.2.1.1 and 3.4.3. respectively), but only partially inhibits total [$^3$H] inositol phosphate generation in response to fusion mix (figs.3.5.3.2. and 3.5.3.1.respectively). A possible explanation for this may be that there is a threshold level of PtdIns 4,5 $P_2$ breakdown required for fusion, below which fusion cannot occur. In this case, TPA could prevent fusion by inhibiting this breakdown so that the required threshold level is not reached. Alternatively, there may be two or more isoforms of PKC present in fusion-competent myoblasts, one of which regulates the breakdown of PtdIns 4,5 $P_2$ by negative control whilst another isoform is involved in the fusion process itself. This may be possible since there are at least seven isozymes of PKC, designated α-, β1 and β2, δ-, ε-, γ– and ζ-, which share common structural features but have distinct biochemical properties (Knopf *et al.*, 1986; Parker *et al.*, 1986; Nishizuka, 1988) in that they exhibit different sensitivities to *sn* -1,2 DAG, phosphatidylserine, fatty acids and $Ca^{2+}$ (Nishizuka, 1988). Although some cell types eg NIH 3T3 cells exhibit only the α subtype (McCaffrey *et al.*, 1987), most co-express several isozymes of PKC in differing ratios (Farago and Nishizuka, 1990) and these differ in their intracellular and cellular locations, depending on the state of growth or differentiation of the cell. PKC isozymes are not equally susceptible to proteolysis (Kishimoto *et al.*, 1989) and are subject to differential down-regulation by TPA (Huang *et al.*, 1989). These differences suggest that PKC isozymes are involved in different aspects of cell regulation and provide support for the proposal that there are at least 2 such isozymes involved in the regulation of fusion-mix stimulated fusion and PtdIns 4,5 $P_2$ hydrolysis.

From section 3.2, long term TPA treatment of myoblasts resulted in a 45·7% decrease in PKC levels. It is thus possible that the isozyme of PKC involved in myoblast fusion was completely down-regulated in response to long term treatment with TPA whilst the other PKC isozyme(s) are either unaffected or down-regulated to a lesser extent. The PKC isozyme which mediates the negative feedback regulation of PtdIns 4,5 $P_2$ breakdown was probably only partially down-regulated since the TPA inhibition of fusion mix stimulated total [$^3$H] inositol phosphate generation was reduced from 38·9 to 22·5%, following long term treatment with TPA. If there was complete down-regulation of this PKC isozyme, then it would be predicted that the agonist-stimulated generation of total [$^3$H] inositol phosphates would be enhanced, due to the loss of the negative feedback control. This has been demonstrated to occur in Swiss 3T3 fibroblasts (Brown *et al.*, 1987) and rat basophilic leukaemia (RBL-2H3) cells (Gat-Yablonski and Sagi-Eisenberg, 1990), following complete down-regulation of PKC by long term TPA treatment. The reduced ability of TPA to inhibit the fusion mix stimulated breakdown of PtdIns 4,5 $P_2$ in myoblasts exposed to 100nM TPA from 24 hours after initial plating in '$Ca^{2+}$-free' medium to fusion-competence, is probably due to TPA activation of the residual PKC isozyme which mediates the negative feedback control of this event.

The putative PKC inhibitors, polymyxin B and staurosporine, inhibited myoblast fusion but were without effect on the stimulation of total [$^3$H] inositol phosphate generation in response to fusion mix. This is consistent with previous studies which have demonstrated that staurosporine has no effect on thrombin stimulated formation of total [$^3$H] inositol phosphates (Watson *et al.*, 1988) and that the putative PKC inhibitor, sphingosine, does not inhibit antigen induced total inositol phosphate generation in rat basophilic leukaemia (RBL-2H3) cells (Gat-Yablonski and Sagi-Eisenberg, 1990). Polymyxin B and staurosporine therefore probably inhibit fusion by exerting their effect on some PKC mediated event that is downstream of PtdIns 4,5 $P_2$ breakdown.

The precise role of PKC in fusion remains to be defined, but it may be involved in the entry of extracellular $Ca^{2+}$ which is essential for and occurs prior to fusion. This entry of $Ca^{2+}$ into myoblasts may be involved in fusion associated rearrangements of the cytoskeleton (Kalderon and Gilula, 1979; Fulton *et al.*, 1981) which are proposed facilitate the local removal of cytoskeletal anchored membrane proteins and thus clear the fusion site to permit mixing of the plasma membranes at their point of contact. A $Ca^{2+}$-activated neutral protease which has been reported to be active at the time of fusion (Kaur and Sanwal., 1984) may play a role in these rearrangements. In this regard, TPA has been recently demonstrated to (i) enhance $Ca^{2+}$ entry into myoblasts as determined using $^{45}Ca^{2+}$ and (ii) to induce precocious fusion (David *et al.*, 1990) over the same time course as the $Ca^{2+}$ ionophore, A23187 (David *et al.*, 1981) i.e. in the 9 hour period prior to the initiation of fusion normally observed in these cultures. However, TPA has also been demonstrated to inhibit $Ca^{2+}$ fluxes into myoblasts (Grove and Schimmel, 1982). These conflicting reports could probably be clarified by use of a fluorescent $Ca^{2+}$ indicator such as Quin 2 or Fura 2 to examine $Ca^{2+}$ fluxes in fusion-competent myoblasts treated with TPA in the presence of extracellular $Ca^{2+}$. If extracellular $Ca^{2+}$ enters myoblasts in a PKC dependent manner then it should (i) occur upon short term treatment with TPA and (ii) be sensitive to treatment with putative PKC inhibitors such as, polymyxin B and staurosporine. The effect of short and long term pretreatment of myoblasts with TPA on the influx of extracellular $Ca^{2+}$, in response to agonist stimulation of fusion-competent myoblasts could also be investigated, to determine whether TPA inhibits myoblast fusion by preventing the entry of extracellular $Ca^{2+}$. In this case, agonist-stimulated generation of *sn* -1,2-DAG in fusion-competent myoblast plasma membranes could therefore initiate fusion by co-ordinating both (i) the formation of lipid fusion intermediates and (ii) $Ca^{2+}$ entry leading to cytoskeletal rearrangements.

In summary, the results presented in this chapter suggest that the generation of *sn* -1,2-DAG upon fusion stimulation, and the consequent activation of PKC, represent key events in the regulation of myoblast fusion.

# Chapter 4
# Guanine nucleotide binding proteins (G proteins) and myoblast differentiation

## Introduction.

The fusion of myoblasts is a receptor mediated event which is accompanied by an increase in the synthesis of muscle specific proteins including the β–adrenergic and nicotinic acetylcholine neurotransmitter receptors. The transfer of information from many such agonist activated cell surface receptors to effector enzymes or ion channels is mediated by a family of guanine nucleotide binding proteins (G-proteins), which are heterotrimeric entities composed of 3 distinct subunits, designated α β and γ. The function and specificity of each G-protein is defined by the nature of its α subunit (Gilman , 1987) whilst the β subunits associate with γ to form βγ subunit complexes which are functionally interchangeable between different G-proteins (Northup *et al.*, 1983; Katada *et al.*, 1984).

G-proteins are known to occur in both adult skeletal muscle (Toutant *et al.*, 1988; Takamori and Yoshikawa, 1989) and in myoblast cell lines (Gardner *et al.*, 1989; Kelvin *et al.*, 1989). The G-proteins present in the membranes of 12-day-old chick embryonic myoblasts were characterised by using two different experimental approaches. These methods both identify Gα subunits and thus define the G-proteins present because these are unique for each G-protein type. In the first approach, G-proteins present in myoblast membranes were examined as possible substrates for the cholera and pertussis toxins which are bacterial toxins, derived from *Vibrio cholerae* and *Bordatella pertussis* respectively. This was carried out because many Gα subunits are substrates for ADP-ribosylation by these toxins, which catalyse the transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide ($NAD^+$) to the α subunit. [$^{32}P$] $NAD^+$ was used as the donor of the ADP-ribose to enable the toxin substrates to be visualised autoradiographically, following resolution by SDS-PAGE. The second approach taken was immunoblotting with a panel of selective anti-G-protein anti-peptide antisera that were raised against sequences unique to the various α subunits of the cholera and pertussis toxin substrates. To date, nine G-proteins have been identified which are currently considered to be involved in signal transduction

pathways. These are listed in Table 4.1 together with their known functions and sensitivity to cholera and pertussis toxins.

**Table 4.1.**

**G-proteins, their functions and sensitivity to pertussis and cholera toxin-catalysed ADP-ribosylation.**

| G-protein | Signal transducing system | Sensitivity to ADP-ribosylation by pertussis and cholera toxins |
|---|---|---|
| $G_s$ | Adenylate cyclase (stimulatory) | Cholera toxin sensitive |
| $G_{olf}$ | Adenylate cyclase (stimulatory) in olfactory sensory neurones | Cholera toxin sensitive |
| $G_i1$ | Unknown | Pertussis toxin sensitive |
| $G_i2$ | Adenylate cyclase (inhibitory) | Pertussis toxin sensitive |
| $G_i3$ | $K^+$ channels | Pertussis toxin sensitive |
| $G_o$ | $Ca^{2+}$ channels | Pertussis toxin sensitive |
| $G_z$ | Unknown, PI-PLC (stimulatory)? | Cholera and pertussis toxin insensitive |
| TD1 | cyclic GMP PDE in rod outer segments | Cholera and pertussis toxin sensitive |
| TD2 | cyclic GMP PDE in cone outer segments | Cholera and pertussis toxin sensitive |

Abbreviations:

cyclic GMP PDE=cyclic guanosine 3',5'monophosphate phosphodiesterase
PI-PLC=phosphoinositide phospholipase C

The addition of the agonist, fusion mix (10% (v/v) heat inactivated horse serum, 5% chick embryo extract and 1.4mM $Ca^{2+}$), to fusion-competent myoblasts results in the stimulation of both inositol phospholipid breakdown and myoblast fusion. This receptor-phosphoinositide phospholipase C (PI-PLC) interaction may, in common with other agonist stimulated events, be G-protein mediated. The possible role of G-proteins in this process was therefore investigated.

Myoblast differentiation is accompanied by the appearance of neurotransmitter receptors such as β–adrenergic receptors (Parent *et al.*, 1980). Such development of hormonal responsiveness is a common feature of the differentiation of various cell types during embryogenesis. Given the major importance of G-proteins in signal transduction, it is probable that there are changes in the types and relative amounts of these proteins during myoblast differentiation. Evidence for this comes, for example, from studies on the neuroblastoma x glioma hybrid cell line NG108-15, where differentiation with dibutyryl cyclic AMP results in changes in the relative amounts of the G-proteins $G_i$ α and $G_o$α (Mullaney *et al.*,1988). Membranes prepared from chick embryonic skeletal myoblasts were therefore studied in order to determine whether there are changes in the relative amounts of the various G-proteins during differentiation.

## Results.

### 4.1 Pertussis-toxin/cholera-toxin catalysed ADP-ribosylation of myoblast membrane proteins.

G-proteins present in myoblast membranes were tested as possible substrates for cholera toxin and pertussis toxin catalysed ADP-ribosylation. The results are shown in figs. 4.1.1, 4.1.2 and 4.1.3.

Fig. 4.1.1 shows membrane samples ADP-ribosylated with 2.5μg/assay thiol preactivated cholera toxin using [$^{32}$P] $NAD^+$ in the presence of exogenous GTP. Under these conditions the α subunit of mammalian $G_s$ is specifically ribosylated (Milligan,1988). Samples were resolved by SDS-PAGE and subsequently autoradiographed.

**Figure 4.1.1.**

**Cholera-toxin-catalysed ADP-ribosylation**

Membrane samples from

a) rat glioma C6 BU1 cells (30μg)

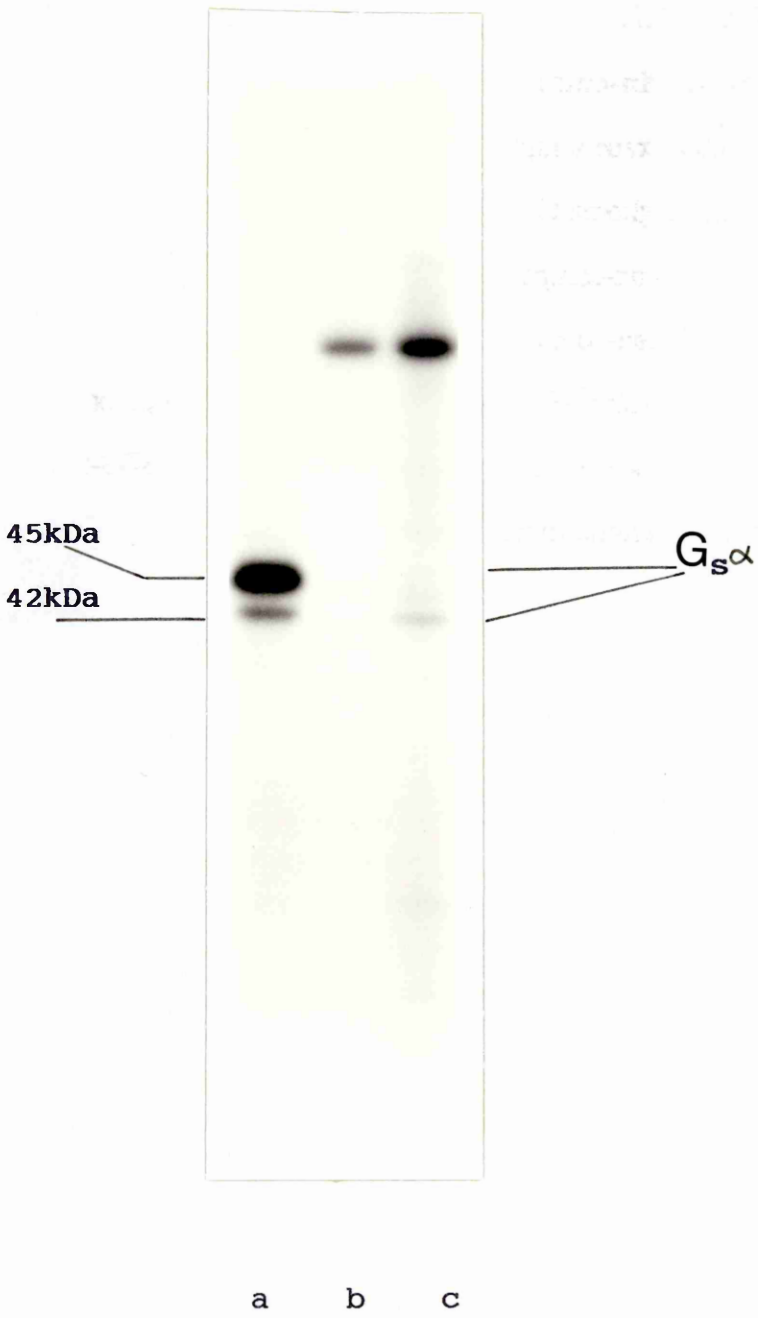
b) fusion-competent myoblasts (30μg)

c) fusion-competent myoblasts (30μg)

were treated with [$^{32}$P] $NAD^+$ and cholera toxin except for sample b) to which no cholera toxin was added. Samples were resolved by SDS-PAGE [10% acrylamide]. The gel was autoradiographed following drying.

Figure 4.1.1.

Molecular Weight



Rat glioma C6 BU1 cells (lane a) possess two forms of $G_s\alpha$ of molecular weights 42kDa and 45kDa. They were used to provide a suitable positive control. Samples in lanes b and c are membranes prepared from fusion-competent myoblasts which were incubated with [$^{32}$P] $NAD^+$ in the presence (lane c) and absence (lane b) of cholera toxin. Comparison of these membrane samples revealed specific cholera-toxin-catalysed ADP-ribosylation of a 42kDa polypeptide.

Figs. 4.1.2 and 4.1.3 show membrane samples ADP-ribosylated with 0.5µg/assay thiol preactivated pertussis toxin and [$^{32}$P] $NAD^+$. Samples were alkylated with N-ethylmaleimide prior to resolution by SDS-PAGE [12.5% acrylamide/0.06% bisacrylamide] because this treatment has been shown to enhance the resolution of mammalian pertussis toxin sustrates (Sternweis and Robishaw,1984). From fig. 4.1.2., (lane b), it can be seen that rat glioma C6 BU1 cells possess a 40kDa polypeptide which was ADP-ribosylated by pertussis toxin. This corresponds to $G_i2\alpha$ which is the major pertussis toxin sensitive sustrate of these cells (Goldsmith *et al.*, 1987). These membranes were used to provide a positive control for pertussis-toxin-catalysed ADP-ribosylation. Samples in lanes a and c are membranes prepared from fusion-competent myoblasts which were incubated with [$^{32}$P] $NAD^+$ in the presence (lane a) or absence (lane c) of pertussis toxin. Comparison of these samples shows that pertussis-toxin-catalysed [$^{32}$P] ADP-ribosylation of myoblast membranes resulted in the specific incorporation of radioactivity into polypeptides of apparent molecular weights of 38kDa and 41kDa. In later experiments, greater resolution of mammalian G-proteins was achieved (fig. 4.1.3.) so that three well resolved pertussis toxin substrates of apparent molecular weights 39kDa, 40kDa and 41kDa were detected in rat cerebral cortex (lane a). These correspond to $G_o\alpha$ $G_i2\alpha$ and $G_i1\alpha$ respectively. (Goldsmith *et al.*, 1987). The 41kDa pertussis toxin substrate present in chick embryonic myoblasts in fig. 4.1.2. was further resolved into two polypeptides of apparent molecular weights 40.5kDa and 41.5kDa. (lane c).

In conclusion, there were apparently one cholera toxin and at least three pertussis toxin substrates for ADP-ribosylation present in myoblast membranes.

**Figure 4.1.2.**

**Pertussis-toxin-catalysed ADP-ribosylation.**

Membrane samples from

a) fusion-competent myoblasts (30μg)

b) fusion-competent myoblasts (30μg)

c) fusion-competent myoblasts (30μg)

d) rat glioma C6 BU1 cells (30μg)

e) rat cerebral cortex (10μg)

were treated with [$^{32}$P] $NAD^+$ and pertussis toxin except for sample c) to which no pertussis toxin was added. Samples were alkylated prior to resolution by SDS-PAGE [12.5% acrylamide/0.06% bisacrylamide]. The gel was autoradiographed following drying.

Figure 4.1.2.

Molecular Weight

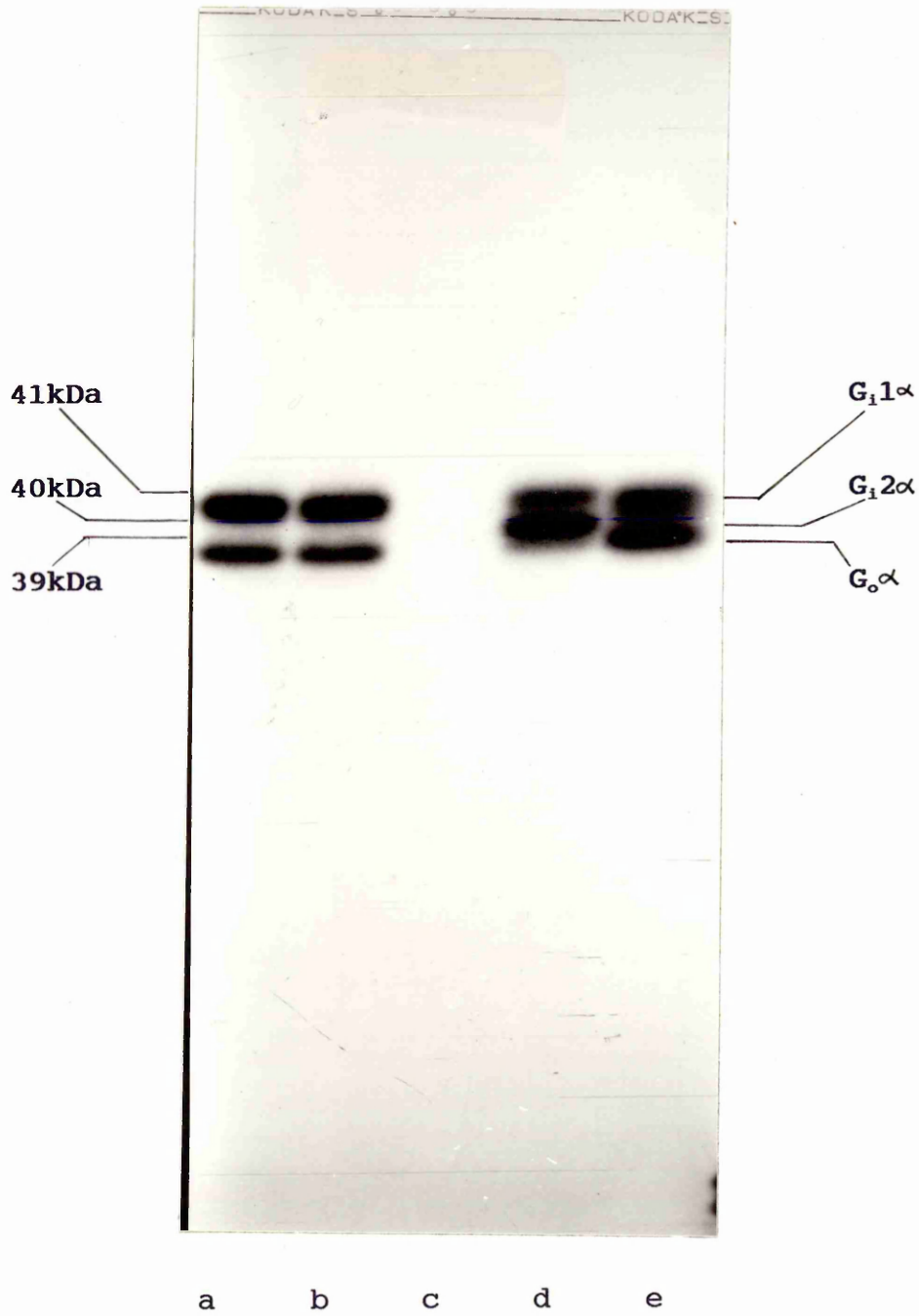


**Figure 4.1.3.**

**Pertussis-toxin-catalysed ADP-ribosylation.**

Membrane samples from

a) rat cerebral cortex (10μg)

b) rat glioma C6 BU1 cells (30μg)

c) fusion competent myoblasts (30μg)

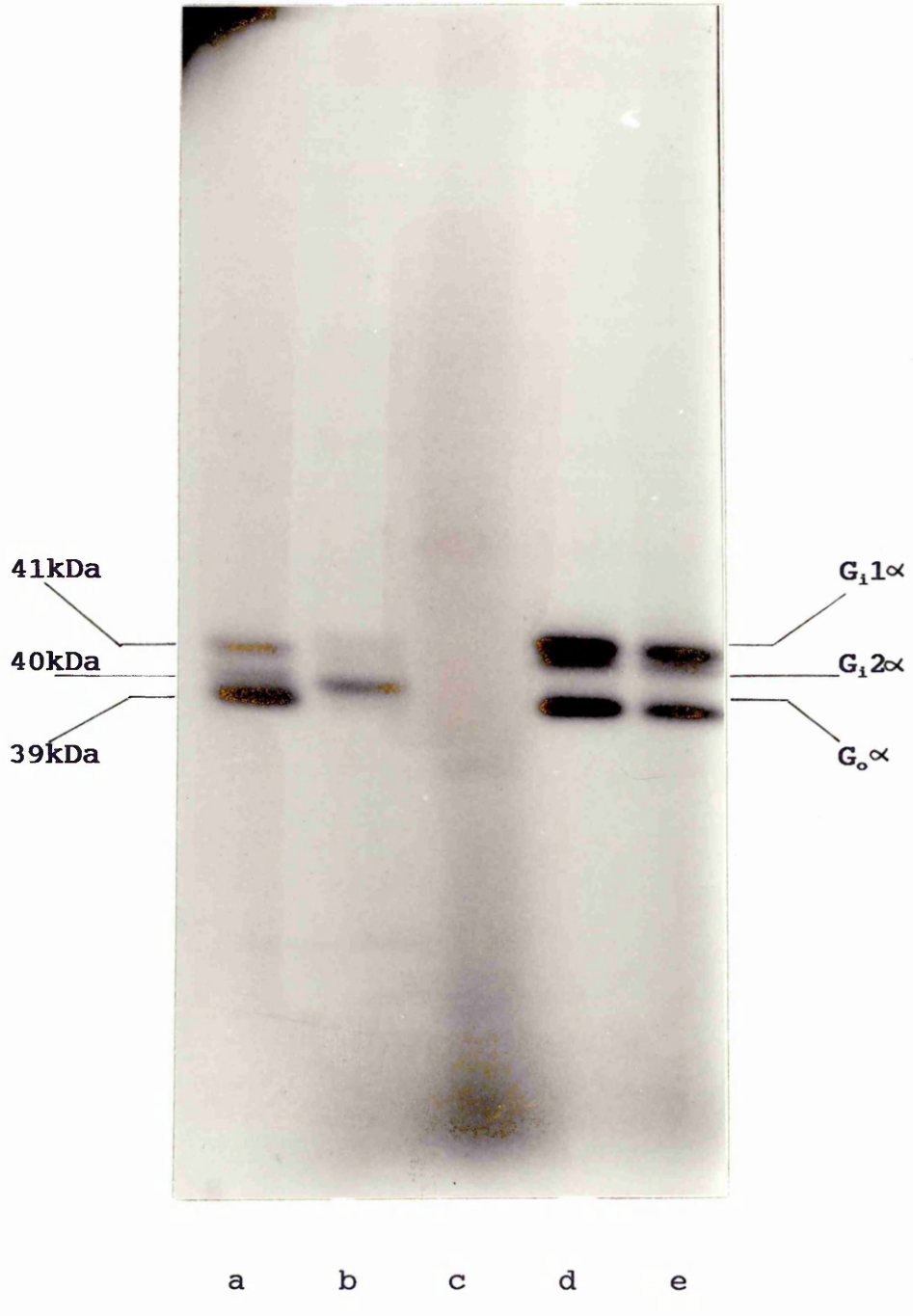
d) fusion-competent myoblasts (30μg)

e) fusion-competent myoblasts (30μg)

were treated with [$^{32}$P] $NAD^+$ and pertussis toxin except for sample c) to which no pertussis toxin was added. Samples were alkylated prior to resolution by SDS-PAGE [12.5% acrylamide/0.06% bisacrylamide]. The gel was autoradiographed following drying.

Figure 4.1.3.

Molecular Weight



## 4.2 Western immunoblotting using anti-peptide anti-G-protein antisera.

Selective anti-G-protein anti-peptide antisera against sequences unique to the various cholera toxin and pertussis toxin substrates were used as the primary reagents. The secondary antiserum was donkey anti-rabbit IgG coupled to horse radish peroxidase and ortho-diansidine hydrochloride was used as the enzyme substrate to detect the antibody complex. The product of this reaction was visualised by its rust-brown colour. Rat cerebral cortex, rat glioma C6 BU1 cells and human monocyte U937 cells were the sources of mammalian G-proteins used to provide suitable positive controls for the various antisera employed in these experiments. Table 4.2.1. list these membrane types together with the G-proteins for which they were used as controls. Table 4.2.2. lists the sequences of the peptides employed as antigens and the specificities of the anti - G-protein antisera generated.

### 4.2.1 Identification of $G_s\alpha$ immunoreactivity.

Fig. 4.2.1.1. shows membrane samples resolved by SDS-PAGE and immunoblotted with the CS1 antiserum. CS1 was raised against the synthetic peptide RMHLRQYELL which corresponds to the C terminal amino acid sequence, (residues 385-394), of mammalian $G_s\alpha$. Rat glioma C6 BU1 cell membranes (lane a) possess two forms of the cholera toxin substrate, $G_s\alpha$, of molecular weights 42kDa and 45kDa. In myoblast membranes (lane b) an immunoreactive polypeptide was identified which co-migrated with the 42kDa form of $G_s\alpha$ from rat glioma C6 BU1 cell membranes.

### 4.2.2. Identification of $G_i1\alpha$ and $G_i2\alpha$ immunoreactivity.

Figs.4.2.2.1. and 4.2.2.2. show membrane samples resolved by SDS-PAGE and immunoblotted with the anti-peptide antiserum SG1. This was raised against the synthetic peptide KENLKDCGLF which corresponds to the amino acid sequence of transducin, residues 341-350, and recognises mammalian $G_i1\alpha$ and $G_i2\alpha$ in non-photoreceptor containing tissues (Mitchell *et al.*, 1989). Mammalian

$G_i1\alpha$ and $G_i2\alpha$ are recognised equally because these proteins possess identical amino acid sequences in the region against which the antiserum was raised.

**Table 4.2.1.**

**Membrane sources of G-proteins used as positive controls for Western Blotting experiments.**

| Membrane type | Gα for which samples were used to provide a suitable control | Molecular weight kDa |
|---|---|---|
| C6 BU1 | $G_s$ | 45 and 42 |
| | $G_i2$ | 40 |
| | $G_i3$ | 41 |
| U937 | $G_i3$ | 41 |
| rat cerebral cortex | $G_i1$ | 41 |
| | $G_i2$ | 40 |
| | $G_i3$ | 41 |
| | $G_o$ | 39 |

$G_s\alpha$ is a substrate for cholera-toxin-catalysed ADP-ribosylation (in the presence of GTP)

$G_i1\alpha$, $G_i2\alpha$, $G_i3\alpha$ and $G_o\alpha$ are substrates for pertussis-toxin-catalysed ADP-ribosylation.

**Table 4.2.2**

**Generation and specificities of the anti-peptide anti-G-protein antisera.**

| Antiserum | Peptide | corresponding G-protein sequence | | α subunit recognition |
|---|---|---|---|---|
| CS1 | RMHLRQYELL | $G_s$ | 385-394 | $G_s$ |
| SG1 | KENLKDCGLF | TD1 | 341-350 | TD1,TD2, $G_i$1 and Gi 2 |
| I3B | KNNLKECGLY | $G_i$3 | 345-354 | $G_i$ 3, $G_o$ |
| ON1 | GCTLSAEERAALERSK | $G_o$ | 1-16 | $G_o$ |
| IM1 | NLKEDGISAAKDVK | $G_o$ | 22-35 | $G_o$ |
| I1C | LDRIAQPNYI | $G_i$ 1 | 159-168 | $G_i$ 1 |
| BN1 | MSELDQLRQE | β | 1-10 | β1 |

TD = Transducin

**Figure 4.2.1.1.**

**Identification of $G_s\alpha$ immunoreactivity in myoblasts.**

Membrane samples from

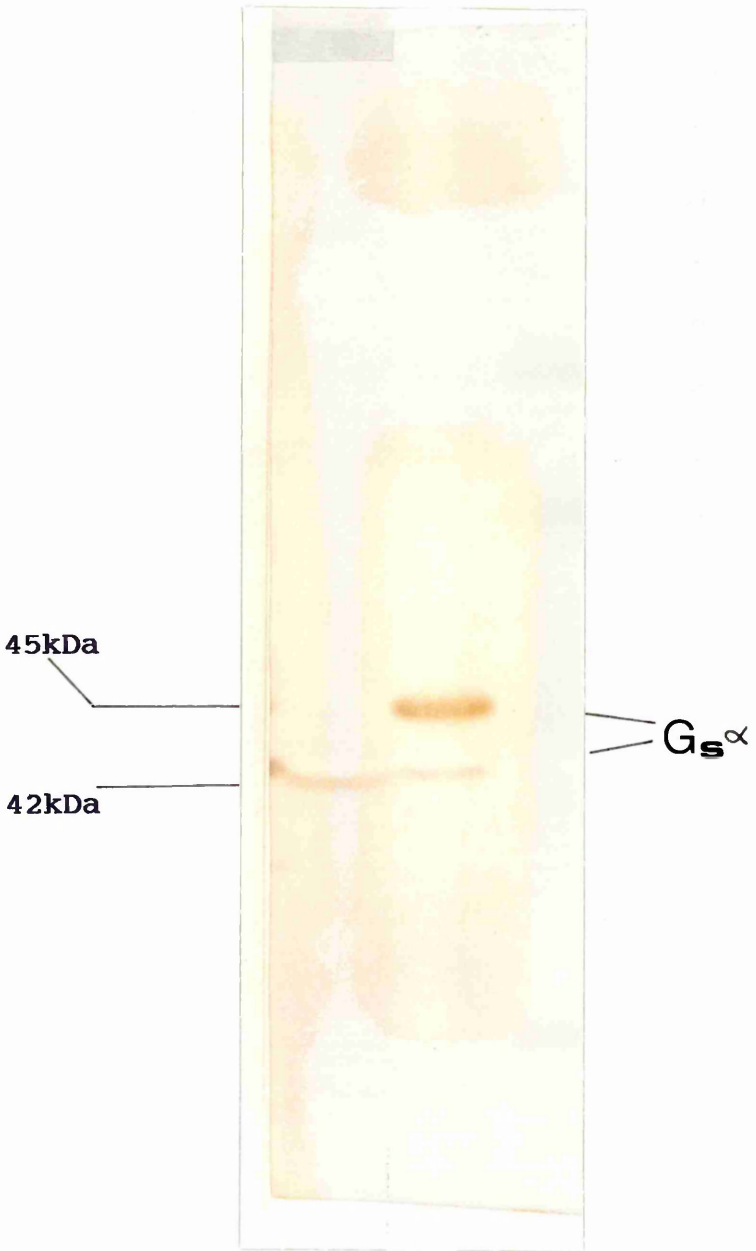
a) fusion-competent myoblasts (250μg)

b) rat glioma C6 BU1 cells (100μg)

were resolved using SDS-PAGE [10% acrylamide] and immunoblotted using the antipeptide antiserum CS1 (1:200) as the primary reagent.

Figure 4.2.1.1.

Molecular Weight




a b

Antiserum SG1 recognised two polypeptides of 41kDa ($G_i1\alpha$) and 40kDa ($G_i2\alpha$) in rat cerebral cortex with $G_i1\alpha$ present in greater abundance than $G_i2\alpha$ (fig. 4.2.2.1., lane b). Rat glioma C6 BU1 cells possess $G_i2\alpha$ (Mitchell *et al.*, 1989) which co-migrated with the 40kDa polypeptide from rat cerebral cortex (fig. 4.2.2.1., lane a). From fig. 4.2.2.1., antiserum SG1 identified two polypeptides of approximately 38kDa and 41kDa, which were present in apparently equal amounts, in chick embryonic myoblast membranes (lane d). A similar pattern of immunoreactivity was also detected for 12-day-old chick embryonic brain membrane samples (lane c). In later experiments, of which that shown in fig. 4.2.2.2. is an example, an additional polypeptide of apparent molecular weight 41.5kDa was identified as being immunoreactive with SG1 (lanes b and c). This may be a reflection of improved resolution by SDS-PAGE as seen in fig. 4.1.3. as these pertussis-toxin -catalysed ADP-ribosylation and immunoblotting experiments were carried out in the same period.

Fig. 4.2.2.3. shows membrane samples resolved by SDS-PAGE and immunoblotted with the anti-peptide antiserum I1C. This was raised against the synthetic peptide LDRIAQPNYI, which corresponds to the amino acid sequence, residues 159-168, of mammalian $G_i1\alpha$. I1C is able to recognise mammalian $G_i1\alpha$ as distinct from $G_i2\alpha$ and it was therefore used to further the conclusions obtained using the SG1 antiserum. From fig. 4.2.2.3., lane a, antiserum I1C recognised a single polypeptide of 41kDa in rat cerebral cortex ($G_i1\alpha$). A single polypeptide of apparent molecular weight 41.5kDa showed immunoreactivity in chick embryonic myoblasts (lane c). A similar pattern of immunoreactivity was seen for 12-day-old chick embryonic brain (lane b).

**Figure 4.2.2.1**

**Identification of $G_i1\alpha$ and $G_i2\alpha$ immunoreactivity in myoblasts (i).**

Membrane samples from

a) rat glioma C6 BU1 cells (100μg)

b) rat cerebral cortex (100μg)

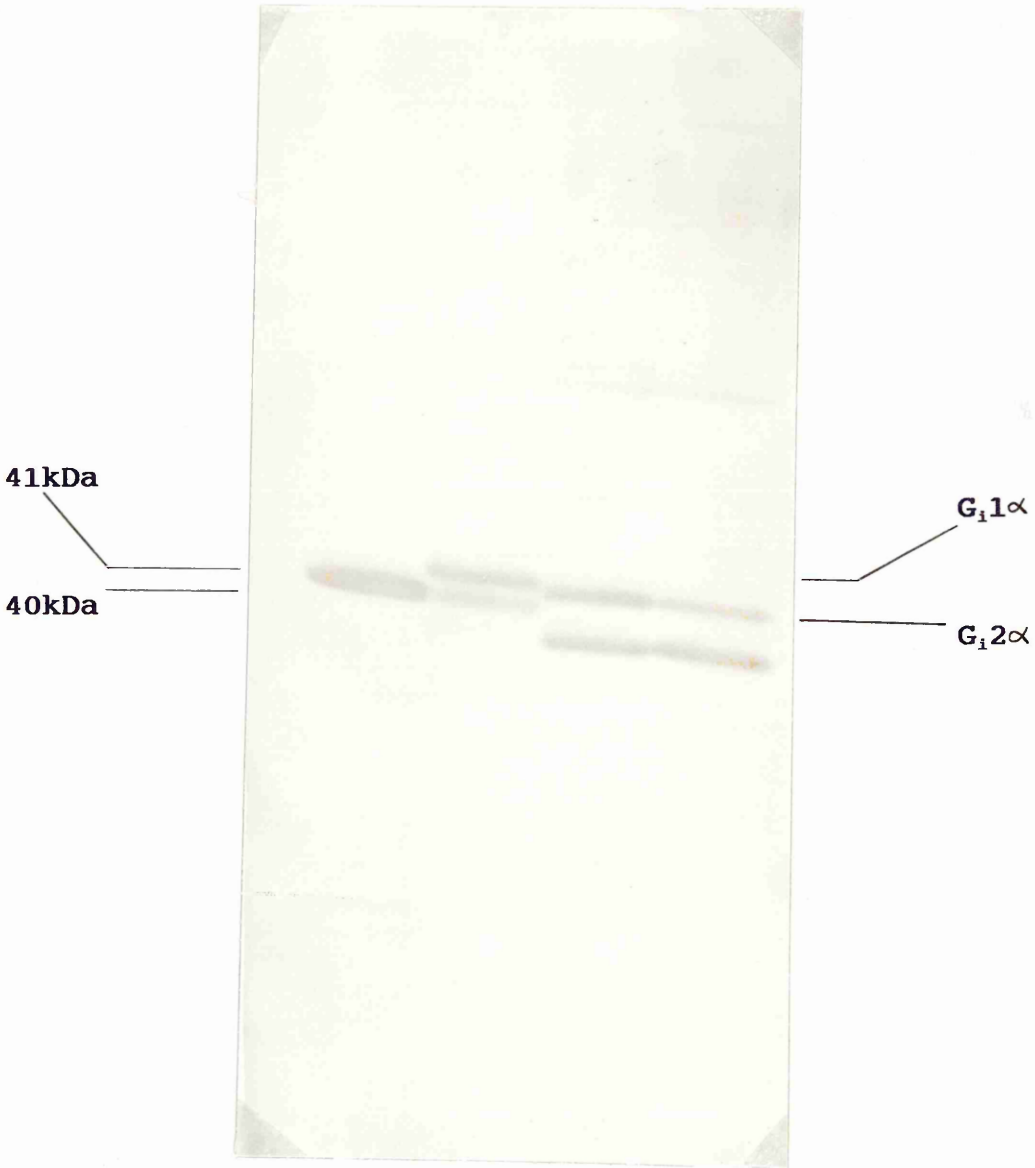
c) 12-day-old chick embryonic brain (100μg)

d) fusion-competent myoblasts (250μg)

were alkylated prior to resolution by SDS-PAGE using a 12.5%/0.06% bisacrylamide slab gel followed by immunoblotting using the anti-peptide antiserum SG1 (1:200) as the primary reagent.

Figure 4.2.2.1.

Molecular Weight




a b c d

**Figure 4.2.2.2**

**Identification of $G_i1\alpha$ and $G_i2\alpha$ immunoreactivity (ii).**

Membrane samples from

a) rat glioma C6 BU1 cells (100μg)

b) 12-day-old chick embryonic brain (100μg)

c) fusion-competent myoblasts (250μg)

d) rat cerebral cortex (100μg)

were alkylated prior to resolution by SDS-PAGE using a 12.5% acrylamide/0.06% bisacrylamide slab gel followed by immunoblotting using the anti-peptide antiserum SG1 (1:200) as the primary reagent.

Figure 4.2.2.2.

Molecular Weight

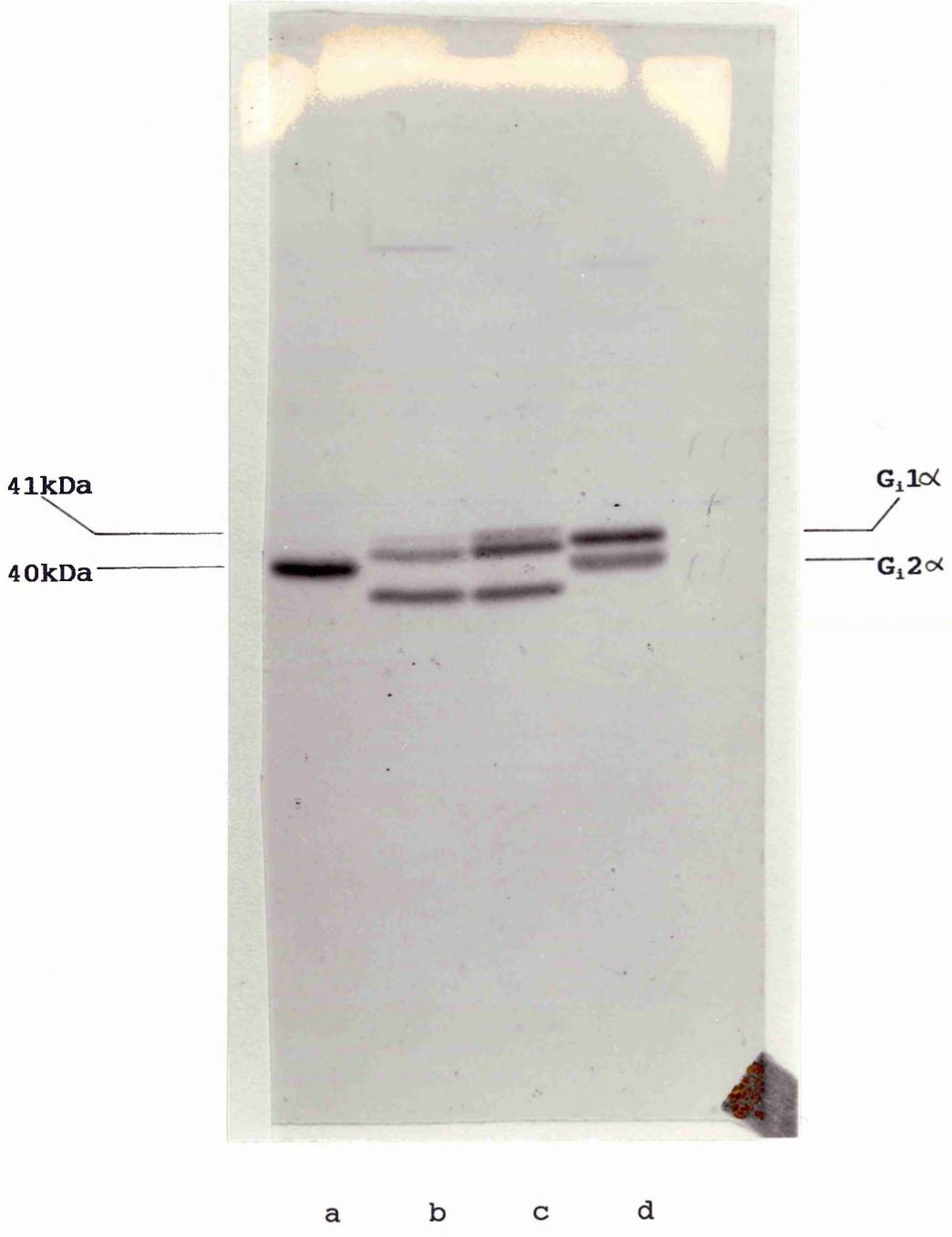


**Figure 4.2.2.3.**

**Identification of $G_i1\alpha$ immunoreactivity in myoblasts.**

Membrane samples from

a) rat cerebral cortex (100μg)

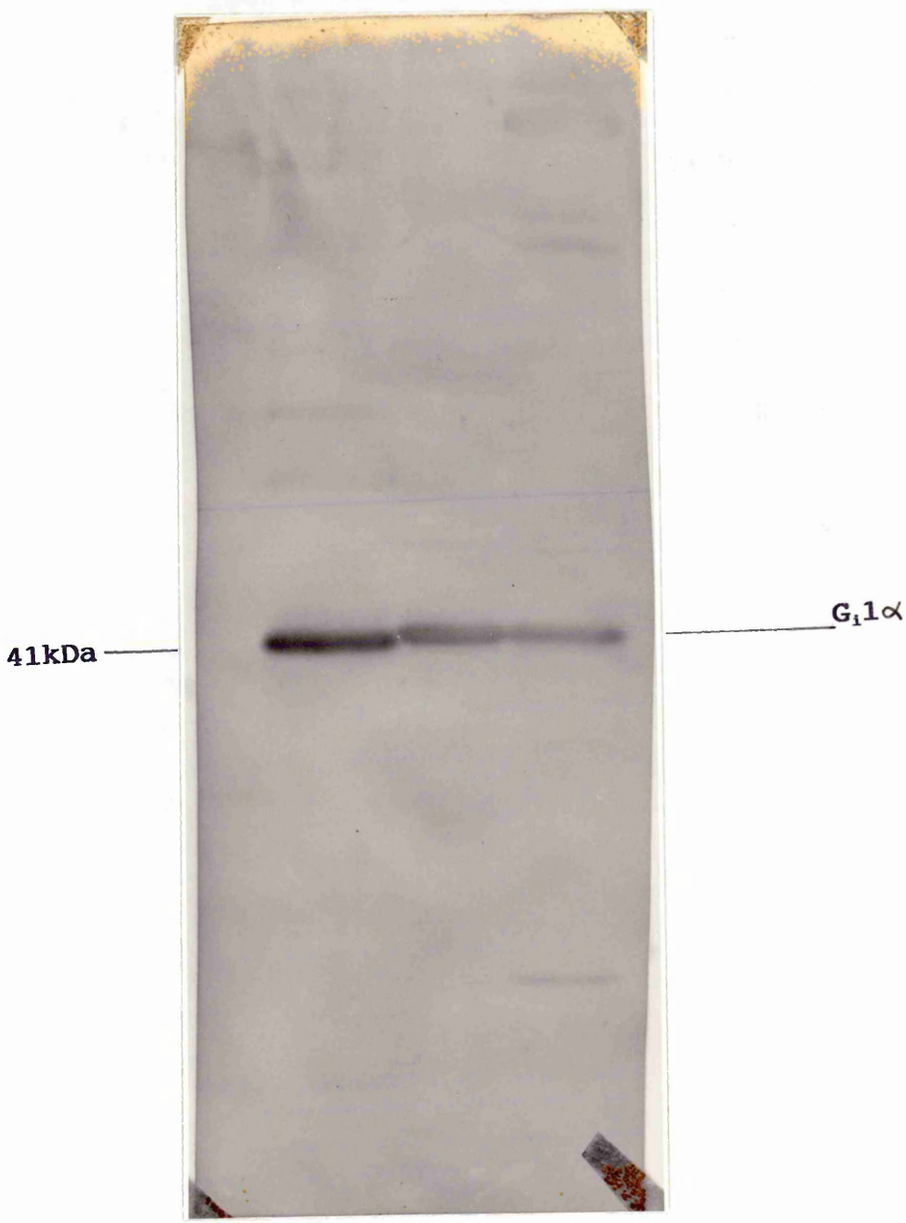
b) 12-day-old chick embryonic brain (100μg)

c) fusion-competent myoblasts (250μg)

were resolved by SDS-PAGE [12.5% acrylamide/0.06% bisacrylamide] followed by immunoblotting using the anti-peptide antiserum I1C (1:200) as the primary reagent.

Figure 4.2.2.3.

Molecular Weight



### 4.2.3 Identification of $G_i3\alpha$ and $G_o\alpha$ immunoreactivity.

Fig. 4.2.3.1. shows samples resolved by SDS-PAGE and immunoblotted with the anti-peptide antiserum I3B. I3B was raised against the synthetic peptide KNNLKECGLY which corresponds to the C terminal sequence, residues 345-354 of mammalian $G_i3\alpha$. It cross-reacts weakly with mammalian $G_o\alpha$ and may therefore be used to detect mammalian $G_i3\alpha$ and $G_o\alpha$. Antiserum I3B detected a single polypeptide of 41kDa ($G_i3\alpha$) in rat glioma C6 BU1 cells and human U937 monocyte cells (lanes b and e respectively). An additional band of 39kDa ($G_o\alpha$) was detected in rat cerebral cortex (lane a). Antiserum I3B recognised a single polypeptide of 41.5kDa in myoblasts (lane c) and two immunoreactive polypeptides of apparent molecular weights 41.5kDa and 40kDa in 12 day old chick embryonic brain (lane d).

Fig. 4.2.3.2. shows membrane samples resolved by SDS-PAGE and immunoblotted with the anti-peptide antiserum ON1. This was raised against the synthetic peptide GCTLSAEERAALERSK, which corresponds to the N terminal amino acid sequence of mammalian $G_o\alpha$, residues 1-16. $G_o\alpha$ was detected as a single polypeptide of 39kDa in rat cerebral cortex (lane c). Antiserum ON1 recognised a single polypeptide of apparent molecular weight 40kDa in chick embryonic myoblasts (lane a). A similar pattern of immunoreactivity was seen for 12-day-old chick embryonic brain (lane b) where it appeared to be present in much greater abundance in comparison to myoblasts.

### 4.2.4. Effect of alkylation on the relative mobilities of mammalian $G_i1\alpha$, $G_i2\alpha$ $G_i3\alpha$ and $G_o\alpha$ in comparison with the corresponding immunoreactive polypeptide(s) in SDS polyacrylamide gels.

Myoblasts and 12 day old chick embryonic brain possess $G_i1\alpha$-,$G_i2\alpha$-, $G_i3\alpha$- and $G_o\alpha$-like immunoreactivity. The mobilities of these polypeptides in SDS polyacrylamide gels are different to those observed for the mammalian forms.

**Figure 4.2.3.1**

**Identification of $G_i3\alpha$ immunoreactivity in myoblasts.**

Membrane samples from

a) rat cerebral cortex (100μg)

b) rat glioma C6 BU1 cells (100μg)

c) fusion-competent myoblasts (250μg)

d) 12-day-old chick embryonic brain (100μg)

e) human monocyte U937 cells (150μg)

were alkylated prior to resolution by SDS-PAGE using a 12.5% acrylamide 0.06% bisacrylamide slab gel followed by immunoblotting using the anti-peptide antiserum I3B (1:200) as the primary reagent.

Figure 4.2.3.1.
Molecular weight

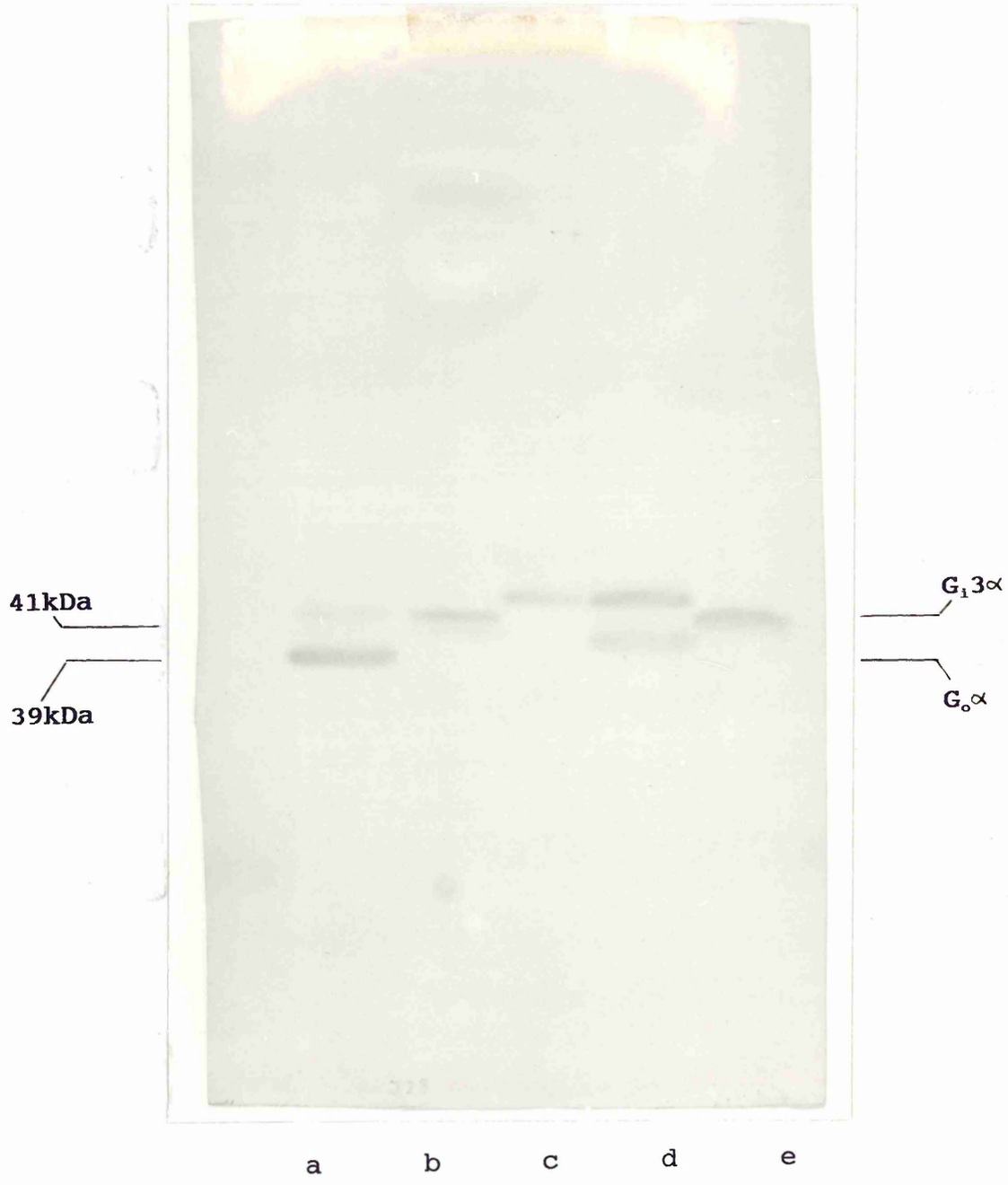


**Figure 4.2.3.2**

**Identification of $G_o\alpha$ immunoreactivity in myoblasts.**

Membrane samples from

a) fusion-competent myoblasts (250μg)

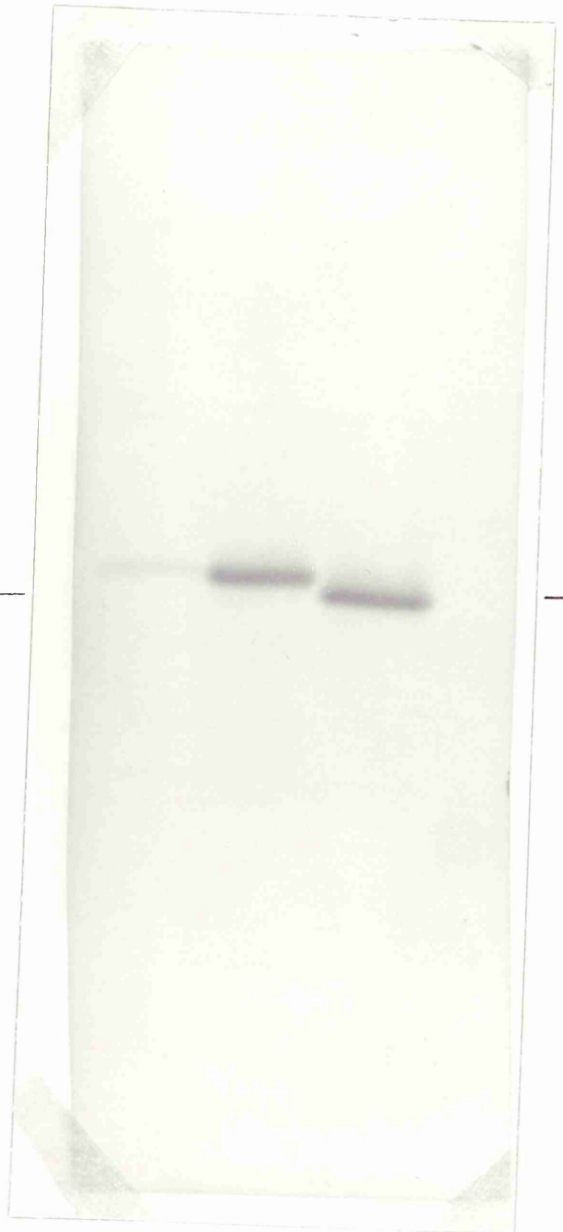
b) 12-day-old chick embryonic brain (10μg)

c) rat cerebral cortex (10μg)

were alkylated prior to resolution by SDS-PAGE using a 12.5% acrylamide/0.06% bisacrylamide slab gel followed by immunoblotting using the anti-peptide antiserum ON1 (1:200) as the primary reagent.

Figure 4.2.3.2.

Molecular Weight



Samples were alkylated using N-ethylmaleimide prior to SDS-PAGE in order to enhance resolution of the various G-proteins. The differing mobilities may therefore reflect differences in the number of cysteine residues available as sites for alkylation. This has been investigated by comparing the effects of alkylation on the relative mobilities of mammalian $G_i1\alpha$, $G_i2\alpha$ $G_i3\alpha$ and $G_o\alpha$ and the corresponding immunoreactive polypeptides present in chick myoblasts and 12-day-old chick embryonic brain.

### 4.2.4.1 Effect of alkylation on the relative mobilities of $G_i1\alpha$ and $G_i2\alpha$ immunoreactive polypeptides.

Fig. 4.2.4.1.1. shows membrane samples resolved by SDS-PAGE and immunoblotted with the anti-peptide antiserum SG1 which recognises equally mammalian $G_i1\alpha$ and $G_i2\alpha$ in non-photoreceptor containing tissues. Membrane samples in lanes a,b,c and d were alkylated prior to SDS-PAGE. Comparison of lanes a with h, b with g, c with f, and d with e shows that alkylation reduced the mobility of all the polypeptides detected by antiserum SG1 but, the relative mobilities of the chick $G_i1\alpha$-like and $G_i2\alpha$-like polypeptides, were unchanged when compared to the mammalian $G_i1\alpha$ and $G_i2\alpha$. Alkylation of myoblast and 12-day-old chick embryonic brain membrane samples did however, result in the enhanced resolution of the 40.5kDa and 41.5kDa immunoreactive polypeptides.

Fig. 4.2.4.1.2. shows membrane samples resolved by SDS-PAGE and immunoblotted with the anti-peptide antiserum I1C which is specific for mammalian $G_i1\alpha$. Membrane samples in lanes d e and f were alkylated prior to SDS-PAGE. Comparison of lanes a with f, b with e and c with d revealed that alkylation reduced the mobility of $G_i1\alpha$ from rat cerebral cortex and the $G_i1\alpha$-like immunoreactive polypeptide, present in myoblasts and 12-day-old chick embryonic brain. The relative mobility of mammalian $G_i1\alpha$- and the $G_i1\alpha$-like immunoreactive polypeptides was however unchanged.

**Figure 4.2.4.1.1.**

**Effect of alkylation on the relative mobilities of the $G_i2\alpha$ and $G_i1\alpha$ immunoreactive polypeptides in SDS-PAGE gels**

Membrane samples from

a) rat cerebral cortex (100μg)

b) 12-day-old chick embryonic brain (100μg)

c) fusion-competent myoblasts (250μg)

d) rat glioma C6 BU1 cells (100μg)

e) rat glioma C6 BU1 cells (100μg)

f) fusion-competent myoblasts (250μg)

g) 12-day-old chick embryonic brain (100μg)

h) rat cerebral cortex (100μg)

were resolved by SDS-PAGE using a 12.5% acrylamide/0.06% bisacrylamide slab gel. Samples a) -d) were alkylated prior to gel electrophoresis. Immunoblotting was performed using the anti-peptide antiserum SG1 (1:200) as the primary reagent.

Figure 4.2.4.1.1.

Molecular Weight

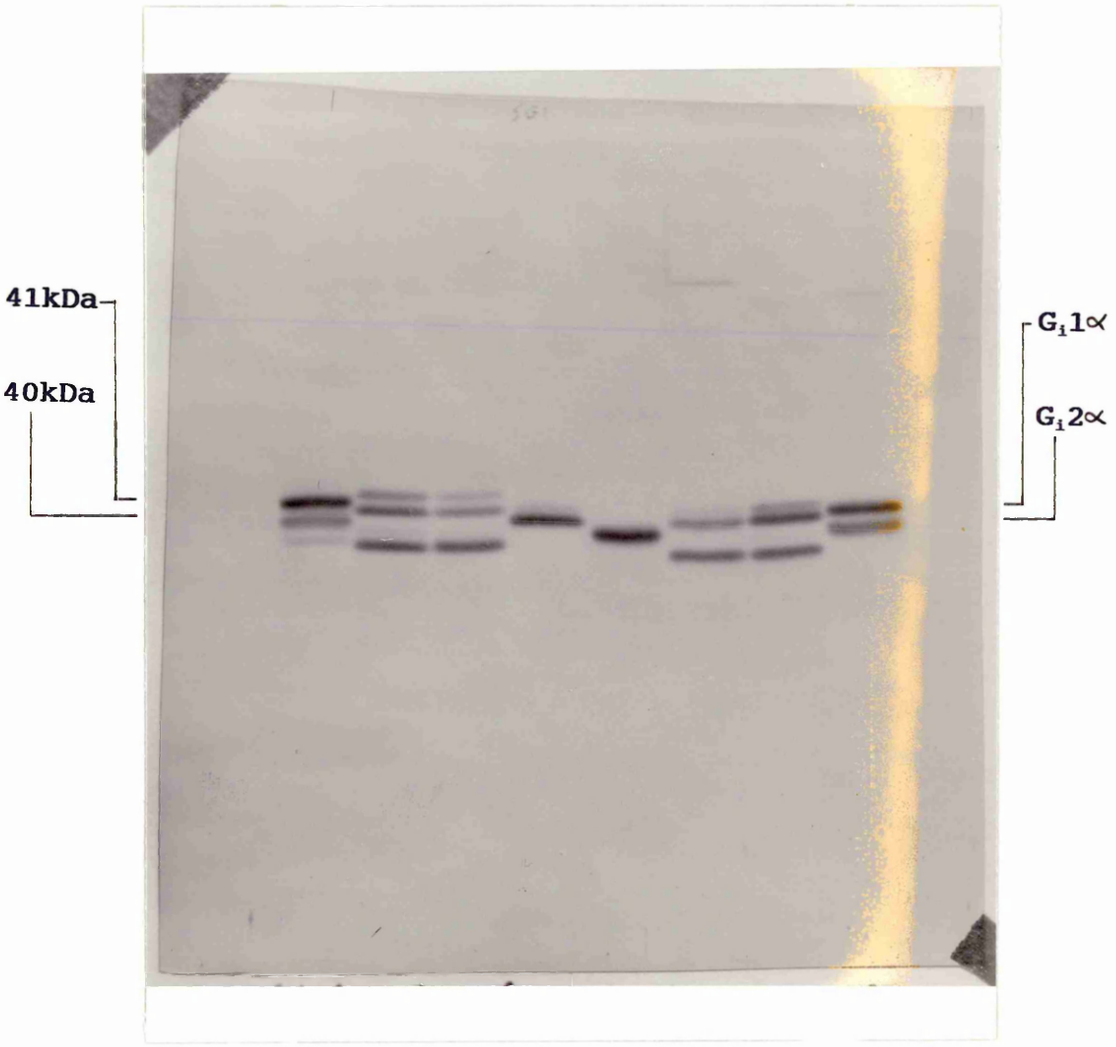


**Figure 4.2.4.1.2.**

**Effect of alkylation on the relative mobilities of the $G_i1\alpha$ immunoreactive polypeptides on SDS-PAGE.**

Membrane samples from

a) rat cerebral cortex (100μg)

b) 12-day-old chick embryonic brain (100μg)

c) fusion-competent myoblasts (250μg)

d) fusion-competent myoblasts (250μg)

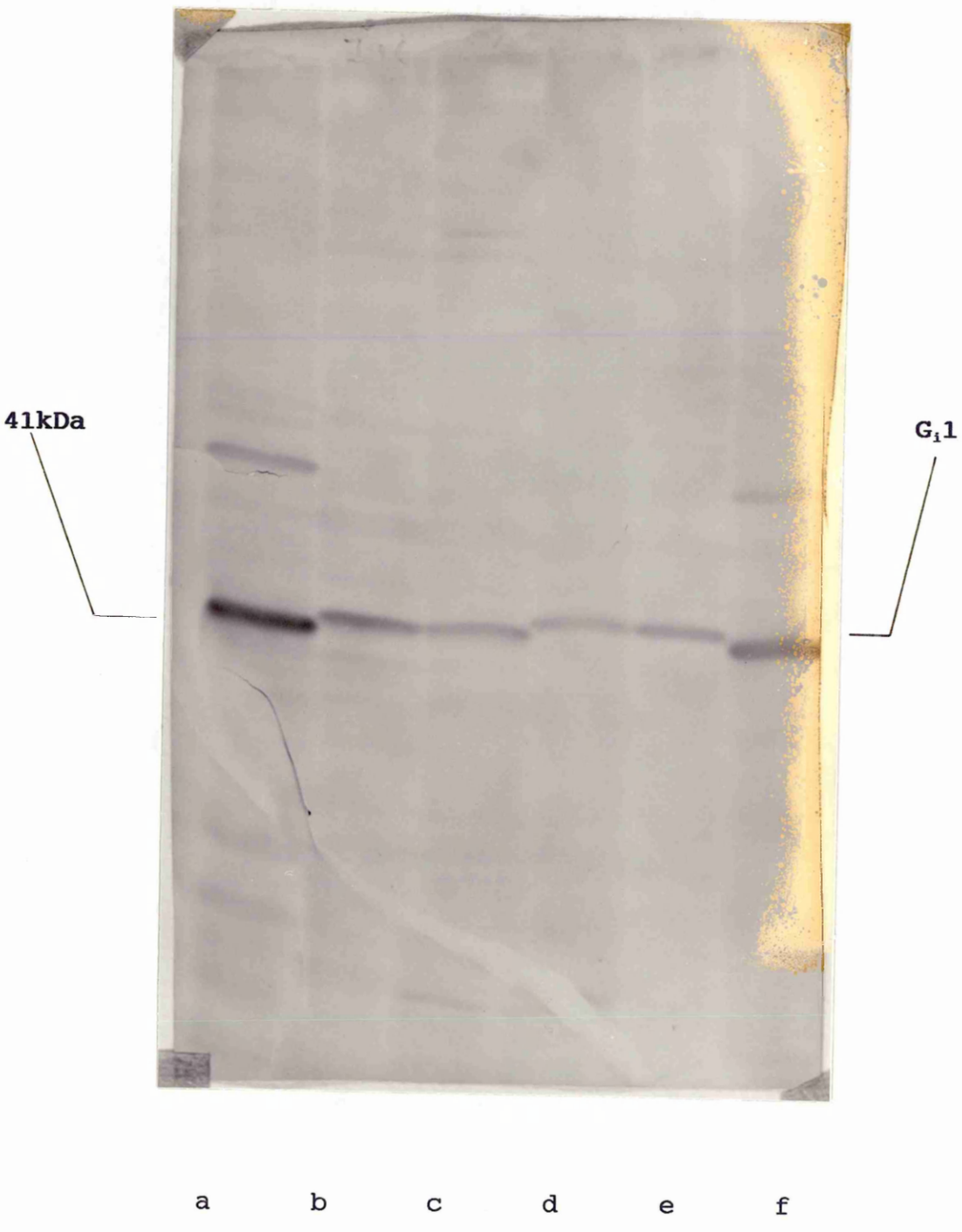
e) 12-day-old chick embryonic brain (100μg)

f) rat cerebral cortex (100μg)

were resolved by SDS-PAGE using a 12.5% acrylamide/0.06% bisacrylamide slab gel. Samples d) - f) were alkylated prior to gel electrophoresis. Immunoblotting was performed using the anti-peptide antiserum I1C (1:200) as the primary reagent.

Figure 4.2.4.1.2.

Molecular Weight



### 4.2.4.2 Effect of alkylation on the relative mobilities of the $G_i3\alpha$ and $G_o\alpha$ immunoreactive polypeptides in SDS polyacrylamide gels.

Fig. 4.2.4.2.1. shows membrane samples resolved by SDS-PAGE and immunoblotted with the anti-peptide antiserum I3B which was raised against mammalian $G_i3\alpha$ and cross reacts weakly with mammalian $G_o\alpha$. Membrane samples in lanes a, b, c d and e were alkylated prior to SDS-PAGE. Comparison of lanes a with j, b with i, c with h, d with g and e with f reveals that alkylation reduced the mobility of mammalian $G_i3\alpha$ together with the $G_i3\alpha$-like and $G_o\alpha$-like immunoreactive polypeptides. The relative mobilities of the mammalian $G_i3\alpha$ and the chick embryonic $G_i3\alpha$-like immunoreactive polypeptide were however unchanged. The non-alkylated forms of mammalian $G_o\alpha$ (lane j) and the chick embryo $G_o\alpha$-like immunoreactive polypeptide (lane i) co-migrated in the SDS-polyacrylamide gel but alkylation caused their mobility to change with respect to each other, so that they no longer co-migrated when resolved by SDS/PAGE.

This observation was confirmed by immunoblotting membrane samples resolved by SDS-PAGE with the IM1 anti-peptide antiserum which recognises $G_o\alpha$ (Mullaney *et al.*, 1988) and is raised against the synthetic peptide NLKEDGISAAKDVK, which corresponds to the amino acid sequence, residues 22-35, of mammalian $G_o\alpha$. Alkylation of the chick embryonic $G_o\alpha$-like immunoreactive polypeptide (fig. 4.2.4.2.2., lane c) reduced its mobility, so that it ran with a higher apparent molecular weight of 40kDa compared to the non-alkylated form (lane b) which co-migrated with mammalian $G_o\alpha$ (lane a) of molecular weight of 39kDa.

**Figure 4.2.4.2.1.**

**Effect of alkylation on the relative mobilities of the $G_i3\alpha$ and $G_o\alpha$ immunoreactive polypeptides in SDS-PAGE gels.**

Membrane samples from

a) rat glioma C6 BU1 cells (100μg)

b) rat cerebral cortex (100μg)

c) 12-day-old chick embryonic brain (100μg)

d) fusion-competent myoblasts (250μg)

e) human monocyte U937 cells (150μg)

f) human monocyte U937 cells (150μg)

g) fusion-competent myoblasts (250μg)

h) rat cerebral cortex (100μg)

i) 12-day-old chick embryonic brain (100μg)

j) rat glioma C6 BU1 cells (100μg)

were resolved by SDS-PAGE using a 12.5% acrylamide/0.06% bisacrylamide slab gel. Samples a) - e) were alkylated prior to gel electrophoresis. Immunoblotting was performed using the anti-peptide antiserum I3B (1:200) as the primary reagent.

Figure 4.2.4.2.1.
Molecular weight

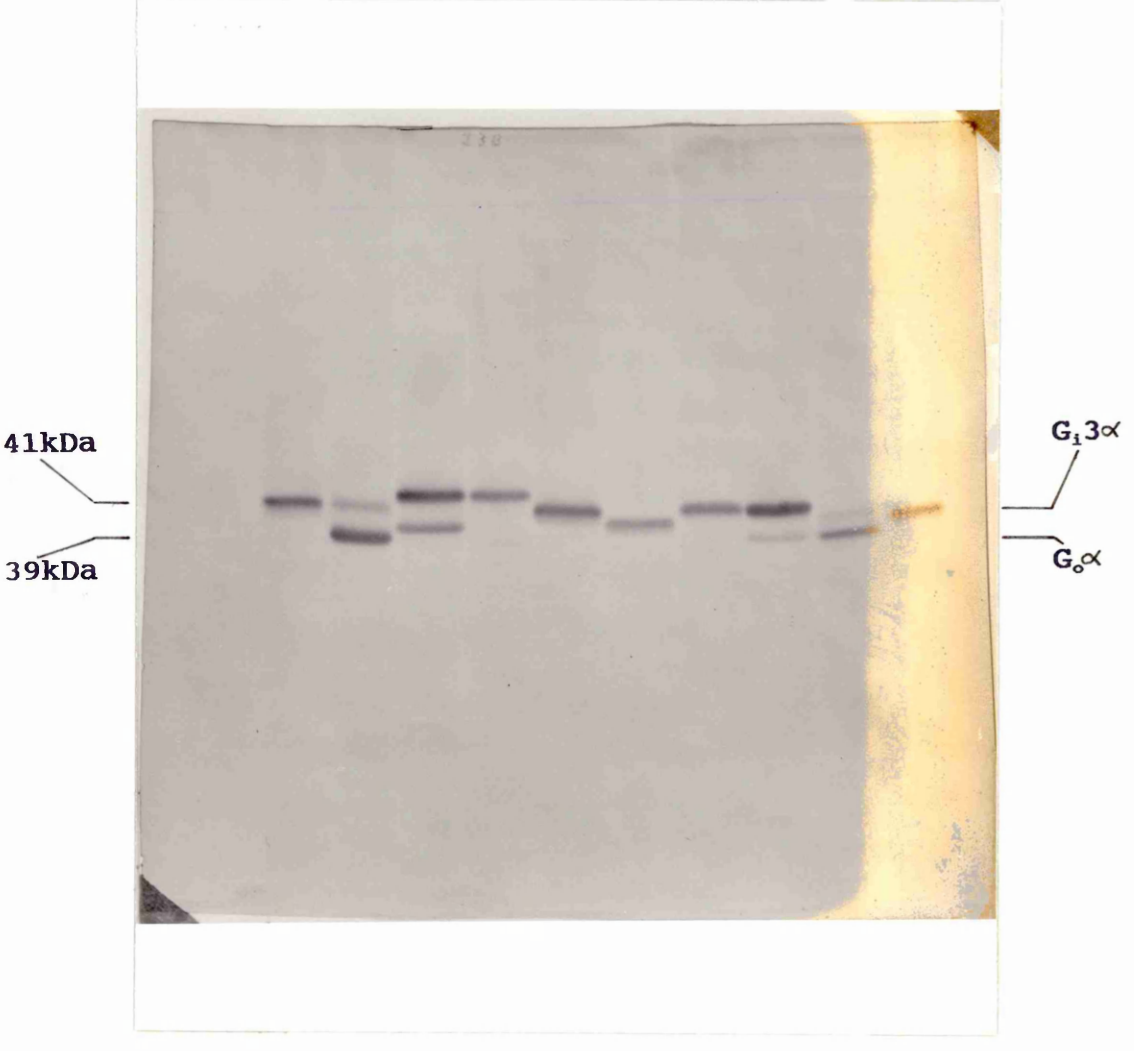


**Figure 4.2.4.2.2.**

**Effect of alkylation on the relative mobilities of $G_o\alpha$ immunoreactive polypeptides on SDS-PAGE.**

Membrane samples from

a) rat cerebral cortex (100μg)

b) 12-day-old chick embryonic brain (100μg)

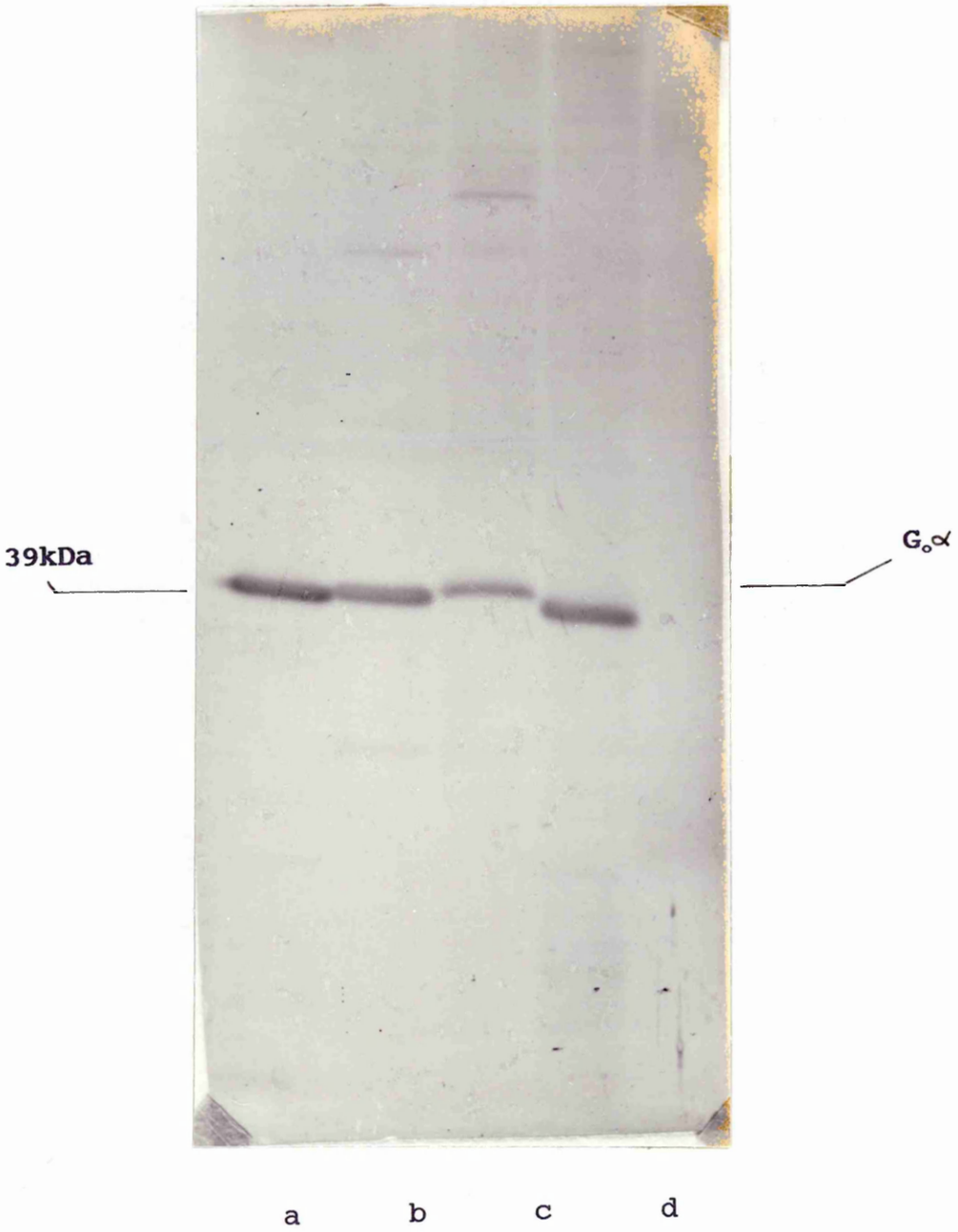
c) 12-day-old chick embryonic brain (100μg)

d) rat cerebral cortex (100μg)

were resolved by SDS-PAGE using a [12.5% acrylamide/0.06% bisacrylamide] slab gel. Samples c) and d) were alkylated prior to gel electrophoresis. Immunoblotting was performed using the anti-peptide antiserum IM1 (1:200) as the primary reagent.

Figure 4.2.4.2.2.

Molecular Weight



### 4.2.5. The effect of protease inhibitors on the immunoreactivity of myoblast membrane polypeptides.

The 40.5kDa and 38kDa immunoreactive polypeptides detected by the SG1 antiserum in membranes of myoblasts and chick embryonic brain may represent proteolytic fragments of the 41.5kDa immunoreactive polypeptide. Similarly the 40kDa polypeptide detected by antiserum I3B may be derived by proteolysis of the 41.5kDa immunoreactive polypeptide. This possibility was investigated by immunoblotting membrane samples from myoblasts and 12-day-old chick embryonic brain which were prepared in the presence or absence of protease inhibitors. The patterns of immunoreactivity were then compared. The protease inhibitors used were benzamidine, soybean trypsin inhibitor and leupeptin because they inhibit the proteolysis of $G_i2\alpha$ and $G_i3\alpha$ in human U937 monocyte cell membranes (Fiona Mitchell, personal communication).

Fig. 4.2.5.1. shows membrane samples resolved by SDS-PAGE and immunoblotted with the SG1 antiserum. Samples in lanes c and e were prepared in the presence of protease inhibitors. Comparison of lanes b with c and d with e reveals that inclusion of protease inhibitors in the membrane sample preparation resulted in no change in the pattern of immunoreactivity detected by SG1.

Fig. 4.2.5.2. shows membrane samples resolved by SDS-PAGE and immunoblotted with the I3B antiserum. Comparison of membrane samples from 12-day-old chick embryonic brain prepared in the presence (lane d) and absence (lane c) of protease inhibitors reveals that the pattern of immunoreactivity detected was unchanged. It should be noted that mammalian $G_i3\alpha$ and $G_o\alpha$ co-migrate in [10% acrylamide] SDS-PAGE but the $G_i3\alpha$- and $G_o\alpha$- like polypeptides in 12-day-old chick embryonic brain, were however, well resolved, in comparison.

**Figure 4.2.5.1.**

**Effect of protease inhibitors on the immunoreactivity of myoblast membrane polypeptides with the antiserum SG1.**

Membrane samples from

a) rat cerebral cortex (100μg)

b) 12-day-old chick embryonic brain (100μg)

c) 12-day-old chick embryonic brain (100μg)+ protease inhibitors

d) fusion-competent myoblasts (250μg)

e) fusion-competent myoblasts (250μg) + protease inhibitors

f) rat glioma C6 BU1 cells (100μg)

were resolved by SDS-PAGE using a 12.5% acrylamide/0.06% bisacrylamide slab gel. Samples c) and e) were prepared in the presence of a cocktail of protease inhibitors(3mM benzamidine, 2mg/ml soybean trypsin inhibitor, 5mg/ml leupeptin). Immunoblotting was performed using the anti-peptide antiserum SG1 (1:200) as the primary reagent.

Figure 4.2.5.1.
Molecular weight

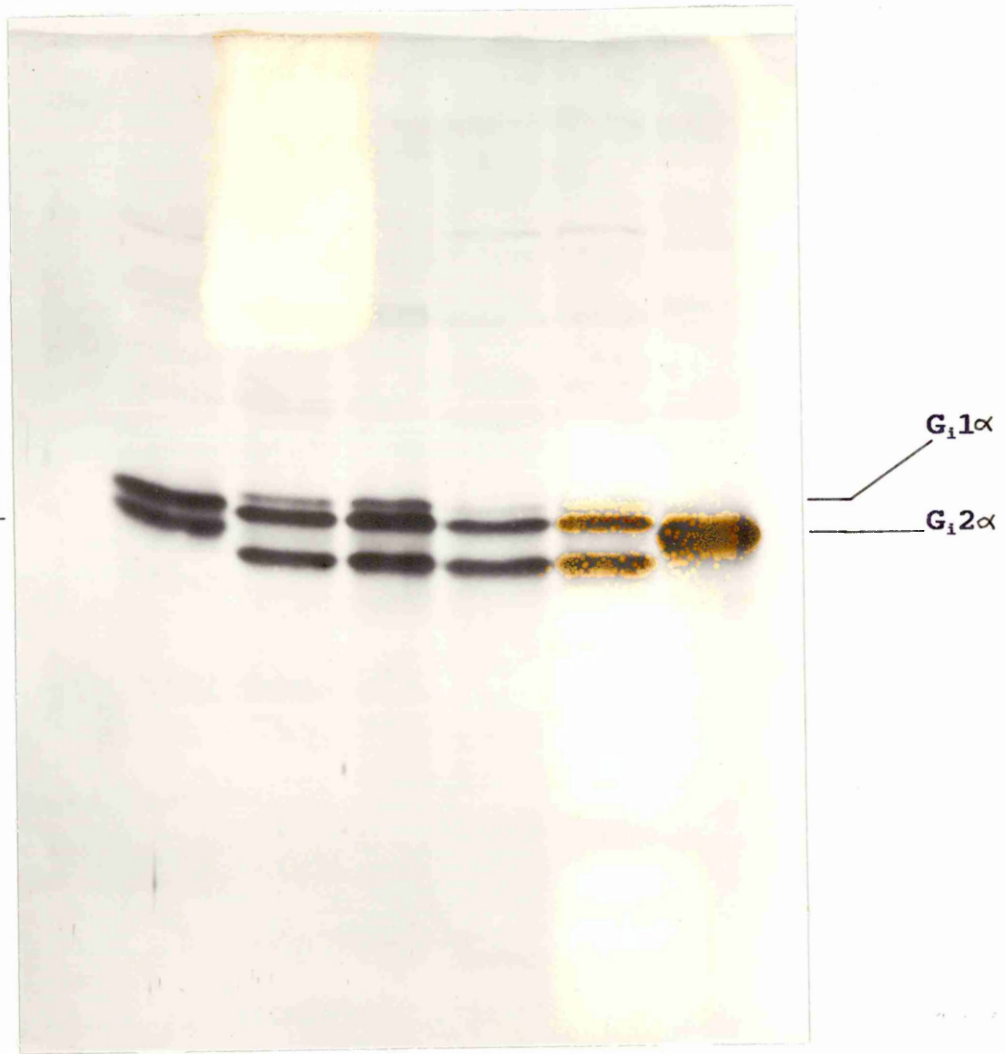


**Figure 4.2.5.2**

**Effect of protease inhibitors on the immunoreactive membrane polypeptides detected by the antiserum I3B.**

Membrane samples from

a) rat cerebral cortex (100μg)

b) fusion-competent myoblasts (250μg)

c) 12-day-old chick embryonic brain (250μg)

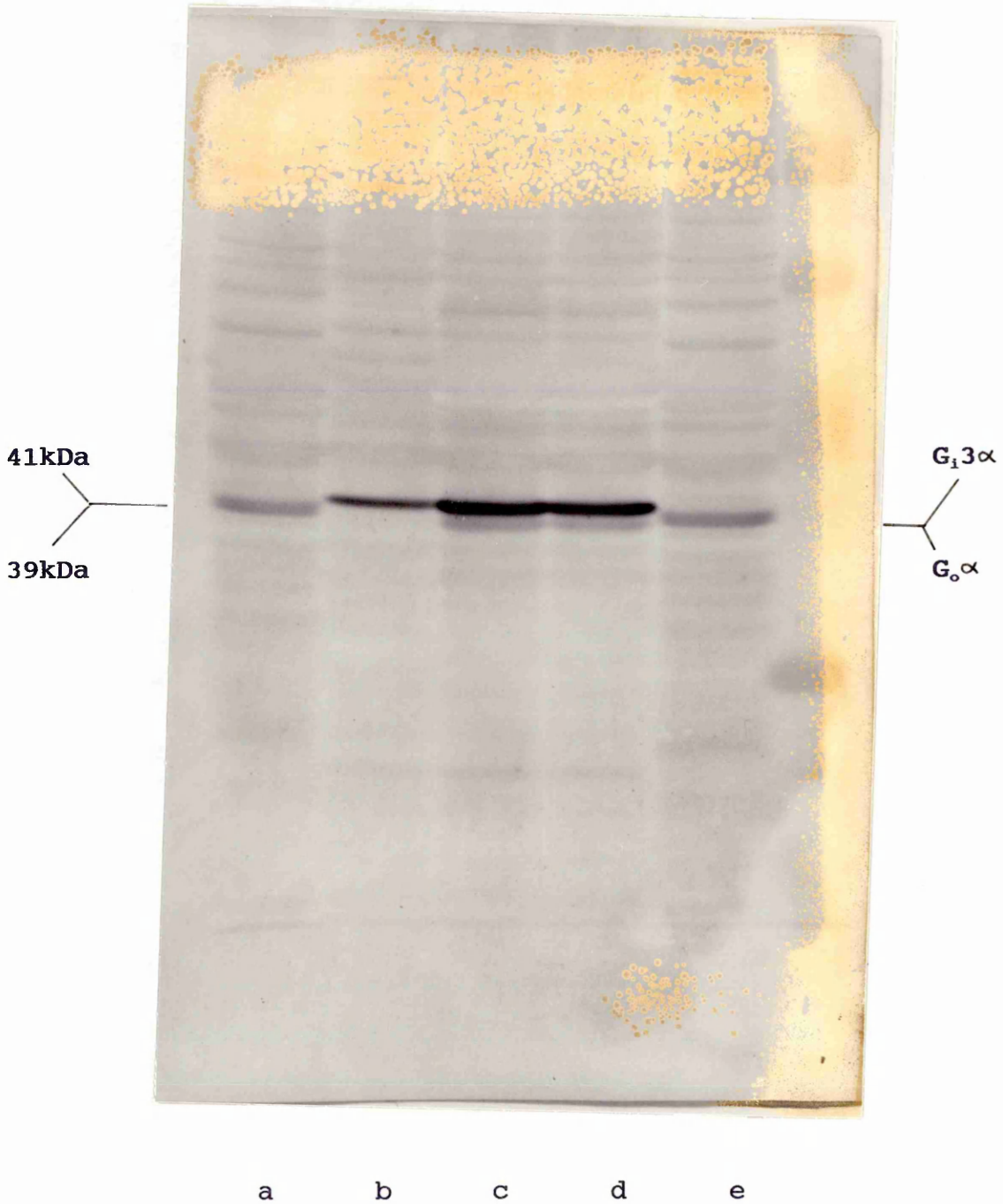
d) 12-day-old chick embryonic brain (250μg)+ protease inhibitors

e) rat glioma C6 BU1 cells (100μg)

were resolved by SDS-PAGE [10% acrylamide]. Membranes in lane d were prepared in the presence of protease inhibitors (3mM benzamidine, 2mg/ml soybean trypsin inhibitor, 5mg/ml leupeptin). Immunoblotting was performed using the anti-peptide antiserum I3B (1:200).

Figure 4.2.5.2.

Molecular Weight



### 4.2.6 Identification of β subunit immunoreactivity in myoblasts

Fig. 4.2.6.1. shows membrane sample resolved by SDS-PAGE and immunoblotted with the BN1 antiserum. This was raised against the synthetic peptide MSELDQLRQE which corresponds to the N terminal sequence, residues 1-10, of the mammalian β1 G-protein β subunit. A single polypeptide of 35kDa was detected in myoblasts (lane d) and 12-day-old chick embryonic brain (lane b). This polypeptide co-migrated with the mammalian β subunit present in rat cerebral cortex (lanes a and e) indicating that the membrane preparations from chick embryonic myoblasts possess β subunit immunoreactive polypeptide(s).

## 4.3. Role of G-proteins in myoblast fusion.

G-proteins mediate agonist regulation of several signal transduction systems, including adenylate cyclase, $K^+$ channels and $Ca^{2+}$ channels. The interaction between receptors linked to inositol phospholipid hydrolysis and PI-PLC is also considered to be G-protein mediated (Cockcroft and Gomperts, 1985; Gilman, 1987). Myoblast fusion represents such an event, where the addition of the agonist, fusion mix, to fusion-competent myoblasts results in the stimulation of both inositol phospholipid breakdown and fusion. The possible involvement of G-proteins in this receptor-PI-PLC interaction was therefore examined and is described in the following section.

### 4.3.1. The ability of fusion mix to stimulate GTPase activity in myoblast membranes.

All known G-proteins bind and hydrolyse GTP. The α subunit possesses an intrinsic GTPase activity which catalyses GTP hydrolysis to generate GDP and inorganic phosphate (Pi). The interaction of an agonist-occupied receptor and a G-protein stimulates this GTPase activity by facilitating the exchange of GDP for GTP, thus increasing the amount of GTP available as substrate. Agonist stimulation of GTPase activity is therefore indicative of receptor G-protein interaction.

**Figure 4.2.6.1**

**Identification of β subunit immunoreactivity in myoblasts.**

Membrane samples

a) rat cerebral cortex (100μg)

b) 12-day-old chick embryonic brain (100μg)

c) 12-day-old chick embryonic brain (100μg)+ protease inhibitors

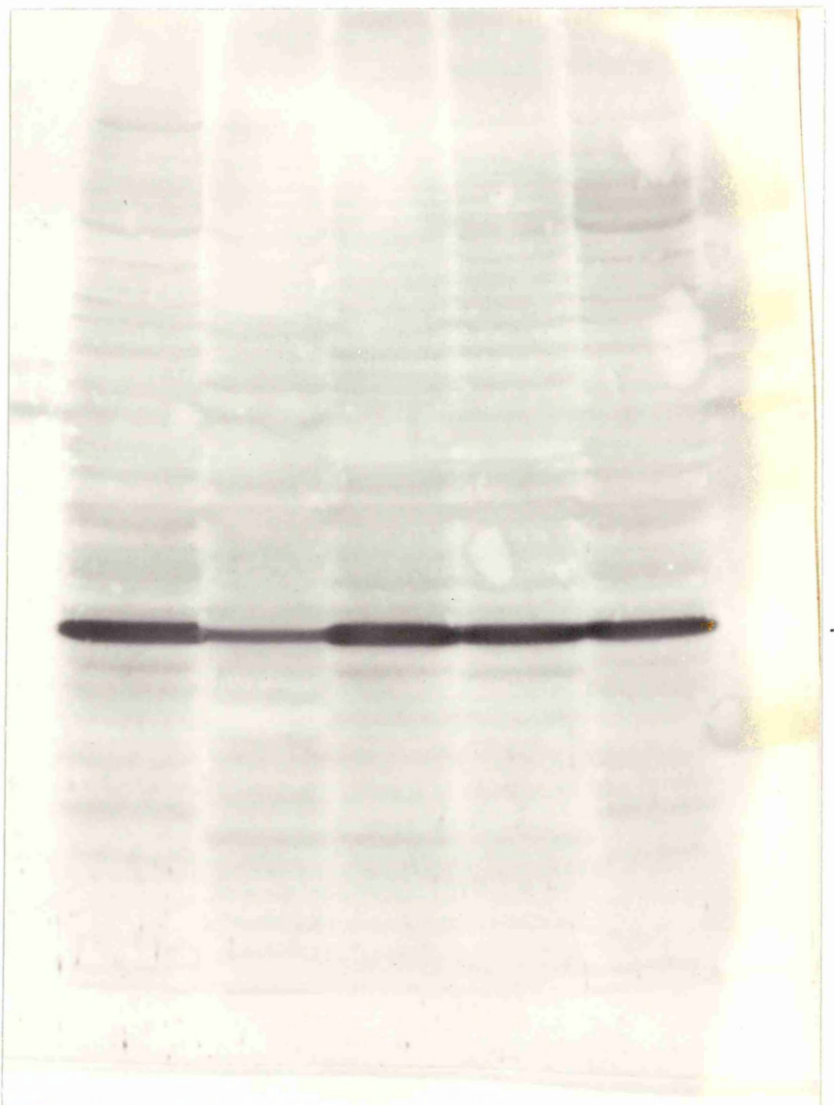
d) fusion-competent myoblasts (250μg)

e) rat brain cerebral cortex (100μg)

were resolved by SDS-PAGE [10% acrylamide] followed by immunoblotting using the anti-peptide antiserum BN1 (1:200) as the primary reagent.

Figure 4.2.6.1.

Molecular Weight



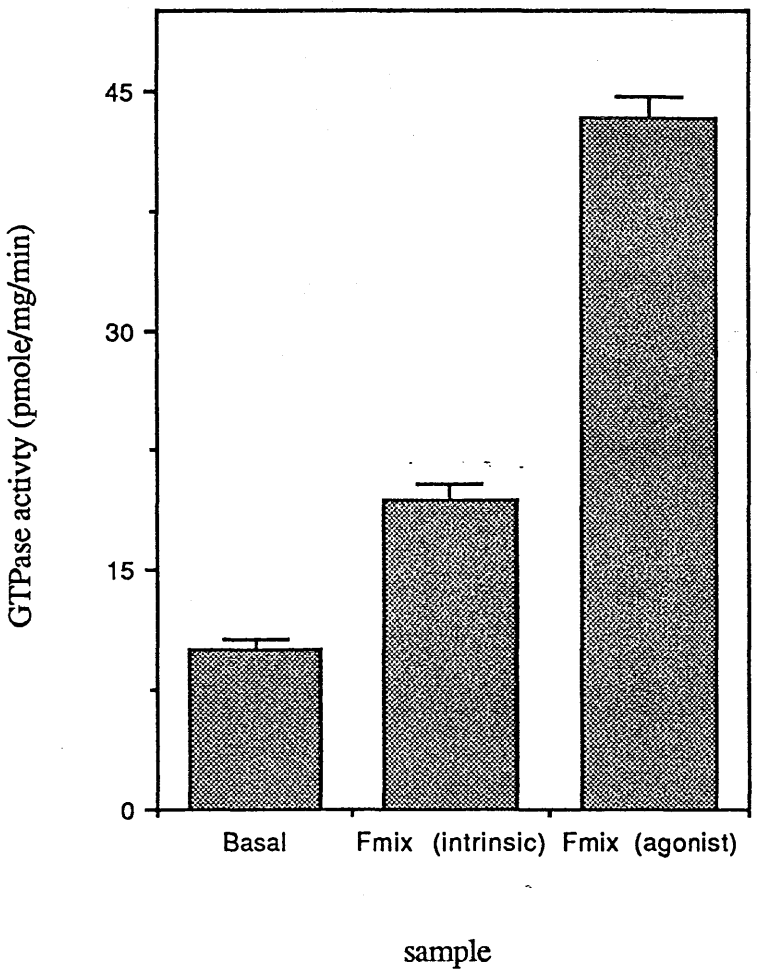

a b c d e

**Figure 4.3.1.1.**

**Fusion mix stimulation of GTPase activity in fusion-competent myoblast membranes.**

Fusion mix was added to GTPase assays of fusion-competent cell membranes. The basal activity was that obtained in the absence of 100μm GTP. Results are means ± S.D. (n=4) from a single experiment typical of 3.

Figure 4.3.1.1.


45
30
15
0
GTPase activty (pmole/mg/min)
Basal
Fmix (intrinsic)
Fmix (agonist)
sample

In order to assess whether fusion mix exerts its effect via G-protein activation, it was tested for its ability to stimulate the hydrolysis of GTP in membranes prepared from fusion-competent myoblasts. GTPase activity was assayed by measuring the release of [$^{32}$P] Pi from [$\gamma$-$^{32}$P] GTP, as described in section 2.10. The results obtained are shown in fig. 4.3.1.1. and the data are expressed as pmoles GTP hydrolysed per minute per mg of membrane protein. The basal and non-specific activities were defined as those obtained in the absence and presence of 100$\mu$M GTP respectively. Fusion mix itself possessed an intrinsic GTPase activity which was defined as that obtained in the absence of the myoblast membrane sample. The 'agonist stimulated' GTPase activity of fusion mix was calculated by subtracting this intrinsic i.e. 'background' GTPase activity from the total GTPase activity that was obtained using fusion mix in the presence of fusion-competent myoblast membranes. From fig. 4.3.1.1, fusion mix stimulated GTPase activity in membranes prepared from fusion-competent myoblasts. GTPase activity was increased 1·93 $\pm$ 0·26 fold over the basal activity ( mean of 3 separate experiments $\pm$ S.E.M.).

### 4.3.3. The effects of GTP$\gamma$S on total [$^3$H] inositol phosphate generation

GTP serves to activate G-proteins by promoting the dissociation of their subunits. Deactivation occurs upon hydrolysis of the bound GTP by the intrinsic GTPase of the $\alpha$ subunit. Thus, analogues of GTP that are resistant to this hydrolysis, result in persistant G-protein activation. Guanosine-5'-Ô-(3-thiotriphosphate), (GTP$\gamma$S) is one such analogue and it provides a biochemical tool with which to directly to activate G-proteins. This molecule is membrane impermeant but was introduced into cells by the technique of electropermeabilisation (section 2.11) which facilitates the rapid entry of small molecules (< 1kDa molecular weight) into the cytosol by producing small pores in the plasma membrane (Knight and Scrutton, 1986). Fig. 4.3.2.1. shows the effect of pulse number on the stimulation of inositol phospholipid breakdown in response to fusion mix.

**Figure 4.3.2.1.**

**Effect of electropermeabilisation on fusion mix stimulated total [$^3$H] inositol phosphate generation.**

Fusion-competent myoblasts that were equilibrium labelled with [$^3$H] inositol were subjected to increasing numbers of discharge pulses (0 - 5) of 1600V/cm. The cells were then incubated in the presence and absence of fusion mix for 30 minutes at 37°C in the presence of 10mM LiCl. Total [$^3$H] inositol phosphates were batch eluted from Dowex formate anion exchange resin. Results are mean ± S.D. (n=3) from a single experiment typical of 3.

Figure 4.3.2.1.

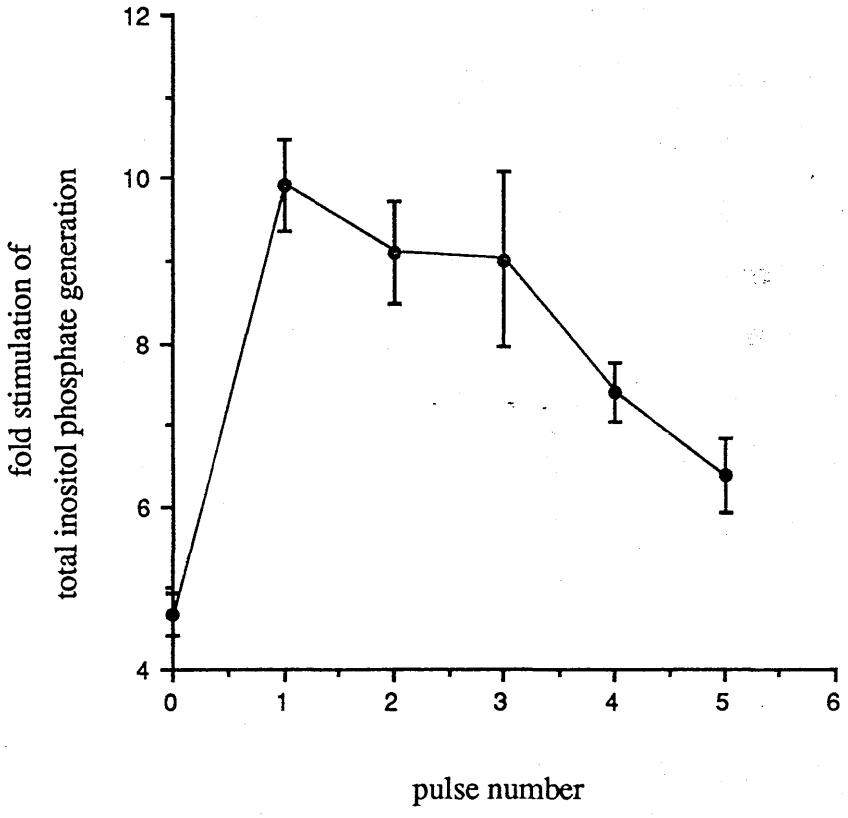

12
10
8
6
4
0
1
2
3
4
5
6
fold stimulation of
total inositol phosphate generation
pulse number

**Figure 4.3.2.2.**

**Effect of GTP[S] on total [$^3$H] inositol phosphate generation in fusion-competent myoblasts.**

Fusion-competent myoblasts were electropermeabilised with 3 discharge pulses of 1600V/cm and incubated with increasing concentrations of GTPγS (GTP[S]) for 30 minutes at 37°C, in the presence of 10mM LiCl. Total [$^3$H] inositol phosphates were batch eluted from Dowex-formate anion exchange resin. Results are means ± S.D. (n=3) from a single experiment typical of 2.

Figure 4.3.2.2.

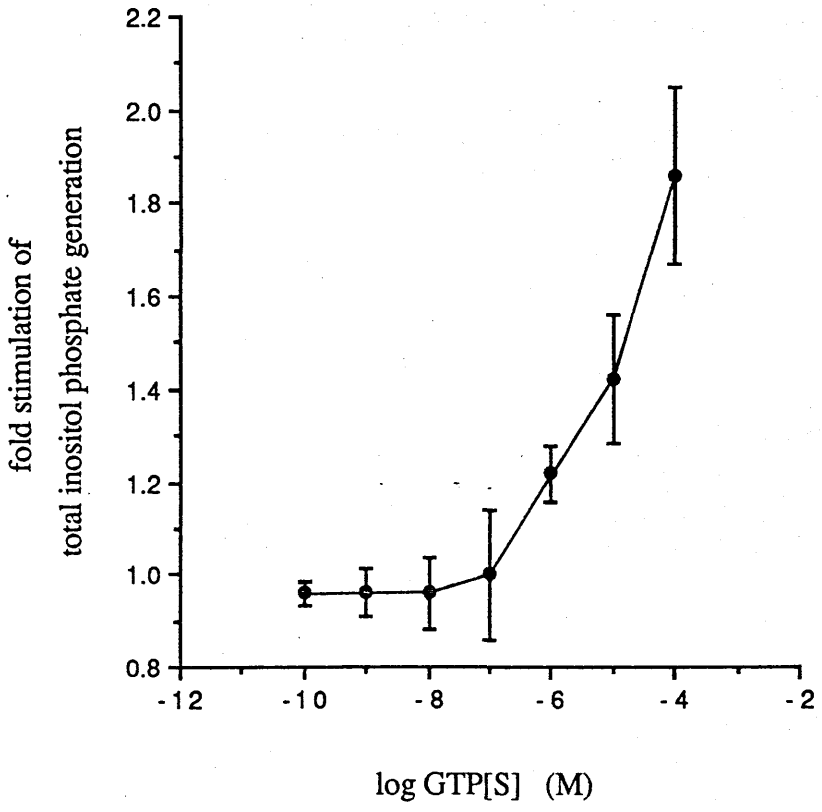
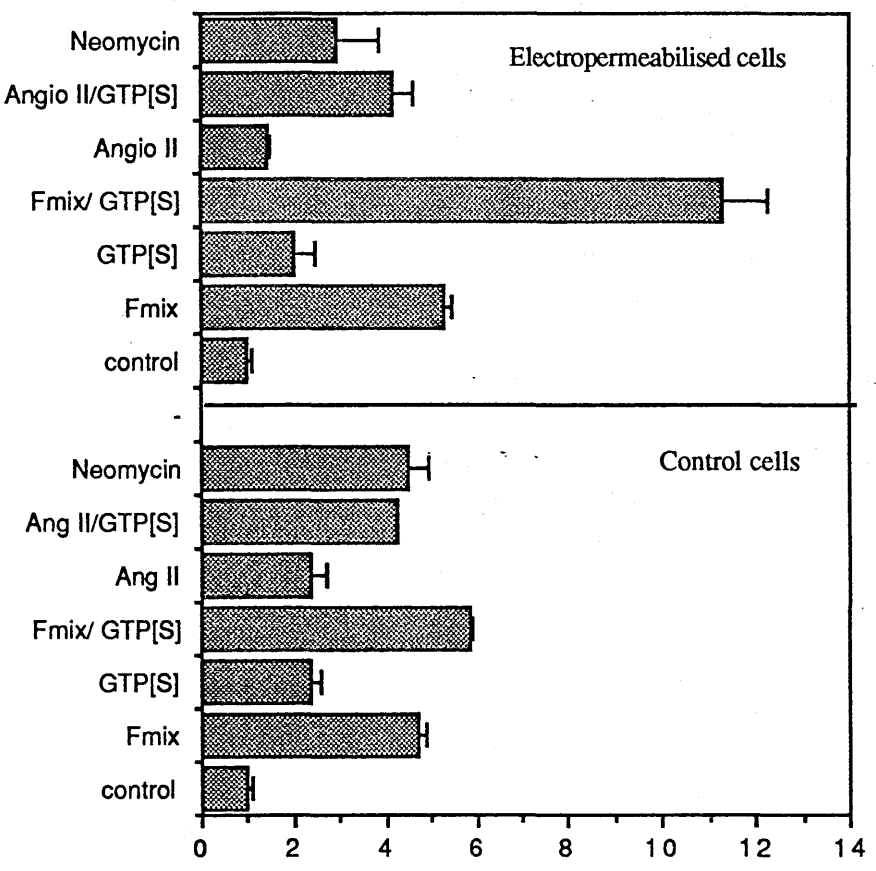

fold stimulation of
total inositol phosphate generation
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
-12
-10
-8
-6
-4
-2
log GTP[S] (M)

**Figure 4.3.2.3.**

**Effect of GTP[S] on agonist stimulated total [$^3$H] inositol phosphate generation in fusion-competent myoblasts.**

Fusion-competent myoblasts labelled to equilibrium with [$^3$H] inositol were electropermeabilised by 3 discharge pulses of 1600V/cm or non-permeabilised (control). Cells were incubated with the agonists, angiotensin II (100nM) or fusion mix, in the presence and absence of 100μM GTPγS (GTP[S]) in buffer containing 10mM LiCl. Total [$^3$H] inositol phosphates were batch eluted from Dowex-formate anion exchange resin. Results are means ± S.D. (n=3) from a single experiment typical of 2.

Figure 4.3.2.3.




fold stimulation of total inositol phosphate generation

Permeabilisation resulted in an increase in the fold stimulation of total [$^3$H] inositol phosphate generation which was maintained for up to 3 pulses. Further pulsing resulted in a decreased response. Thus, for subsequent experiments, the pulse number chosen was 3 because, under these conditions of electropermeabilisation, the cells were 100% permeabilised (as assessed by uptake of a membrane-impermeant molecule, propidium iodide, which is fluorescent upon intercalation into nucleic acids and thus identified cells whose membrane integrity was compromised) and fusion mix stimulated total [$^3$H] inositol phosphate generation was unimpaired (fig. 4.3.2.1). Fig. 4.3.2.2. shows the effect of GTPγS on total [$^3$H] inositol phosphate generation. It can be seen that GTPγS stimulated the generation of total [$^3$H] inositol phosphates in a dose-dependent manner and 100mM GTPγS resulted in a fold increase in total [$^3$H] inositol phosphates of 1·49 ± 0·13 (mean ± S.E.M.). Fig.4.3.2.3. shows the effect of 100mM GTPγS, both alone and in combination with fusion mix or 100nM angiotensin II , on total [$^3$H] inositol phosphate generation. GTPγS was synergistic with fusion mix and angiotensin II in stimulating total [$^3$H] inositol phosphate generation.

### 4.3.3. Sensitivity of fusion mix stimulated total [$^3$H] inositol phosphate generation and fusion to pertussis and cholera toxins.

Many G-protein α subunits are substrates for pertussis or cholera toxin-catalysed ADP-ribosylation. In some cells, pretreatment with these toxins leads to an inhibition of agonist stimulated inositol phosphate generation (Lo and Hughes, 1987; Paris and Pouyssegur, 1986) and this is proposed to result from ADP-ribosylation of the G-protein coupling the receptor to PI-PLC. The effects of pretreatment with pertussis or cholera toxins on fusion mix stimulation of myoblast fusion and the generation of total [$^3$H] inositol phosphates were therefore examined, in order to indicate whether a pertussis or cholera toxin sensitive G-protein is involved.

Myoblasts were cultured in '$Ca^{2+}$-free' medium and cholera toxin (100ng/ml), pertussis toxin (100ng/ml) or no addition were added to myoblasts from 34 hours after initial plating to fusion-competence (i.e. for 16 hours). At this time, the cells were

harvested and stimulated to fuse by plating in fresh '$Ca^{2+}$-free' DMEM in the presence of fusion mix with no addition or with cholera or pertussis toxin (100ng/ml). The results are shown in fig. 4.3.3.1. and the data are expressed in terms of fusion index. The percentage fusion obtained for myoblasts cultured in the absence of cholera or pertussis toxin, was arbitrarily assigned a fusion index of 1. This is the control for fusion and represents the amount of fusion obtained in the absence of these toxins. It may be seen that cholera and pertussis toxins were unable to inhibit myoblast fusion.

To test the effects of cholera and pertussis toxin pretreatment on the agonist stimulation of total [$^3$H] inositol phosphate generation, myoblasts were treated as above until fusion-competence. Following harvesting, the cells were incubated in the presence and absence of fusion mix and total [$^3$H] inositol phosphate generation was determined using the $Li^+$ assay. The results are shown in fig. 4.3.3.2. and expressed as fold stimulation over the control sample which was incubated in the absence of the agonist, fusion mix. From the fig., it may be seen that cholera and pertussis toxin did not inhibit fusion mix stimulated total [$^3$H] inositol phosphate generation.

Cholera and pertussis toxin pretreatments may not have resulted in the complete ADP-ribosylation of all the available G-protein substrates. This possibility was investigated, by challenging membranes prepared from cholera or pertussis toxin pretreated (as above) myoblasts with fresh toxin and [$^{32}$P] $NAD^+$. Any G-proteins that were not ADP-ribosylated by these toxins during the *in vivo* pretreatment will be available for this modification *in vitro*. The samples were resolved by SDS-PAGE [12.5% acrylamide] and the resulting gel was dried and autoradiographed. The autoradiograph is shown in figs. 4.3.3.3. and 4.3.3.4. Membranes from rat cerebral cortex and rat glioma C6 BU1 cells were used to provide positive controls for the cholera and pertussis toxin ADP-ribosylation. The incubation of myoblasts with cholera toxin (100ng/ml) or pertussis toxin (100ng/ml), for 16 hours prior to fusion-competence, prevented the incorporation of radioactivity into their substrates, when membranes of these pretreated cells were challenged *in vitro* with [$^{32}$P] $NAD^+$ and fresh toxin. Thus there were no available substrates for further ADP-ribosylation.

**Figure 4.3.3.1.**

**Effect of cholera and pertussis toxin treatment on myoblast fusion.**

Myoblasts were treated for 16 hours prior to fusion-competence with either no addition (control), 100ng/ml cholera toxin or 100ng/ml pertussis toxin. At this time, myoblasts were plated into medium containing fusion mix in the presence and absence of 100ng/ml cholera toxin or 100ng/ml pertussis toxin. Fusion was assessed 20 hours later. Results are means ± S.D. (n=3) from a single experiment typical of 3.

Figure 4.3.3.1.

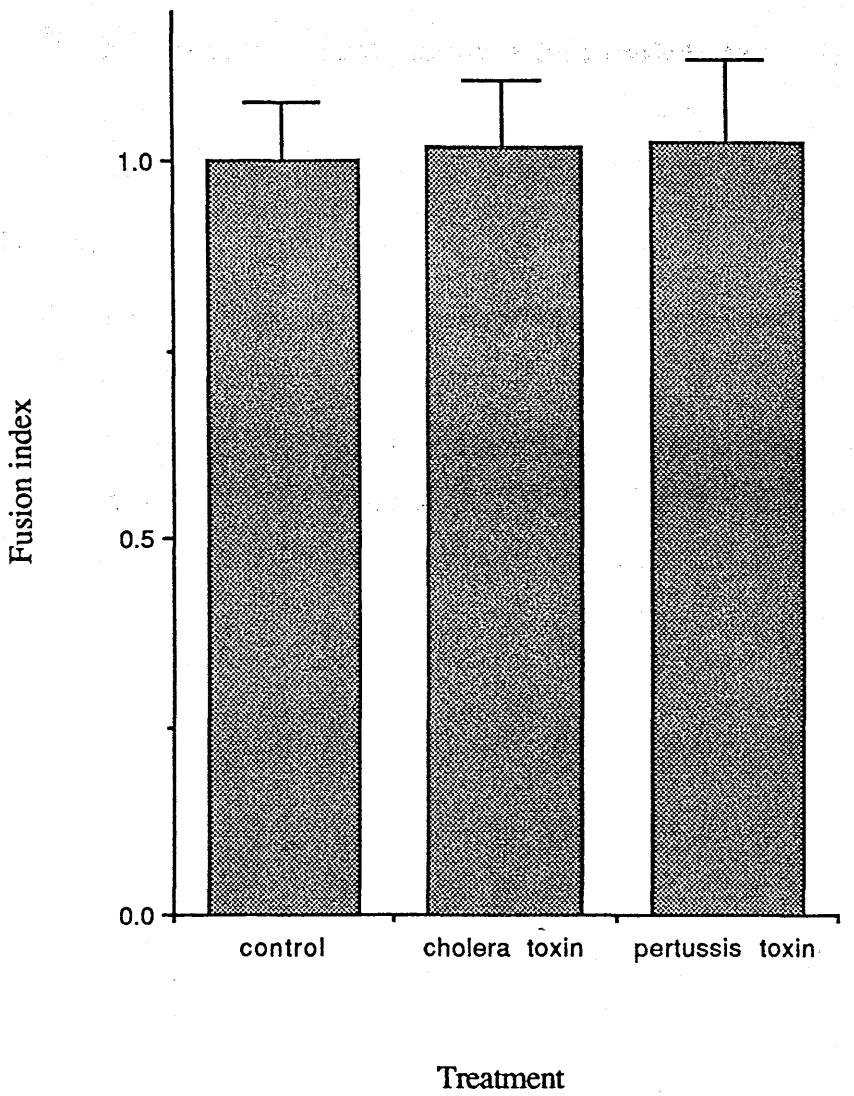

Fusion index
1.0
0.5
0.0
control
cholera toxin
pertussis toxin
Treatment

**Figure 4.3.3.2.**

**Effect of cholera and pertussis toxin treatment on fusion mix stimulated total [$^3$H] inositol phosphate generation in fusion-competent myoblasts.**

Myoblasts were labelled with [$^3$H] inositol (from 24 hours in culture) and treated for 16 hours prior to fusion-competence with either no addition (control), 100ng/ml cholera toxin or 100ng/ml pertussis toxin. At this time, myoblasts were washed and incubated, in the presence and absence of fusion mix, for 30 minutes at 37$^o$C, in buffer containing 10mM LiCl. Total [$^3$H] inositol phosphates were batch eluted from Dowex-formate anion exchange resin. Results are means ± S.D. (n=3) from a single experiment typical of 3.

Figure 4.3.3.2.

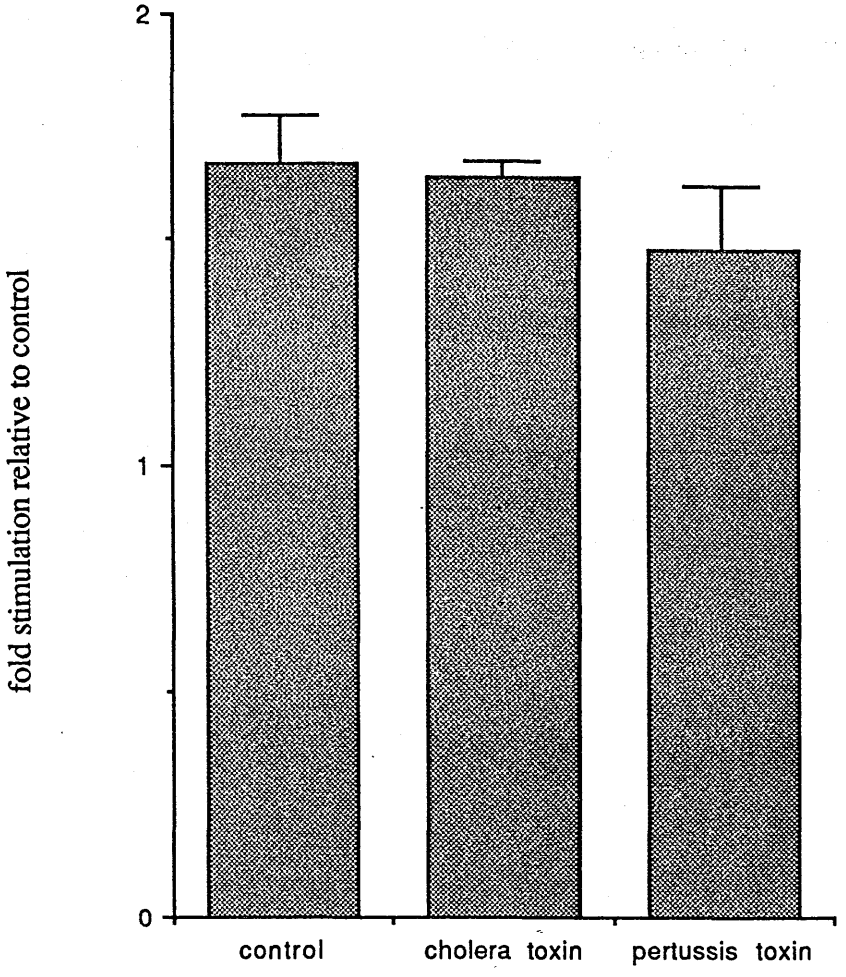

2
1
0
fold stimulation relative to control
control
cholera toxin
pertussis toxin
Treatment

**Figure 4.3.3.3**

**Cholera toxin catalysed ADP-ribosylation of myoblast G-proteins in primary culture.**

Membrane samples from

a) C6 BU1 rat glioma cells (30μg)

b) fusion-competent myoblasts (50μg)

c) fusion-competent myoblasts (50μg)

d) cholera toxin pretreated fusion-competent myoblasts (50μg)

e) cholera toxin pretreated fusion-competent myoblasts (50μg)

f) cholera toxin pretreated fusion-competent myoblasts (50μg)

g) fusion-competent myoblasts (50μg)

were treated with [$^{32}$P] $NAD^+$ and cholera toxin except for sample f to which no toxin was added. Samples were resolved by SDS-PAGE [12.5% acrylamide/0.06% bisacrylamide] and subjected to autoradiography.

Figure 4.3.3.3.
Molecular weight

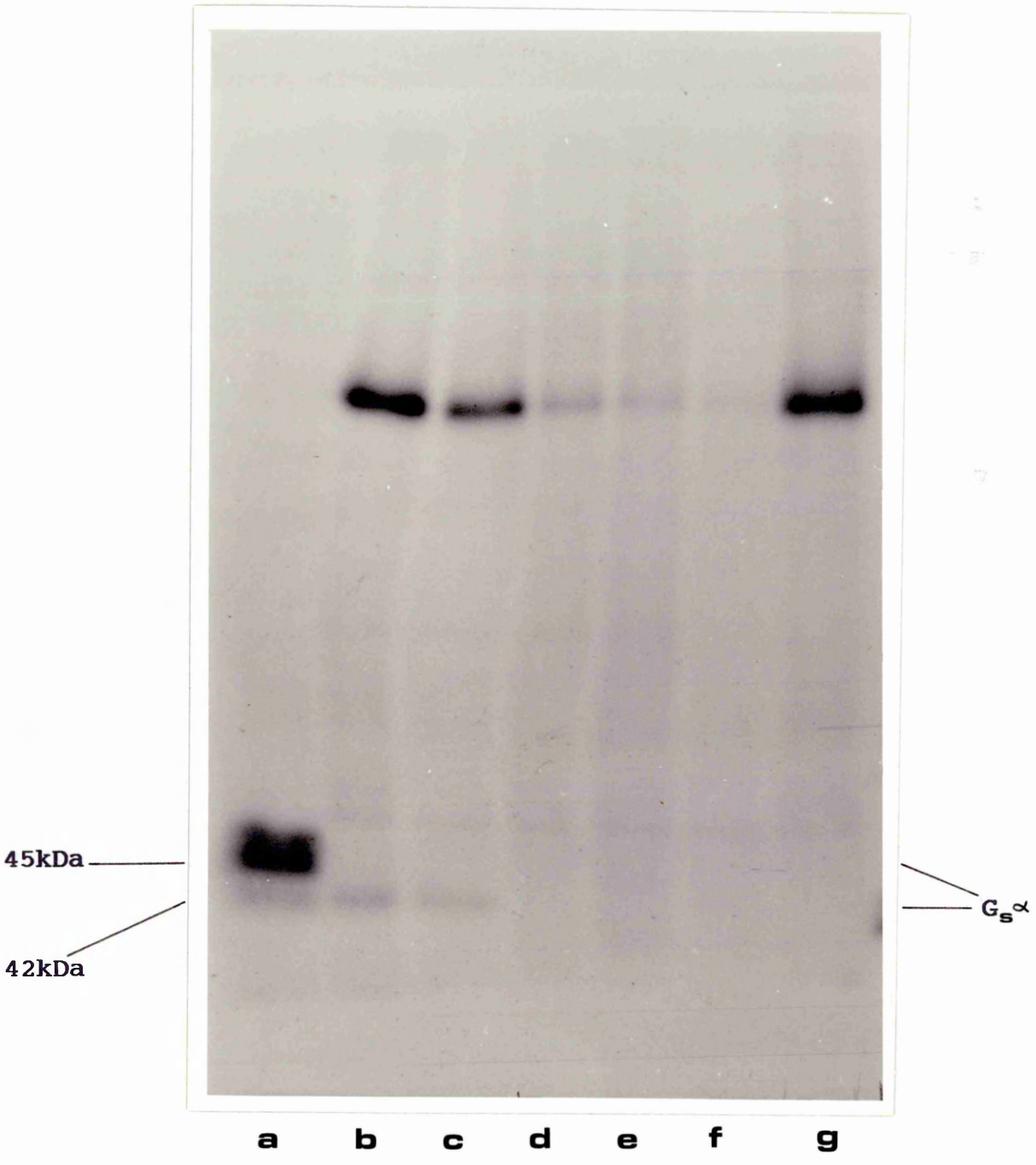


**Figure 4.3.3.4.**

**Pertussis toxin catalysed ADP-ribosylation of myoblast G-proteins in primary culture.**

Membrane samples from

a) fusion-competent myoblasts (50μg)

b) pertussis toxin pretreated fusion-competent myoblasts (50μg)

c) pertussis toxin pretreated fusion-competent myoblasts (50μg)

d) cholera toxin pretreated fusion-competent myoblasts (50μg)

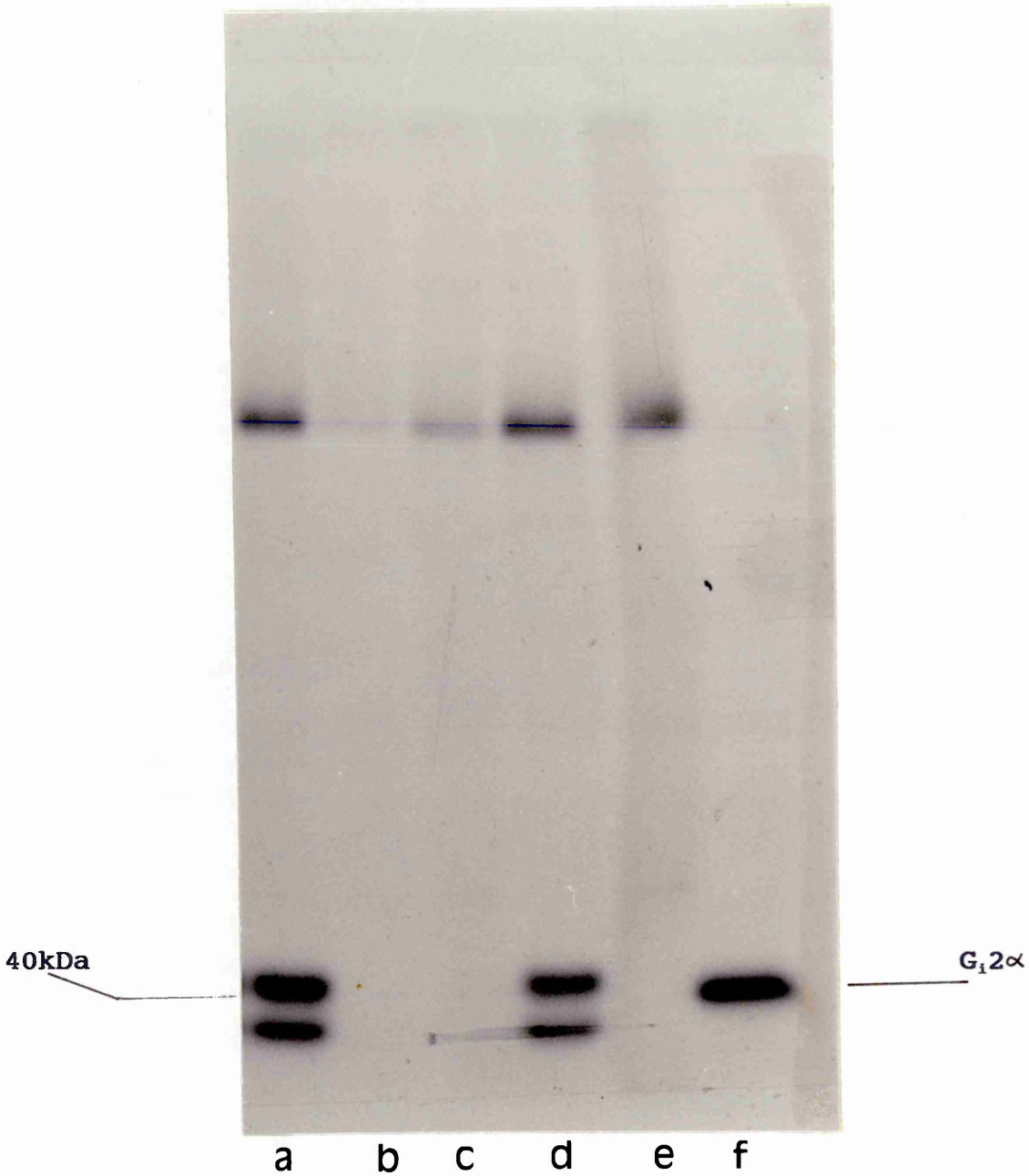
e) fusion competent myoblasts (50μg)

f) C6 BU1 rat glioma cells (30μg)

were treated with [$^{32}$P] NAD$^{+}$ and pertussis toxin except for sample e to which no toxin was added. Samples were resolved by SDS-PAGE [12.5% acrylamide/0.06% bisacrylamide].and subjected to autoradiography.

Figure 4.3.3.4.

Molecular Weight



## 4.4. Changes in levels of myoblast G-proteins on differentiation.

Membrane samples of myoblasts at various stages of differentiation were examined for changes in the levels of pertussis toxin and cholera toxin sensitive G-proteins. These samples were prepared from myoblasts cultured for 24, 50 and 98 hours in '$Ca^{2+}$-free' medium. At these times myoblasts are predominantly proliferating, fusion-competent and non-fused respectively. Myoblast fusion was initiated by addition of 1.4mM $Ca^{2+}$ to fusion-competent myoblasts and the resulting myotubes were further cultured for 48 hours before harvesting (total time in culture 98 hours). These samples were incubated with cholera or pertussis toxin in the presence of [$^{32}$P] $NAD^+$, resolved by SDS-PAGE and autoradiographed. The results are shown in fig. 4.4.1., from which it may be seen that there was an increase in the cholera toxin substrate and pertussis toxin sustrates upon differentiation of fusion-competent myoblasts (lanes b) into myotubes (lanes c ). This increase was apparently dependent on the presence of extracellular $Ca^{2+}$ since it was not seen in the non-fused samples (lanes d).

These changes may result from the activation of second messenger system(s) i.e. be mediated by second messenger signalling molecule(s). This possibility was investigated by treating myoblasts with (i) forskolin which activates the catalytic unit of adenylate cyclase directly to elevate intracellular levels of cyclic adenosine 5' monophosphate(cyclic AMP) and (ii) TPA which activates protein kinase C (PKC) directly and (iii) polymyxin B which is a putative of inhibitor PKC.

Figs. 4.4.2. and 4.4.3. show pertussis toxin and cholera toxin catalysed ADP-ribosylation of membranes from fusion-competent myoblasts and myotubes respectively. Myoblasts were treated with various agents at fusion-competence and harvested 48 hours later. Membranes from the same sets of samples were used in both experiments. Comparison of these two figures revealed that the increases in the pertussis toxin and cholera toxin substrates, which occur on differentiation (lane h), were mimicked in the presence of the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA) (lanes k and l) but inhibited by polymyxin B (lane m). Treatment with forskolin (lanes d and e) mimicked the increase in the cholera toxin substrate but not that of the pertussis toxin substrates.

**Figure 4.4.1.**

**Cholera and pertussis toxin-catalysed ADP-ribosylation of myoblast membranes from differentiating myoblasts.**

Membrane samples (50μg) from

a) a) proliferating myoblasts

b) fusion-competent myoblasts

c) fused myoblasts i.e. predominantly myotubes

d) non-fused myoblasts

Membrane samples (75μg) from

b) a) proliferating myoblasts

b) fusion-competent myoblasts

c) fused myoblasts i.e. predominantly myotubes

d) non-fused myoblasts

were incubated with [$^{32}$P] $NAD^+$ and either I) cholera toxin or II) pertussis toxin. Samples were resolved by SDS-PAGE [10% acrylamide]. The gel was dried and subjected to autoradiography.

Figure 4.4.1.a) and b)
Molecular weight

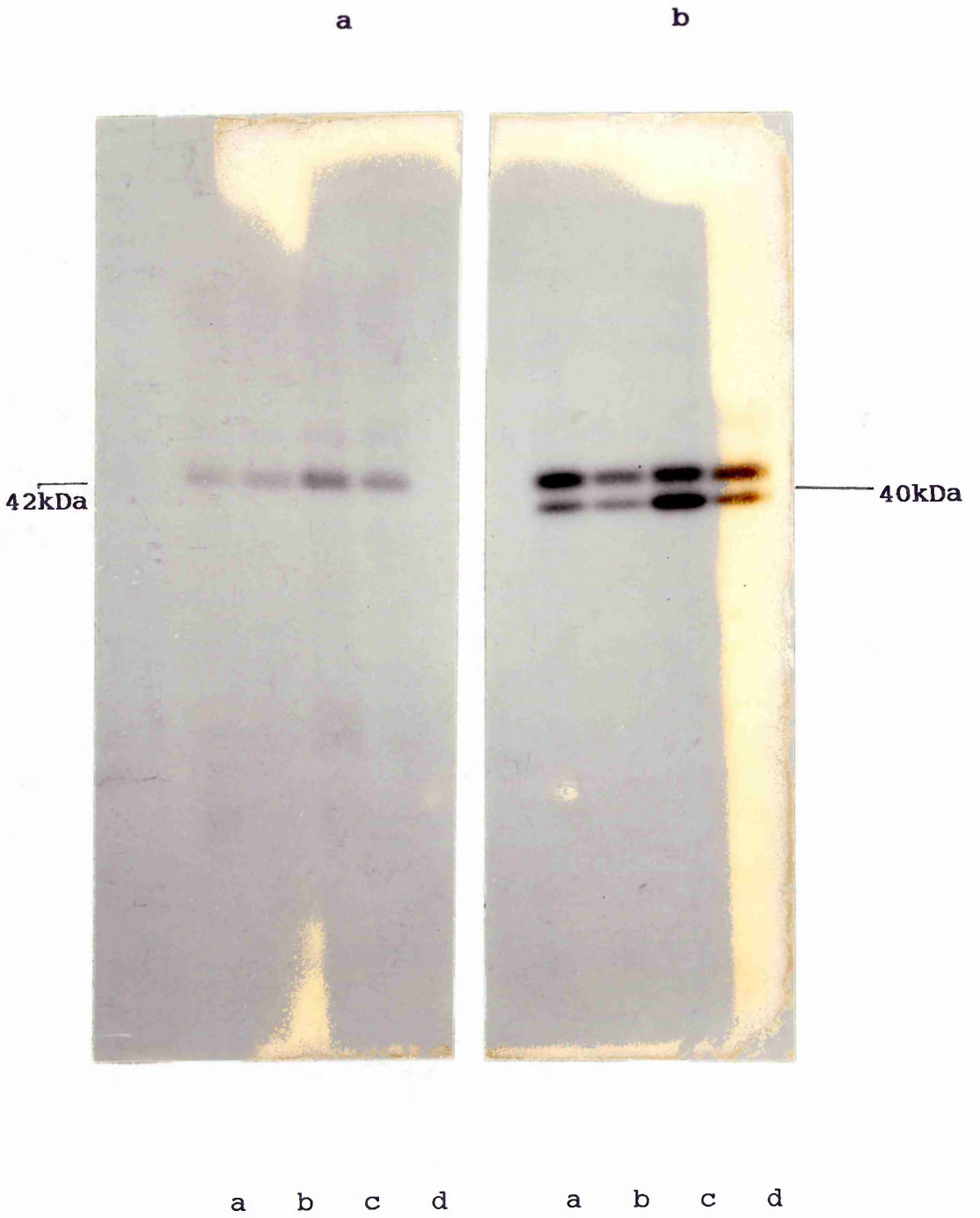


**Figure 4.4.2.**

**The effect of various pharmacological agents on the differentiation-related increase in pertussis toxin substrates.**

Membrane samples from

a) rat glioma C6 BU1 cells (30μg)

b) - n) myoblasts treated after 50 hours in culture (i.e. at fusion-competence) with various agents and harvested 48 hours later (50μg)

b) and c) 10μM $PGE_1$

d) and e) 10μM forskolin

f) and g) fusion-competent myoblasts harvested 50 hours after initial culture in '$Ca^{2+}$-free' medium.

h) 1.4mM $Ca^{2+}$/10% (v/v) horse serum and 5% (v/v) chick embryo extract i.e. myotubes.

i) and j) '$Ca^{2+}$-free' medium i.e. non-fused myoblasts

k) and l) treatment with 100nM TPA for 15 minutes prior to theaddition of 1.4mM $Ca^{2+}$/10% (v/v) horse serum and 5% (v/v) chick embryo extract

m) treatment with 30μM polymyxin B for 15 minutes prior to the addition of 1.4mM $Ca^{2+}$/10% (v/v) horse serum and 5% (v/v) chick embryo extract

n) rat cerebral cortex (10μg)

o) fusion-competent myoblast

were incubated with [$^{32}$P] $NAD^+$ and pertussis toxin except for sample o) to which no toxin was added. Samples were resolved by SDS-PAGE [12.5% acrylamide/0.06% bisacrylamide]. The gel was dried and autoradiographed.

Figure 4.4.2
Molecular weight

**Figure 4.4.3.**

**The effect of various pharmacological agents on the differentiation-related increase in cholera toxin substrates.**

Membrane samples from

a) rat glioma C6 BU1 cells (30μg)

b) - n) myoblasts treated after 50 hours in culture (i.e. at fusion-competence) with various agents and harvested 48 hours later (50μg)

b) and c) 10μM $PGE_1$

d) and e) 10μM forskolin

f) and g) fusion-competent myoblasts harvested 50 hours after initial culture in '$Ca^{2+}$-free' medium.

h) 1.4mM $Ca^{2+}$/10% (v/v) horse serum and 5% (v/v) chick embryo extract i.e. myotubes.

i) and j) '$Ca^{2+}$-free' medium i.e. non-fused myoblasts

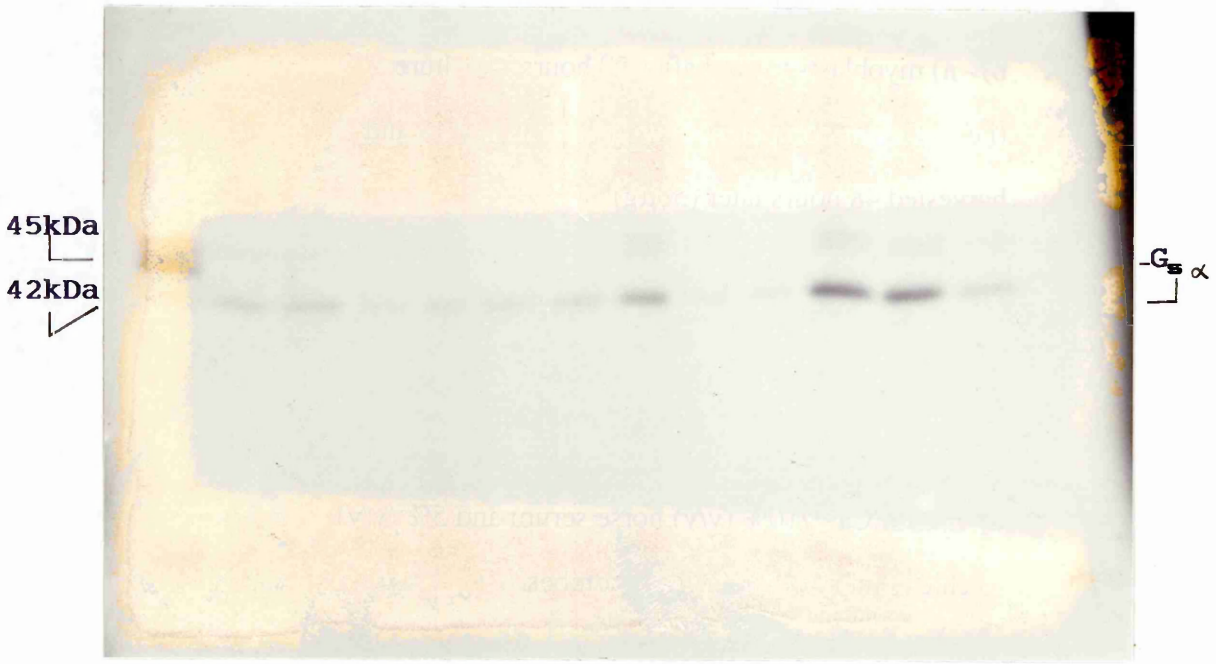
k) and l) treatment with 100nM TPA for 15 minutes prior to theaddition of 1.4mM $Ca^{2+}$/10% (v/v) horse serum and 5% (v/v) chick embryo extract

m) treatment with 30μM polymyxin B for 15 minutes prior to the addition of 1.4mM $Ca^{2+}$/10% (v/v) horse serum and 5% (v/v) chick embryo extract

n) fusion-competent myoblasts

were incubated with [$^{32}P$] $NAD^+$ and cholera toxin except for sample n) to which no toxin was added. Samples were resolved by SDS-PAGE [12.5% acrylamide/0.06% bisacrylamide]. The gel was dried and autoradiographed.

Figure 4.4.3.
Molecular weight




a b c d e f g h i j k l m n

## Discussion

### 4.5. Characterisation of G-proteins present in myoblast membranes

Membrane samples of myoblasts prepared from chick embryonic skeletal muscle were studied in order to characterise the G-proteins present. Two experimental approaches were adopted to define the Gα subunits present since they are unique for each G-protein type. These were (i) cholera toxin or pertussis toxin catalysed ADP-ribosylation and (ii) western immunoblotting using a panel of selective anti-G-protein anti-peptide antisera raised against amino acid sequences unique to the various α subunits of these toxin substrates.

Cholera and pertussis toxins are multi subunit proteins which catalyse the ADP-ribosylation of Gα protein subunits in both whole cells and membrane preparations. Cholera toxin is composed of 2 subunit types and exists as A1B5 multimer. It binds its cell surface receptor, the ganglioside GM1, via the B subunits. This leads to the formation of a pore through which the catalytically active A subunit is extruded into the cytosol. Pertussis toxin consists of 6 subunits of which 5 are dissimilar. The S1 subunit contains the ADP-ribosyltransterase activity whilst the others mediate its attachment and entry into cells (Foster and Kinney, 1984). Both these toxins are inactive in the holomeric form and thus require preactivation (to dissociate the catalytically active subunits) for use with membrane preparations. This was achieved by treatment with the reducing agent dithiothreitol.

Cholera-toxin-catalysed ADP-ribosylation was performed in the presence of guanine nucleotides which resulted in the specific ADP-ribosylation of the α subunit of $G_s$. A 42kDa form of $G_s\alpha$ was present in myoblasts, as determined by cholera-toxin - catalysed ADP-ribosylation (fig. 4.1.1.) and immunoblotting with the anti-peptide antiserum CS1 which identifies the C terminus of mammalian $G_s\alpha$ (fig. 4.2.1.1.). Three pertussis toxin sensitive substrates were present in myoblast membranes (fig. 4.1.3.) with apparent molecular weights of 38kDa, 40.5kDa and 41kDa.

In order to further characterise the G-proteins present in myoblasts, membrane samples were immunoblotted using selective anti -G-protein antisera. Immunoblotting

with the antiserum SG1, which identifies the C terminal sequence of mammalian $G_i1\alpha$ and $G_i2\alpha$, in non-photo-receptor containing tissues, identified three immunoreactive polypeptides of 38kDa, 40.5kDa and 41.5kDa (fig. 4.2.2.2.). Antiserum I1C was used to further characterise these $G_i1\alpha$ and $G_i2\alpha$ immunoreactive polypeptides, since it is capable of discriminating between mammalian $G_i1\alpha$ and $G_i2\alpha$. Using this antiserum a $G_i1\alpha$ -like polypeptide of 41.5kDa was detected (fig. 4.2.2.3.).

Antiserum I3B was raised against the C terminal region of mammalian $G_i3\alpha$ but it cross-reacts weakly with mammalian $G_o\alpha$ and therefore recognises both mammalian $G_i3\alpha$ and $G_o\alpha$. Western blotting with this antiserum identified a single immunoreactive polypeptide of 41.5kDa in membranes prepared from myoblasts and 12-day-old chick embryonic brain. An additional immunoreactive polypeptide of 40kDa was also detected in 12-day-old chick embryonic brain membranes (fig. 4.2.3.1.). Antiserum ON1, which recognises the N terminal amino acid sequence of mammalian $G_o\alpha$, identified this 40kDa polypeptide as a $G_o\alpha$ like polypeptide. It also identified a similar immunoreactive polypeptide in myoblast membranes.(fig. 4.2.3.2.). This polypeptide was only detectable in trace amounts and was probably recognised by ON1, and not antiserum I3B, because this antiserum is a much more sensitive probe for $G_o\alpha$ immunoreactivity than I3B. In conclusion, these results demonstrated that chick myoblasts possess $G_i1\alpha$-, $G_i2\alpha$- $G_i3\alpha$- and $G_o\alpha$-like immunoreactivity.

The mobilities of these $G_i1\alpha$, $G_i2\alpha$ $G_i3\alpha$ and $G_o\alpha$ immunoreactive proteins in SDS polyacrylamide gels were different to those for the mammalian forms. These membrane samples were alkylated prior to SDS-PAGE since this treatment has been shown to enhance the resolution of mammalian pertussis toxin substrates (Sternweis and Robishaw, 1984). Alkylation of proteins results in an increase in their molecular weight so that they are less mobile in SDS-polyacrylamide gels. The differing mobilities may therefore reflect differences in amino acid sequence between the mammalian and chick G-proteins, since this could result in relatively more or less cysteine residues available for alkylation. This is probably true for the $G_o\alpha$ immunoreactive polypeptide, since alkylation caused it to be less mobile upon SDS-

PAGE than the non alkylated form which has a similar mobility to mammalian $G_o\alpha$ (figs. 4.2.4.2.1. and 4.2.4.2.2.). Alkylation, however, does not explain the difference in the relative moblities of the $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ immunoreactive polypeptides when compared to the mammalian G-proteins (see figs. 4.2.4.1.1., 4.2.4.1.2. and 4.2.4.2.1.). These differences in mobilities are seen, in both myoblasts and 12 day old chick embryonic brain, and may reflect species differences in the amino acid sequences of mammalian and chick G-proteins. Unfortunately, this could not be further investigated as, to date, there is no avian G-protein amino acid sequence data available for comparison with mammalian G-proteins.

In order to determine whether the polypeptides recognised by the antisera SG1 and I3B represent distinct immunoreactive entities, membrane samples from myoblasts and 12-day-old chick embryonic brain were prepared in the presence and absence of protease inhibitors and immunoblotted using these antisera. The pattern of immunoreactivity detected for each membrane type was the same for both sets of samples (figs. 4.2.5.1. and 4.2.5.2.). The polypeptides recognised by these antisera thus represent distinct immunoreactive entities and the differences in their molecular weights are not a consequence of proteolytic cleavage of the 41.5kDa polypeptide.

Membrane samples from myoblasts and 12-day-old chick embryonic brain were also immunoblotted using the antiserum BN1 which is raised against the N terminal amino acid sequence of the mammalian $\beta$ G-protein subunit. An immunoreactive polypeptide of 35kDa which co-migrated with the mammalian $\beta$ subunit was detected (fig. 4.2.6.1.) which suggests that chick embryonic myoblast G-proteins probably exist as classical heterotrimers.

## 4.6. Role of G-proteins in myoblast fusion.

G-proteins are involved in the signal tranduction of many hormones and agonists. These systems are membrane associated and comprise an agonist-specific cell surface receptor, G-protein and an effector which may be an intracellular second messenger forming enzyme or an ion channel. The receptor and effector interaction is

mediated by G-proteins, which are heterotrimeric proteins, consisting of α, β and γ subunits. The primary specificity for both the receptor and target effector is determined by the unique nature of the α subunit. The structural variability which is essential for their function, is found only in the regions involved in receptor and effector recognition, whilst the structure of the guanine nucleotide binding site is conserved.

Agonist occupation of the receptor promotes a GTP and $Mg^{2+}$-dependent dissociation of the oligomer into a βγ subunit complex and an activated α subunit which transduces information from the receptor to the specific target effector. Subsequent hydrolysis of GTP to GDP and Pi by the intrinsic GTPase of the α subunit, causes deactivation and dissociation of the α subunit from the effector. It then reassociates with the βγ subunit complex and returns to the 'inactive' state. The presence of a receptor-controlled GTPase activity and its function in the termination of an agonist stimulated response was first demonstrated for the β–adrenergic receptor in turkey erythrocytes (Cassel and Selinger 1975), where agonist occupation of these receptors caused a GTP dependent activation of adenylate cyclase and an increase in the high affinity GTPase activity. Agonist stimulated GTPase activity is indicative of receptor- G-protein coupling. Fusion mix was tested and found to stimulate GTPase activity in the membranes prepared from fusion-competent myobasts (fig. 4.3.2.1.).

GTPγS, a non-hydrolysable analog of GTP, stimulated total [$^3$H] inositol phosphate generation in electropermeabilised myoblasts (fig. 4.3.2.2.) and was synergistic with fusion mix in stimulating total [$^3$H] inositol phosphate generation in these cells (fig. 4.3.2.3) which suggests that they share a common mechanism i.e. G-protein activation, for promoting the activation of PI-PLC. GTPγS alone was also able to stimulate total [$^3$H] inositol phosphate generation in non-permeabilised cells and may achieve this either by acting at a $P_2$-purinergic receptor or by entering 'leaky' cells to directly activate G-proteins. In order to establish whether this stimulation of total [$^3$H] inositol phosphate generation is due to activation of a purinergic receptor, these experiments should be repeated in the presence of a specific $P_2$ receptor antagonist, for

example, arylazido-aminopropionyl ATP (ANAPP3), a photo-affinity ligand which irreversibly blocks $P_2$-purinergic receptors (Katsuragi and Furakawa, 1985).

## 4.7. Effects of cholera and pertussis toxin pretreatment.

Evidence for the involvement of G-proteins in the regulation of agonist stimulated PI-PLC activity has come from studies using cholera and pertussis toxins. Pertussis toxin catalyses the ADP-ribosylation of a cysteine residue that is positioned 4 amino acids from the carboxy terminus of the $\alpha$ subunit (Hurley *et al.*, 1984; West *et al.*, 1985). ADP-ribosylation at this site prevents the stimulation of G-protein subunit dissociation that normally occurs on agonist occupation of the receptor (Haga *et al.*, 1985). Agonists which mediate their effects through G-proteins, that are substrates for pertussis-toxin-catalysed ADP-ribosylation, are therefore prevented from doing so by pretreatment with pertussis toxin. For example, pertussis toxin pretreatment inhibits the stimulation of inositol phosphate generation by fmet-leu-phe in mast cells (Ohta *et al.*, 1985) and by platelet activating factor in macrophages (Huang *et al.*, 1988).

Cholera toxin catalyses ADP-ribosylation of specific arginine residue in the $\alpha$ subunit of $G_s$ (van Dop *et al.*, 1984). This leads to a much reduced GTPase activity which maintains the $\alpha$ subunit in the activated state (Cassel and Selinger, 1977). Cholera toxin pretreatment has been reported to inhibit receptor stimulated inositol phosphate generation in human pituitary clonal cells, and on this basis, it was suggested that the receptor-PI-PLC interaction was mediated by a novel cholera toxin sensitive G-protein (Lo and Hughes, 1987). Alternatively, the observed inhibition may be cyclic AMP mediated since cholera-toxin-catalysed ADP-ribosylation of $G_s$, would lead to permanent activation of adenylate cyclase and thus elevate intracellular levels of cyclic AMP. This has been demonstrated to be the case for cholera toxin inhibition of vasopressin stimulation of renal glomerulosa cells (Guillon *et al.*, 1988) and L6 skeletal myoblasts (Gardner *et al.*, 1989), where cholera toxin treatment led to a down regulation of the vasopressin receptors. This probably resulted from activation of the cyclic AMP dependent protein kinase, protein kinase A, since this effect was mimicked

by other agents which increase cyclic AMP levels. Thus cholera toxin mediated inhibition of agonist stimulated inositol phospholipid breakdown is not necessarily indicative of receptor-PI-PLC coupling via a cholera toxin sensitive G-protein. In many cells however, pretreatment with cholera or pertussis toxin does not modify agonist stimulated total [$^3$H] inositol phosphate generation. This suggests that, in these cells, the G-protein involved in receptor - PI-PLC coupling is not a substrate for either pertussis toxin or cholera toxin-catalysed ADP-ribosylation.

The receptor-mediated stimulation of myoblast fusion and inositol phospholipid breakdown by the agonist, fusion mix were examined for their sensitivity to pertussis and cholera toxins. Myoblasts were pretreated from 24 hours after initial plating to fusion-competence with pertussis toxin, cholera toxin or no addition (control) and their effect on fusion and fusion mix stimulated total [$^3$H] inositol phoshate generation determined. From figs. 4.3.3.1. and 4.3.3.2., it may be concluded that these events are pertussis and cholera toxin insensitive.

If these events are G-protein mediated, and involve a pertussis or cholera toxin sensitive substrate(s), then the lack of inhibition obtained with these toxins may be due to incomplete ADP-ribosylation of the available G-protein. This may be due to insufficient transport of the toxin across the cell membrane into the cytosol or alternatively, the cellular levels of $NAD^+$ may be inadequate for complete ADP-ribosylation. This possibility was examined by pretreating myoblasts with no addition (control), pertussis toxin or cholera toxin from 34 hours after initial plating until fusion-competence. Membranes were then prepared from these cells and subsequently challenged with pertussis toxin or cholera toxin, in order to determine whether any of their toxin sensitive G-protein substrates were available for further ADP-ribosylation. Pretreatment of myoblasts with pertussis and cholera toxins resulted in the complete ADP-ribosylation of their available substrates. (fig. 4.3.3.3. and 4.3.3.4.). A cholera or pertussis toxin sensitive G-protein is therefore unlikely to be involved in fusion mix stimulated total [$^3$H] inositol phosphate generation and myoblast fusion. Thus, at this

time, there is no convenient probe available with which to further identify the G-protein involved.

## 4.8. Changes in the levels of myoblast G-proteins upon differentiation.

The main event in muscle differentiation is the fusion of monucleated myoblasts to form myotubes with concomitant synthesis of several muscle specific proteins. These processes may be either causally related, or initiated simultaneously, but occuring independently. Fusion-negative mutants of the L6 and L8 rat myoblast cell lines have been obtained which are capable of producing some, but not all, muscle specific proteins (Devlin and Emerson, 1978). It would therefore appear that the synthesis of all the muscle specific proteins is not co-ordinated. However, there are subsets of muscle specific proteins, the synthesis of which is co-ordinately regulated during the differentiation of chick myoblasts (Schudt *et al.*, 1975). Myoblast differentiation thus provides an experimental system in which to examine co-ordinate protein synthesis.

Changes in the levels of myoblast G-proteins during differentiation have been examined using the low $Ca^{2+}$ culture system in which rapid synchronous fusion occurs on addition of 1.4mM $Ca^{2+}$ to fusion-competent myoblasts. Membranes were prepared from proliferating, fusion-competent and non-fused myoblasts and from myotubes and subjected to pertussis toxin and cholera toxin catalysed ADP-ribosylation. There was an increase in the amounts of the cholera toxin and pertussis toxin substrates upon myoblast differentiation (fig. 4.4.1.) which was dependent on extracellular $Ca^{2+}$. These increases in cholera toxin and pertussis toxin substrates may be regulated by alterations in the intracellular levels of second messenger molecules since myoblast fusion is a receptor mediated event that is apparently coupled to inositol phospholipid breakdown (Wakelam and Pette, 1984). The hydrolysis of phosphatidylinositol 4,5 bisphosphate (PtdIns 4,5 $P_2$) by PI-PLC results in the generation of the second messengers, inositol trisphosphate ($IP_3$) and *sn* -1,2-diacylglycerol (*sn* -1,2-DAG). $IP_3$ causes a increase in the levels of intracellular $Ca^{2+}$ by releasing $Ca^{2+}$ from

intracellular stores (Berridge and Irvine, 1984). *Sn* -1,2-DAG is a physiological acivator of protein kinase C (PKC) which is a $Ca^{2+}$/phospholipid dependent serine threonine protein kinase Nishizuka, 1984). Following fusion, there is an increase in adenylate cyclase activity (Shainberg *et al.*, 1971) and the levels of its product, the second messenger, cyclic AMP have been demonstrated to rise following fusion (Schutzle *et al.*, 1984).

The effects of various pharmacological agents on the increases in cholera and pertussis toxin substrates were examined in an attempt to identify the second messenger signalling pathways involved. The role of PKC in this differentiation linked event, was investigated by the addition of TPA to fusion-competent myoblasts, in order to directly activate PKC and it was found to mimic the increases in cholera and pertussis toxin substrates which occured on differentiation (figs. 4.4.2. and 4.4.3.). In addition these increases were inhibited in the presence of the putative PKC inhibitor, polymyxin B (figs. 4.4.2. and 4.4.3.), which provides further support for PKC involvement. The increase in pertussis toxin substrates (fig. 4.4.2.), but not the cholera toxin substrate (fig. 4.4.3.), was mimicked by treatment of fusion-competent myoblasts with forskolin. This diterpene directly activates the catalytic unit of adenylate cyclase thus raising intracellular levels of cyclic AMP. Therefore synthesis of the pertussis toxin substrates appears to be stimulated by an increase in intracellular levels of cyclic AMP. These results suggest the involvement of PKC and adenylate cyclase activation in the changes in levels of G-proteins during myoblast differentiation.

Second messenger signalling systems have been demonstrated, in number of reports, to play a role in muscle specific protein synthesis. Evidence for an involvement of PKC in the synthesis of muscle specific proteins comes from experiments using the phorbol ester, TPA, to stimulate PKC directly. Treatment of myoblasts with TPA causes an increase in hexose transport which is due to increased mRNA and protein synthesis of the saturable component of the hexose transport system (Schimmel and Keldie, 1980). In addition, TPA has been demonstrated to stimulate synthesis of plasminogen activator in chick myotubes (Miskin *et al.*, 1978). The $IP_3$

mediated release of $Ca^{2+}$ from intracellular stores will also effect biochemical events regulated by calmodulin (CaM) which is the intracellular $Ca^{2+}$ receptor protein. CaM mediates many calcium-dependent processes in a variety of cell types (Cheung, 1980) but the physiological relevance of CaM-dependent interactions in relation to the synthesis of muscle specific protein synthesis remains to be determined. It has been implicated as a regulator of the intracellular levels of the second messenger cyclic AMP and may (i) increase cyclic AMP levels by stimulating adenylate cyclase, the enzyme present in the plasma membrane which converts ATP to cyclic AMP and (ii) cause a decrease in the levels of cyclic AMP by activation of the CaM dependent phosphodiesterase which degrades cyclic AMP to 5'-AMP. The rise in cyclic AMP which occurs following the onset of fusion has been implicated in the stimulation of the synthesis of a muscle specific protein, creatine kinase, since it precedes the appearance of this enzyme (Schutzle *et al.*, 1984). Cyclic AMP may also be the second messenger involved in the calcitonin gene-related peptide (CGRP) stimulated synthesis of surface nicotinic acetylcholine receptor (AChR) in primary cultures of chick myotubes (Fontaine *et al.*, 1986, New and Mudge, 1986) since CGRP has been shown to increase the levels of cyclic AMP in cultured myotubes, and to activate adenylate cyclase in chick muscle membranes (Laufer and Changeux, 1987). Additional evidence for cyclic AMP involvement comes from the demonstration that the increase in AChR was mimicked by cholera toxin, which persistently activates adenylate cyclase, thus elevating levels of cyclic AMP (Blosser and Appel, 1980). This is further supported by the demonstration that the cell permeant cyclic nucleotide derivative, dibutyryl cyclic AMP, elevates intracellular levels of cyclic AMP and increases the synthesis of AChR (Betz and Changeux 1979).

As discussed in chapter 3, protein kinase C activation is a key event in the process of myoblast fusion. A role for PKC in the increases in toxin sensitive G-proteins may be argued since i) direct activation of this enzyme by TPA mimicked these increases and ii) pretreatment of fusion-competent myoblasts with the putative PKC inhibitor polymyxin B resulted in inhibition. The generation of *sn* -1,2-DAG in the

plasma membrane and consequent activation of PKC therefore represent critical biochemical steps in the mechanisms underlying i) the fusion event itself, which involves transient changes in membrane structure and cytoskeletal rearrangements (ii) in the regulation of associated muscle specific protein synthesis. Activation of PKC thus provides a mechanism by which myoblast fusion and protein synthesis may be co-ordinately regulated.

In summary, the results presented in this chapter demonstrate that myoblasts possess one cholera toxin substrate and 3 pertussis toxin substrates for ADP-ribosylation together with $G_s\alpha$-, $G_i1\alpha$-, $G_i2\alpha$-, $G_i3\alpha$- and $G_o\alpha$- immunoreactive polypeptides. The role of G-proteins in myoblast fusion was investigated and it may be mediated by a G-protein that is cholera and pertussis toxin insensitive.

The levels of the cholera and pertussis toxin substrates increase upon differentiation and these increases are mimicked by TPA. The increase in the pertussis toxin substrates but not the cholera toxin substrate was also mimicked by forskolin. These results suggest that the increases in G-protein levels are second messenger mediated.

# Chapter 5
# The developmental regulation of myoblast differentiation

## Introduction

Myoblast differentiation *in vitro* requires the addition of supplements to a chemically defined medium (Konigsberg, 1963). These are, most commonly, heat-inactivated horse serum and chick embryo extract. Such factor(s) may also be required for the differentiation of myoblasts *in vivo*, and of possible physiological relevance is the observation that chick embryo extract is essential for the fusion of chick embryonic myoblasts in primary culture (Wakelam and Pette, 1982). It apparently contains fusion stimulating factor(s) (FSF(s)), which are proposed to be neuronal in origin because chick embryonic brain and spinal cord extracts can substitute for the whole extract in promoting fusion (Oh, 1975; Wakelam, 1986). The differentiation of myoblasts *in vivo* is generally considered to be under neuronal control (Bonner and Adams, 1982); skeletal muscle is innervated by motor neurones derived from the spinal cord which exert a trophic influence on its morphological and biochemical properties. Such influence is proposed to be mediated by trophic substance(s) released from the nerve (Guth, 1968; Hoffmann *et al.*, 1972; Oh *et al.*, 1972). This proposal is supported by the observation that the neuropeptides, arginine vasopressin (AVP) and angiotensin II can stimulate fusion in the absence of chick embryo embryo extract (Wakelam and Pette, 1984 a,b).

In order to determine the nature of the factor(s) in spinal cord which stimulate myoblast fusion, chick embryonic spinal cord extract was fractionated by gel filtration chromatography and the ability of the fractions collected to stimulate fusion tested in a bioassay using primary cultures of chick myoblasts prepared from 12-day-old chick embryonic breast muscle. These cells were cultured in '$Ca^{2+}$-free' medium for 50 hours until fusion-competent, washed in '$Ca^{2+}$-free' medium to remove the chick embryo extract and horse serum and plated into DMEM containing 1.4mM $Ca^{2+}$. Aliquots of the test fractions were added to fusion-competent myoblasts and their ability to stimulate fusion i.e. to replace chick embryo extract determined. Several FSFs were found to stimulate fusion including some of low molecular weight (<4kDa). The nature of these low molecular weight factors was further investigated and an alternative

method of assaying fusion was assessed to determine its suitability for rapid screening of possible FSF(s).

## 5.1. Characterisation of FSF(s) in spinal cord.

### 5.1.1. Fractionation by gel filtration chromatography.

Chick spinal cord extract (prepared as described in section 2.1.3.6) was fractionated by gel filtration chromatography on a column of Sephadex G100 (90 x 1.6cm) using 10mM ammonium acetate (pH 4.0 with acetic acid) as the eluent buffer. 1.5ml fractions were collected and elution was monitored by measuring the absorbance at 280nm.

This method allowed fractionation on the basis of molecular weight, with molecules eluted from the column in order of decreasing molecular size. Protein standards of known molecular weight were used to calibrate the column as described in section 2.4 which had a fractionation range of 4-150kDa. The fractions collected were freeze-dried to remove the eluent buffer and thus concentrate the sample, dissolved in 0.5ml 1mM acetic acid and the fusion stimulating ability assessed by adding 75µl of each fraction to be tested to myoblasts in culture. Fusion was determined 20 hours later and a profile of fusion stimulating ability is shown in fig. 5.1.1.1., together with the protein concentration. The fusion stimulating ability is expressed in terms of fusion index. The fusion index of the 'non-fused', control sample of myoblasts, which were cultured in DMEM in the absence of any fractions, was arbitrarily assigned a value of 1. The fusion index of each fraction was defined as

$$\frac{\%\text{ fusion for myoblasts cultured in the presence of test fraction and DMEM}}{\%\text{ fusion for myoblasts cultured in the presence of DMEM only}}$$

There were four main regions of fusion stimulating ability in fractions 41-44, 61, 71 and 110-128 which, by reference to the calibration curve, correspond to molecules of apparent molecular weights of >150 kDa, 37kDa, 19kDa and <4kDa.

**Figure 5.1.1.1.**

**Fractionation of chick embryonic spinal cord extract by gel filtration chromatography.**

Chick embryonic spinal cord extract was applied to the Sephadex G100 gel filtration column (90 x 1.6cm) and eluted with 10mM ammonium acetate (pH 4.0 with acetic acid). The fractions collected were tested for fusion stimulating ability ( —— ) and protein content ( ..... ).

Figure 5.1.1.1.

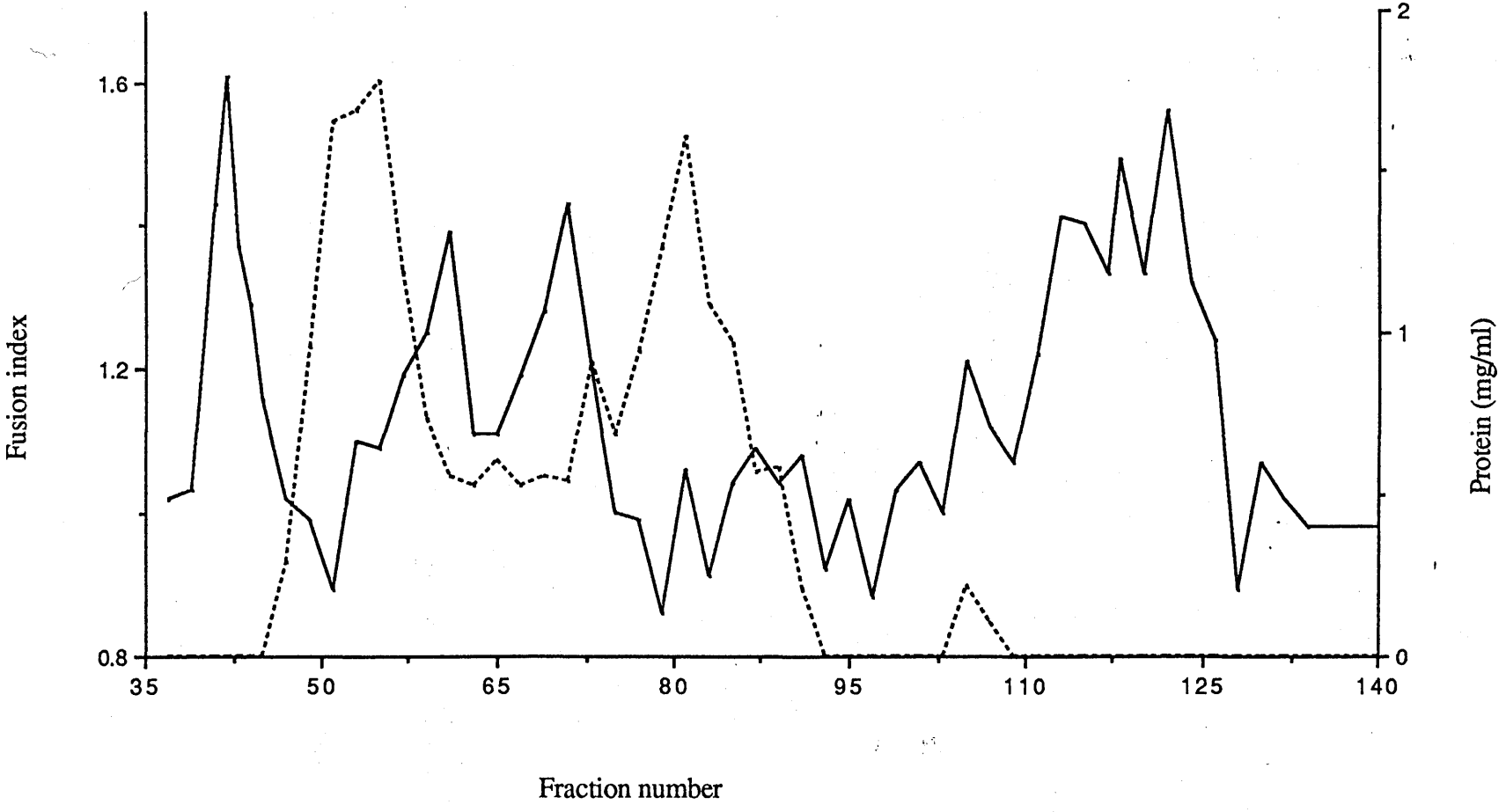
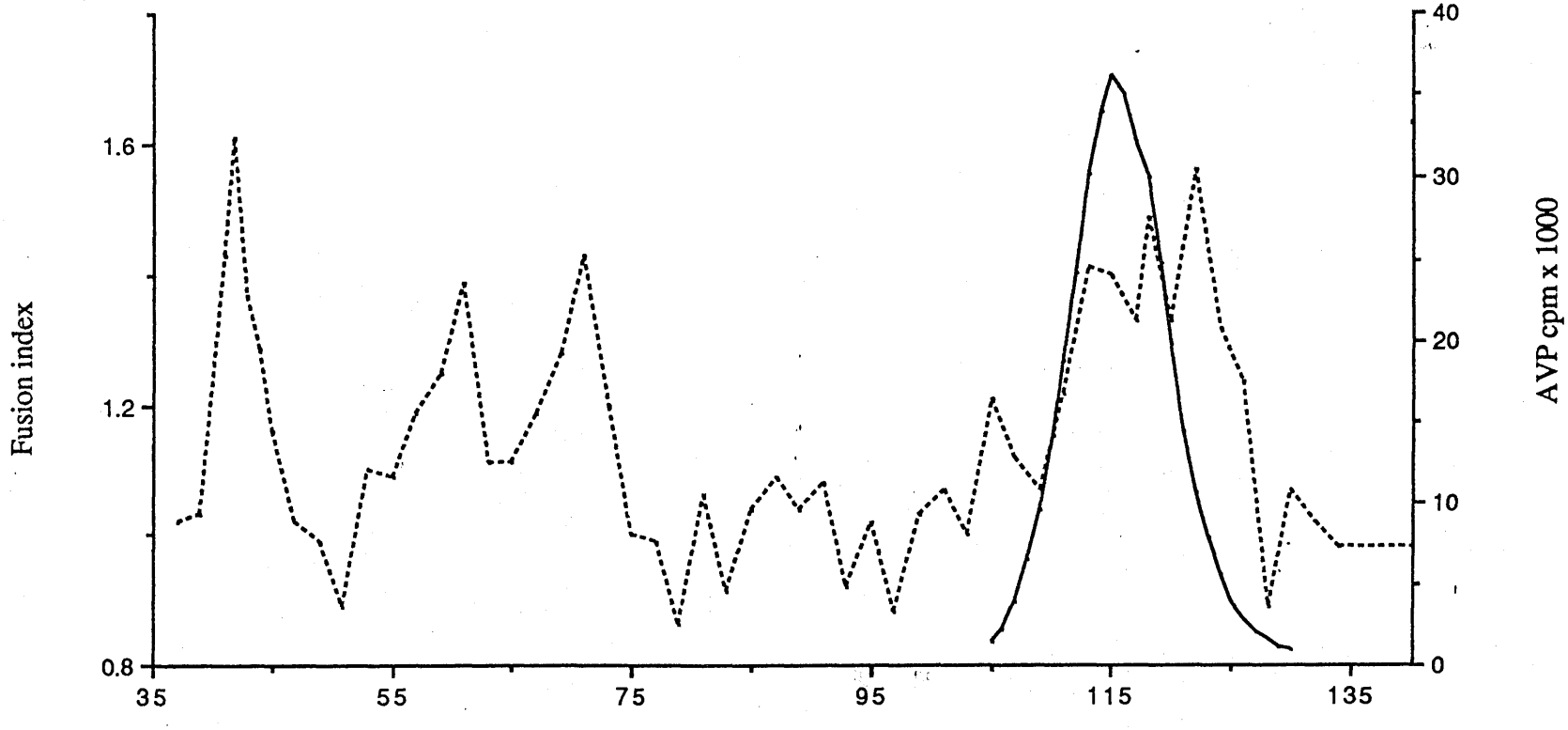

Fusion index
1.6
1.2
0.8
35
50
65
80
95
110
125
140
Fraction number
2
1
0
Protein (mg/ml)

**Figure 5.1.1.2.**

**The elution profile of [$^3$H] AVP.**

The sample of [$^3$H] AVP was applied to the Sephadex G100 gel filtration column and eluted with 10mM ammonium acetate (pH 4.0 with acetic acid). The fractions were collected and their associated radioactivity determined ( —— ). The profile of fusion stimulating ability obtained for chick embryonic spinal cord is shown for comparison ( ..... ).

Figure 5.1.1.2.


Fusion index
1.6
1.2
0.8
35
55
75
95
115
135
40
30
20
10
0
AVP cpm x 1000
Fraction number

The FSF(s) in fractions 41-44 elute in the same fractions as β–amylase (200kDa) and alcohol dehydrogenase (150kDa) which correspond to the void volume of the column. The molecular weight of the factor(s) in fractions 110-128 is less than 4kDa since the elution volume is around that of the total column volume. This factor(s) elutes in a broad peak of activity. The elution profile of a tritiated neuropeptide, [$^3$H] AVP was determined for comparison and is shown in fig. 5.1.1.2., together with the profile of fusion stimulating ability obtained for chick embryonic spinal cord extract. [$^3$H] AVP also has a broad elution peak, from fractions 108 - 124.

In view of the ability of the neuropeptides AVP and angiotensin II to stimulate fusion, attention was focused onto the 'low molecular weight' fractions (less than 4kDa). These were pooled, freeze-dried, dissolved into 1ml PBS and further characterised.

### 5.1.2. Biochemical tests performed on the pooled fractions.

Various biochemical tests were carried out on the 'low molecular weight' FSF(s). The sample was subjected to boiling, dialysis and to treatments with trichloroacetic acid (TCA) and diethyl ether. The results are shown in Table 5.1.2.1. and the data expressed in terms of fusion index calculated relative to the control level of fusion observed for the untreated pooled fractions which was arbitrarily assigned a value of 1. From this, the fusion stimulating ability of the factor(s) is stable to boiling and treatment with TCA and ether but lost upon dialysis.

The sensitivity of the FSF(s) to enzyme digestion was examined by treating aliquots (10µl) of the pooled fractions with trypsin, chymotrypsin, leucine aminopeptidase and carboxypeptidase Y. The results are shown in Table 5.1.2.2. and the data expressed in terms of fusion index calculated relative to the control level of fusion observed for the untreated pooled fractions. The percentage fusion obtained in the presence of 10µl untreated pooled fractions was arbitrarily assigned a fusion index of 1 and all results were calculated relative to this value.

**Table 5.1.2.1.**
**Biochemical properties of the 'low molecular weight' FSFs**

| Test | Fusion index | % inhibition relative to control |
|---|---|---|
| Boiling | 0.98 | 6.8 |
| Dialysis | 0.48 | 100 |
| TCA | 0.97 | 6.8 |
| Ether | 0.95 | 11 |

**Table 5.1.2.2.**
**Enzymic sensitivities of the 'low molecular weight' FSFs.**

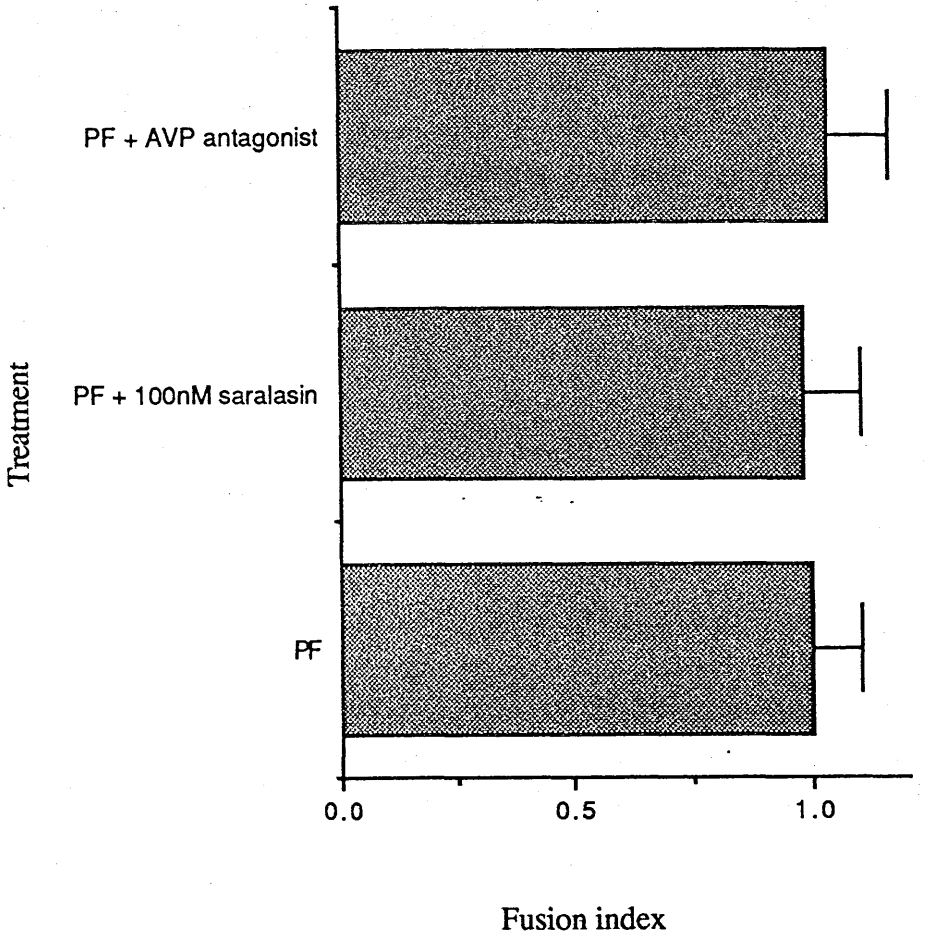
| Enzyme treatment | Fusion index | % inhibition of fusion relative to the control |
|---|---|---|
| Control | 0.96 | 8 |
| Trypsin | 0.5 | 100 |
| Chymotrypsin | 0.99 | 2.7 |
| Control | 0.95 | 1 |
| Leucine aminopeptidase | 0.94 | 12 |
| Control | 0.94 | 12 |
| Carboxy-peptidase Y | 0.94 | 12 |

**Figure 5.1.2.3.**

**The effect of AVP and angiotensin II antagonists on the fusion stimulating ability of the 'low molecular weight ' FSF(s).**

Myoblasts were cultured in '$Ca^{2+}$-free' medium until fusion-competence, washed and then plated into medium containing 1.4mM $Ca^{2+}$ and the pooled 'low molecular weight' FSF(s), designated PF, in the presence and absence of the AVP and angiotensin II receptor antagonists. Fusion was determined 20 hours later.

figure 5.1.2.3.


PF + AVP antagonist
PF + 100nM saralasin
PF
Treatment
0.0
0.5
1.0
Fusion index

The neuropeptides AVP and angiotensin II can stimulate fusion and the factor(s) present in the pooled fractions was thus tested for sensitivity to the AVP receptor antagonist, [1-β–mercapto-β,β cyclopentamethylene propionic acid, 2 (O-methyl) tyrosine) arginine$^8$ vasopressin (10μM) and the angiotensin II receptor antagonist, saralasin (10μM). The results are shown in fig. 5.1.2.3. and are expressed in terms of fusion index. Fusion stimulating ability was not inhibited by these antagonists and is therefore unlikely to be either AVP or angiotensin II.

### 5.1.3. RP-HPLC profile of the low molecular weight fraction.

Low molecular weight fractions were prepared from extracts of brain, eye and spinal cord (50% (w/v) in 50mM $NH_4HCO_3$). These samples were centrifuged for 40 hours at 100,000g, to remove nucleic acids and high molecular weight proteins, the supernatents were collected and concentrated by freeze-drying to remove $NH_4CO_3$. They were then dissolved in 1ml $NH_4CO_3$ and cenfrifuged at 5000g through a 10kDa molecular weight cut-off membrane filter (Amicon). The filtrate obtained was collected, freeze-dried and dissolved into 100ml 0.1% trifluoroacetic acid (TFA) in deionised distilled water (buffer A): 0.1% TFA in propan-2-ol (buffer B), (90:10) and analysed by RP-HPLC using a Waters μ bondapack C18 column (4.6mm x 250mm, 10μm pore size). A linear gradient of buffer A:buffer B from 90:10 to 10:90 over 80 minutes was used to elute the sample. Elution was determined by monitoring the absorbance at 215nm and the elution profiles obtained for neuropeptide standards and the low molecular weight fractions are shown in fig. 5.1.3.1.

**Figure 5.1.3.1.**

**Reverse phase (RP) - HPLC profiles of 'low molecular weight' fractions prepared from brain, spinal cord and eye extracts.**

The samples were analysed on a Waters μ bondapack C18 column equilibrated in 0.1% (v/v) TFA in double distilled $H_2O$ (A): 0.1% (v/v) TFA in propan-2-ol (B) (90:10). Samples were eluted using a linear gradient of A:B from 90:10 to 10:90 over 80 min with a flow rate of 1ml/min. Elution was monitored by continuous measurement of absorbance at 215nm ($A_{215}$). The elution times of neuropeptide standards are shown for comparison

a) Arginine vasopressin

b) Neuromedin C

c) Angiotensin II

d) Bradykinin

e) Substance P

f) Somatostatin

g) Calcitonin Gene Related Peptide.

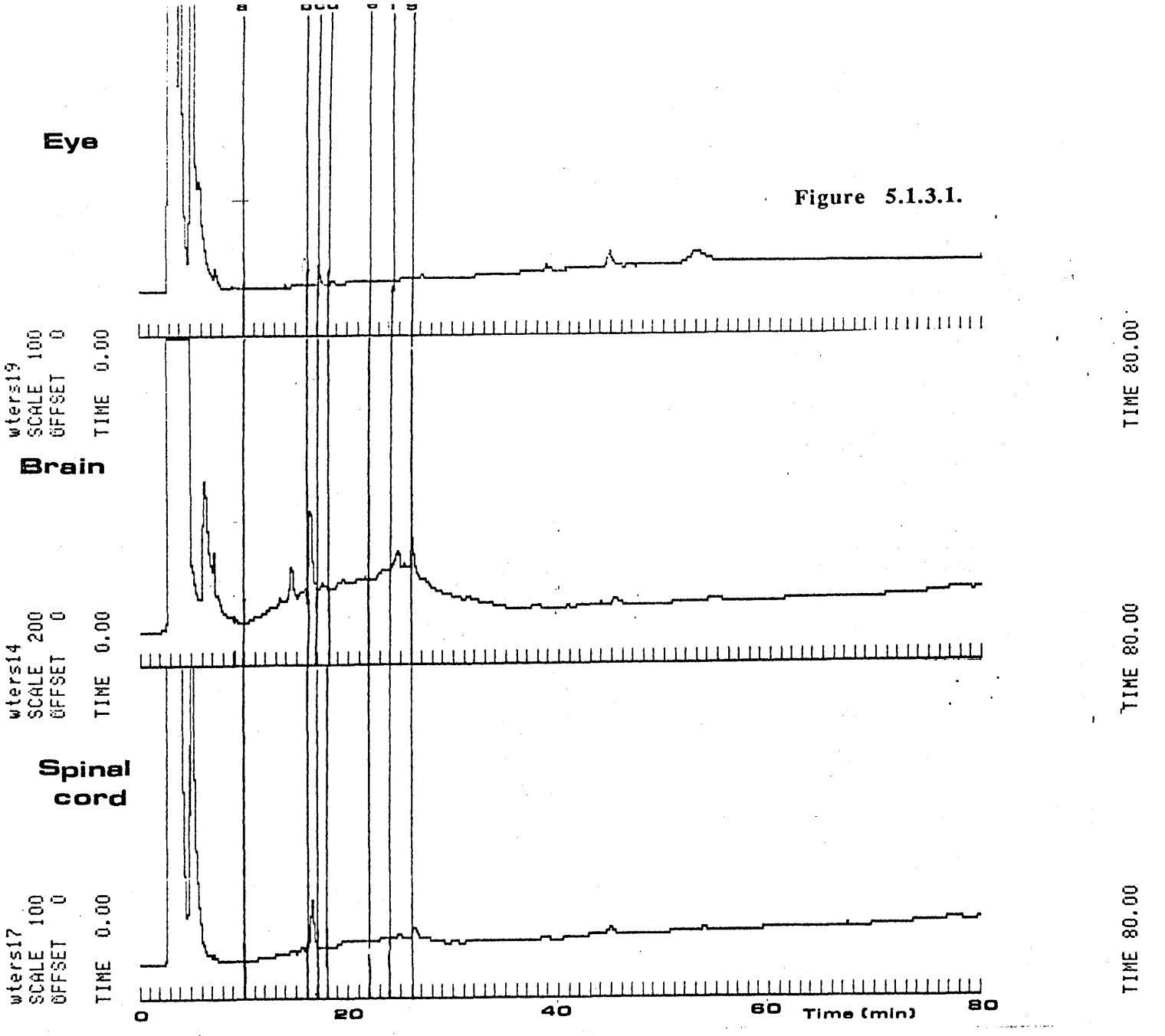

Eye
wters19
SCALE 100
OFFSET 0
TIME 0.00
TIME 80.00
Brain
wters14
SCALE 200
OFFSET 0
TIME 0.00
TIME 80.00
Spinal cord
wters17
SCALE 100
OFFSET 0
TIME 0.00
TIME 80.00
0
20
40
60
80
Time (min)

Figure 5.1.3.1.

### 5.2.1. Fusion assay.

The determination of fusion by counting cell nuclei to assess the development of multinucleated myotubes is time consuming and subjective and therefore not ideal for rapid screening of possible FSFs. An alternative method for assaying fusion was therefore investigated.

Fusion between myoblasts results in the formation of a myotube which, in addition to multinucleate morphology, has an increased cell volume relative to those of its individual myoblast components. The size distribution profile of the myoblast population should therefore change as a result of fusion. This provides an alternative criterion for fusion and is the basis of the fusion assay of Neff and Horwitz (1982) which used a Coulter® electronic particle counter to count myoblast populations on the basis of size. Samples of 'control' (non-fused) and 'fused' myoblasts were prepared and analysed using this method with the modification that the Coulter® counter was interfaced to a Channelyser® multichannel analyser and that monolayer samples of myoblasts were used rather than in suspension. This equipment rapidly and reproducibly counted cell populations to generate histograms of cell number against cell size, thus providing a method to compare the size distributions of 'control' and 'fused' myoblast populations.

Myoblasts were plated in '$Ca^{2+}$-free' medium and cultured as monolayers in 35mm tissue culture dishes for 50 hours until fusion-competent. At this time, cells were either maintained in '$Ca^{2+}$-free' medium ('control' sample) or cultured in the presence of 1.4mM $Ca^{2+}$ and 5% (v/v) embryo extract to generate a sample of 'fused' cells. The cells were analysed 16 hours later using the Coulter® counter. The samples were rinsed with 2 x 1ml PBS to remove non-attached cells and then removed from the dishes by trypsinisation for 10 seconds. Treatment with trypsin also served to disperse cells that were aggregated but not fused (Knudsen and Horwitz, 1977) and provided a collection of mononucleate and multinucleated cells that were suitable for Coulter® counter analysis (Neff and Horwitz, 1982). Trypsin treatment was terminated by the addition of (1mg/ml) soybean trypsin inhibitor. The samples were diluted 1ml in 10ml

into the counting fluid 'Isoton®' (Coulter Inc.) and analysed. The size distribution profiles obtained for the 'control' and 'fused' cell samples are shown in fig. 5.2.1.1. and the data are expressed as cell number against cell diameter. The 'control' and 'fused' samples exhibited different and heterogeneous size distribution profiles.

The Coulter® counter-Channelyser® collected data in a series of electronic 'gates' or channels, each of which corresponds to a particular diameter, d, which ranged between 7.99μm and 15.36μm. The cell diameter measured by each channel (i) may be defined as $d_i$. The total number of cells analysed (R) is the sum of the number of cells in each channel ($R_i$)

$$\text{i.e. } R = \sum_{d=7.99\mu m}^{15.36\mu m} R_i$$

The relative frequency or % probability of possessing diameter d (Pi) is equal to

$$\frac{Ri}{R} \times 100$$

and

$$\sum_{d=7.99\mu m}^{15.36\mu m} Pi = 100$$

The mean cell diameter, $d_{av}$, can be calculated as

$$\sum_{d=7.99\mu m}^{15.36\mu m} Pi\, d_i = d_{av}$$

and

$$\sum_{d=7.99\mu m}^{N} Pi = P_N$$

may also be determined by adding up the Pi values for each successive diameter up to Nμm ($d_N$). When $P_N = 50$, 50% of the cells have a diameter smaller than $d_N$. The 'control' and 'fused' cell populations can thus be compared in this respect.

**Figure 5.2.1.1.**

**Size distribution profiles obtained using the Coulter counter® - Channelyser®**

Representative profiles obtained for samples of 'control (non-fused)' ( ..... ) and 'fused' ( —— ) myoblast populations.

Figure 5.2.1.1.

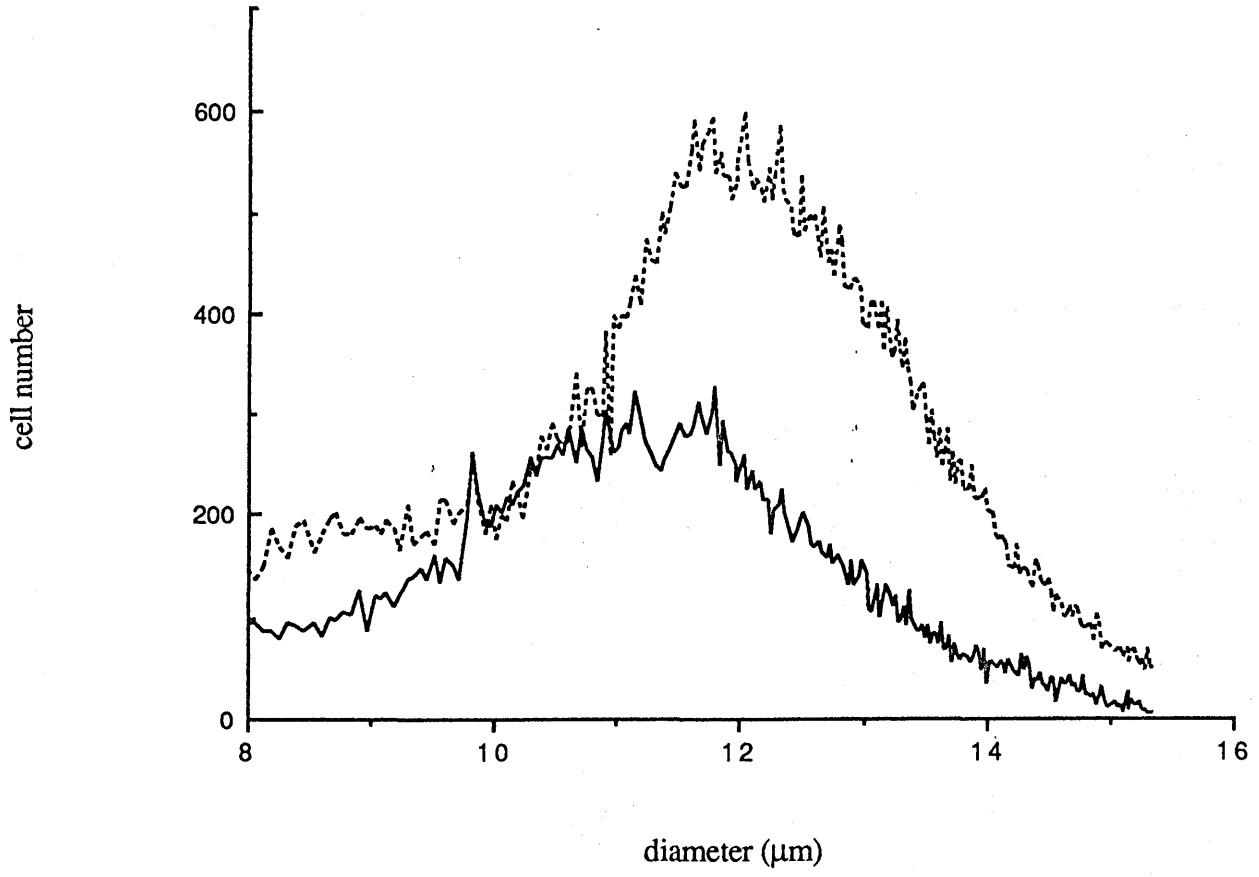


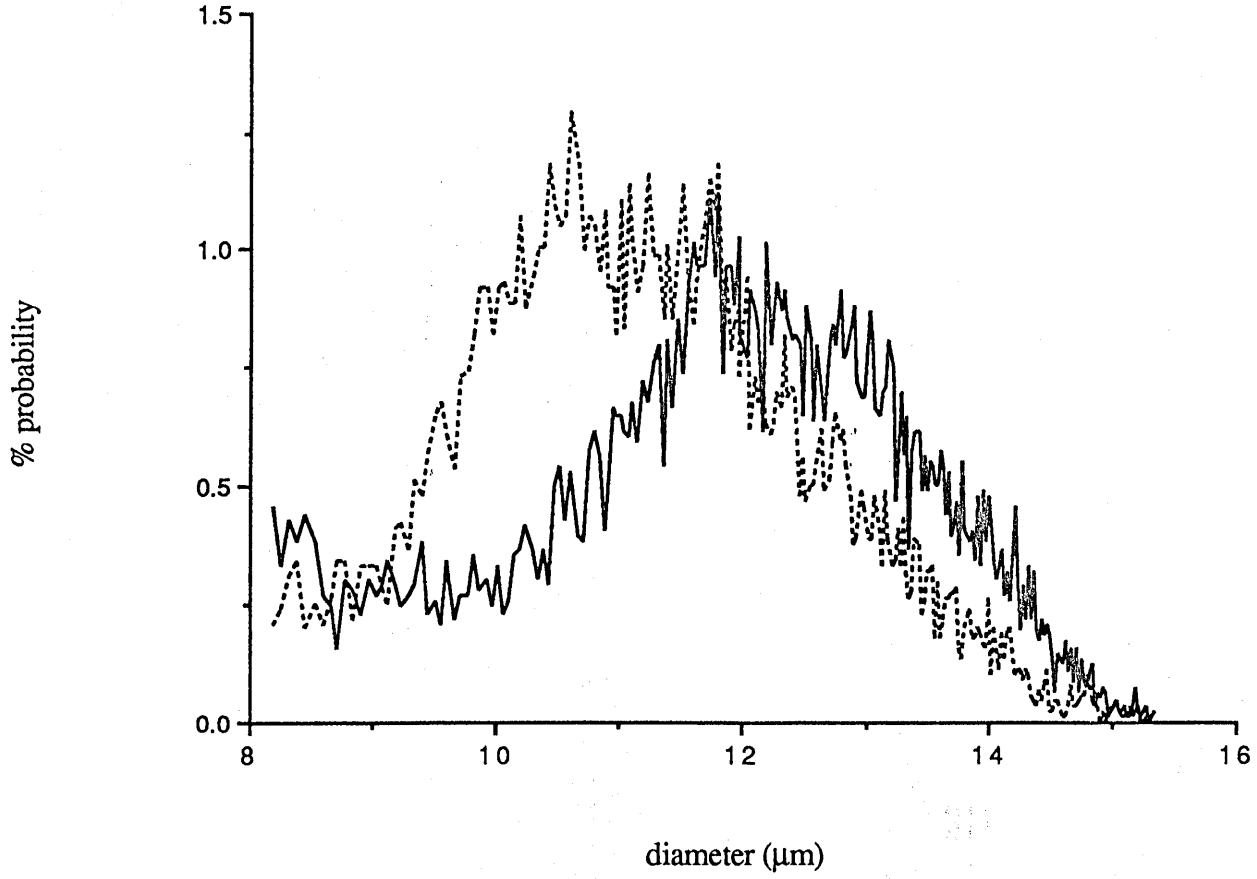
**Figure 5.2.1.2.**

**Profiles of the size distribution of myoblast populations analysed using the Coulter counter® - Channelyser®**

Representative profiles of 'control (non-fused)' ( ..... ) and 'fused' ( —— ) myoblast populations, where % probability =

$$\frac{R_i}{R} = \frac{\text{number of cells in channel i}}{\text{total number of cell analysed}}$$

Figure 5.2.1.2.



Representative plots of % probability against cell diameter obtained for 'control' and 'fused' samples are shown in fig. 5.2.1.2. respectively. It may be seen that there was a change in the size distribution upon myoblast fusion with a net shift of the profile of the 'fused' sample to the right with respect to the 'control' sample. The $d_{av}$ of the 'control' and 'fused' populations were calculated to be 11·64 ± 0·092μm and 12·227 ± 0·056μm respectively. The values of d corresponding to $P_N$ = 50% were 11·64 ± 0·186μm and 12·32 ± 0·26μm for the 'control' and 'fused' samples respectively (means of 3 separate experiments ± S.E.M.). These values are significantly different ($p < 0.01$). The cells in the 'fused' population were therefore increased in size relative to the 'control' population. This method is further discussed, in comparison with that of Neff and Horwitz (1982) in section 5.4.

## Discussion

### 5.3. Investigation of FSF(s).

As discussed in the introduction, chick embryo extract contains FSF(s) which may be neuronal in origin. The differentiation of myoblasts is generally considered to be under neuronal control (Oh and Markelonis, 1980: Bonner and Adams, 1982), a conclusion which is supported by the demonstrations that chorioallantoic grafting of spinal cord tissue stimulates myoblast differentiation *in vivo* (Avery *et al.*, 1956), co-culturing of spinal cord tissue explants and myoblasts accelerates myoblast growth and differentiation (Murray, 1965) and chick embryonic spinal cord extract can replace chick embryo extract in stimulating fusion (Oh, 1975; Wakelam 1986). Furthermore, the neuropeptides AVP and angiotensin II are also able to stimulate fusion. The possible nature of these FSF(s) was investigated by biochemical fractionation of an extract prepared from chick embryonic spinal cord.

This was achieved by gel filtration chromatography using a Sephadex G100 column (90cm x 1.6cm) which fractionates molecules on the basis of their molecular weight with a fractionation range of 4kDa - 150kDa. The ability of the fractions collected to stimulate fusion was assessed by means of a bioassay using myoblasts in

primary culture. There were 4 main peaks of fusion stimulating ability (fig. 5.1.1.1.) corresponding to apparent molecular weights of >150kDa, 37kDa, 19kDa and >4kDa. The neuropeptides, AVP and angiotensin II are capable of stimulating fusion in the absence of chick embryo extract (molecular weights are 1.084kDa and 1.046kDa respectively). Attention was therefore focussed onto the fusion stimulating activity of >4kDa. Fractions containing these 'low molecular weight FSF(s)' were pooled and further analysed.

They were subjected to boiling, dialysis and treatment with TCA and ether. From Table 5.2.1.1., the fusion stimulating activity was stable to both boiling and ether treatment. It was therefore heat stable and not lipid in nature. TCA treatment was without effect indicating that it may be a peptide, since peptides lack the complex secondary structure of polypeptides which would be destroyed by such treatment. Its activity was however dialysable. The molecular weight of the factor(s) was therefore less than 3kDa since this is the size limit of the retention ability of the membrane.

Enzymic treatments performed on the factor(s) in the pooled fractions showed the fusion stimulating ability to be trypsin sensitive but it was unaffected by digestion with chymotrypsin, leucine aminopeptidase and carboxypeptidase Y. Trypsin cleaves peptide bonds on the C terminal side of the amino acids, lysine and arginine. The factor(s) therefore probably contains a lysine or arginine residue and, from its apparent molecular weight, is probably a peptide. Chymotrypsin cleaves peptides and proteins at the C terminal side of tryptophan, tyrosine and phenylalanine, leucine and methionine residues with occasional cleavage at other sites. The activity may have been retained because there was no appropriate cleavage site for this enzyme. Alternatively, cleavage at one or more of these sites may occur, with the fragment(s) produced retaining activity.

Treatment with leucine aminopeptidase did not inhibit the fusion stimulating ability of the factor(s). This enzyme cleaves N terminal amino acids. Peptide bonds involving the secondary amino groups of proline are hydrolysed at a negligible rate, and thus proline effectively blocks the N terminus. It is possible either that the N

terminal amino acid of the factor(s) was adjacent to a proline residue or, alternatively, the N terminus may be blocked by acetylation or cyclisation of N terminal glutamyl residues to pyrrolidonecarboxyl (pyroglutamyl). Another possibility is that the N terminal amino acid may have been cleaved, but was non-essential for fusion.

The fusion stimulating ability of the factor(s) was also insensitive to treatment with carboxypeptidase Y which cleaves the C terminal peptide bond. This could be due to C terminal cleavage of an amino acid residue that is non-essential for its activity. Alternatively, the C terminus may be blocked, for example by amidation thus preventing cleavage by this enzyme.

In previous experiments, chick embryonic brain extract was similarly fractionated by gel filtration using Sepahdex G100 (Caroline Evans, BSc Hons project) and the fractions collected tested for their ability to stimulate myoblast fusion using the bioassay described above. FSFs of apparent molecular weights of at least 150kDa, 10.7kDa and less than 4kDa were found. The fractions containing the low molecular weight (less than 4kDa) factor(s) were pooled and further charaterised and in common with the low molecular weight factor(s) from spinal cord, the activity was dialysable but stable to boiling and treatments with TCA and ether. The factor(s) is probably also peptide in nature since its activity was lost on trypsin digestion. Its fusion stimulating ability was insensitive to treatment with chymotrypsin, leucine aminopeptidase and carboxypeptidase Y. Thus the 'low molecular weight FSFs' from brain and spinal cord share similar properties and enzyme sensitivities.

The evidence of Wakelam and Pette (1984a,b) that the neuropeptides, AVP and angiotensin II can replace embryo extract in stimulating fusion suggests a possible physiological role for these 'low molecular weight' factors. It is however unlikely that they are either of these peptides since their activity was not blocked in the presence of the AVP or angiotensin II antagonists (fig. 5.1.2.3.).

Skeletal muscle is innervated by motor neurones derived from the spinal cord and possibly also receives innervation from sensory sympathetic and parasympathetic ganglia. 'Developmental factor(s)' may be released from these nerves during

embryonic development *in vivo* (Guth *et al.*, 1968; Hoffman *et al.*, 1972; Oh *et al.*, 1972). In this regard, L6 rat muscle cells have been demonstrated to possess AVP receptors of the V1 subtype which are coupled to inositol phospholipid breakdown. In addition, CGRP-I immunoreactivity has been detected in a subpopulation of motoneurones innervating the chick embryonic limb bud and the time course of the development of this immunoreactivity paralleled that of the formation of neuromuscular synapses (New and Mudge, 1986). CGRP is also able to increase synthesis of the nicotinic acetylcholine receptor (Fontaine *et al.*, 1986; New and Mudge, 1986) and is therefore a candidate for a muscle trophic factor that is released from motoneurone terminals *in vivo*.

The RP-HPLC protocol described provides a means to separate the peptide components of the low molecular weight fractions (< 10kDa) prepared from chick embryonic brain, spinal cord and eye extracts. It is based on that of Morris *et al.*,. (1984) with the modification of trifluoroacetic acid (TFA) replacing acetic acid to allow sample elution to be followed by monitoring the absorbance of the carboxyl group of the peptide bond at 215nm, rather than the absorbance of aromatic amino acid residues at 280nm, which may not be present in all peptides. The carboxyl group of acetic acid interferes with absorbance measurement at 215nm, but TFA is suitable since its intrinsic absorbance at this wavelength is much lower due to the presence of the fluorine atoms. The presence of TFA in the eluent ensured complete protonation of the peptides which is required to prevent tailing of their elution profiles (Morris *et al.*, 1984). Peptides thus elute from the column as a function of their solubility in propan-2-ol. Using this method, it was possible to resolve mixtures of μg amounts of neuropeptides including for example, angiotensin II, AVP, CGRP and substance P (fig. 5.3.1.) and it was thus sensitive at the nanomole level of detection. This method is thus suitable for use as a step in a protocol for purification of possible FSF(s). In addition, the eluent was volatile so could be easily removed by freeze drying to generate samples suitable for bioassay. A limiting factor for purification is the amount of

starting material available since chick embryos are comparatively small and thus only yield gram quantities of starting material.

The RP-HPLC profiles of brain spinal cord and eye extracts show that there are several peptide components, one of which co-eluted with CGRP. This may correspond to the CGRP I immunoreactive peptide which was found to exist in a subpopulation of motorneurones innervating the chick embryonic limb bud (New and Mudge, 1986). CGRP is however probably not a FSF *in vivo* since it was unable to increase total [$^3$H] inositol phosphate generation in fusion-competent myoblasts (data not shown) and the CGRP receptor is coupled to the adenylate cyclase signalling system in myotubes (Laufer and Changeux, 1987) and muscle (Takamori and Yoshikawa, 1989). The ability of CGRP to stimualte the fusion of myoblasts in primary culture remains to be determined since myoblasts lose viability when cultured with this or other neuropeptides alone. The original experiments with AVP and angiotensin II were performed in the presence of a non-fusogenic batch of horse serum. However, all batches of horse serum used in this project were found to be fusogenic. Thus, such potential FSFD should be tested in a medium comprising additional 'viability promoting' components. Preliminary experiments in this direction using ovotransferrin were unsuccessful and further experimental work is therefore required to resolve this. Altenatively, a short term fusion assay to determine early fusion events (approximately 30 minutes to 2 hours) could be developed to circumvent this problem.

## 5.4. The assessment of an alternative fusion assay.

The bioassay employed to determine fusion stimulating ability is based on the counting of cell nuclei. This requires staining of the cells followed by visual scoring to determine the number of nuclei present in the fused cells as a percentage of the total number of nuclei in the sample. This method is time consuming and therefore not ideal for rapid screening of FSFs. An alternative method for assaying fusion using a Coulter® particle counter (Neff and Horwitz, 1982) was therefore investigated. This method is based on the fact that myoblasts fuse to form myotubes whose cell volume is

greater than any of their individual myoblast components. A Coulter® particle counter was employed since it is able to determine the number and size of particles in a conductive liquid. This is achieved by monitoring the electrical current between 2 electrodes, on either side of a small aperture, through which the liquid flows. Each particle passing through the aperture is detected as a change in the resistance between the electrodes which generates an electrical impulse proportional to the particle size. Neff and Horwitz (1982) have previously used a Coulter® counter to compare 'non-fused' and 'fused' populations of myoblasts. It was used to measure the number of cells above (and thus exclude those below) a certain threshold or size limit and cell counts were obtained for two such size limits. The lower size limit was chosen to accurately count all the cells in the population whilst the upper value corresponded to the largest difference in cell counts obtained. This upper value was determined empirically and the number of cells counted were calculated as the percentage of the number of cells counted at the lower threshold/size limit i.e. as a percentage of the total cell count. This percentage was found to increase upon fusion since the 'fused' cell population contained cells of larger volume than the 'non-fused' population, resulting in an increased cell count at the upper size limit relative to the total cell count. Unfortunately this method gave variable results. The method was therefore modified by using the Coulter® counter coupled to a Channelyser® to obtain histograms of cell number against cell diameter and thus quantitatively analyse the size distribution profiles of samples of 'control' (unfused) and 'fused' myoblasts. The 'control' and 'fused' populations are denoted in inverted commas since, as discussed in chapter 3, the percentage fusion in the control sample is never zero, due to the presence of preformed myotubes at the time of plating and the percentage fusion in the 'fused' sample is never 100%. This is due to presence of satellite cells in culture which do not fuse during embryonic development, but have the ability to do so in response to muscle injury, resulting in the repair of damaged muscle.

If it is assumed that

(i) the cells in suspension are spherical, the cell volume (V) of each cell is equivalent to

$$\frac{4\pi r^3}{3} = V$$ where r is the radius of the cell.

(ii) fusion between 2 or more myoblasts generates a cell whose volume ($V_f$) is the sum of their volumes prior to fusion. Considering for example, the simplest case of fusion between 2 myoblasts of volumes $V_1$ and $V_2$ and radii $r_1$ and $r_2$ respectively, the volume of the fused cell of radius $r_f$ will be

$$V_f = V_1 + V_2$$

Substituting for V with $\frac{4\pi r^3}{3}$ gives the following equation:

$$\frac{4\pi r_f^3}{3} = \frac{4\pi r_1^3}{3} + \frac{4\pi r_2^3}{3} = \frac{4\pi}{3}(r_1^3 + r_2^3)$$

which can be simplified to

$$r_f^3 = r_1^3 + r_2^3$$

therefore

$$r_f = \sqrt[3]{r_1^3 + r_2^3}$$

In general therefore, for fusion between n cells, where $n \geq 3$

$$r_f = \sqrt[3]{r_1^3 + r_2^3 ..... + r_n^3}$$

The cell diameter d is equal to 2r, thus the diameter of the fused cell ($d_f$) is

$$d_f = (\sqrt[3]{r_1^3 + r_2^3 ..... + r_n^3}) \times 2$$

The 'control' and 'fused' cell populations prepared from primary chick myoblast cultures were heterogenous in cell size and the size distribution profile of the 'fused' population was shifted to the right with respect to that of the 'control' sample (figs. 5.2.1.1. and 5.2.1.2.). The mean diameters, $d_{av}$, of the 'control' and 'fused'

samples, calculated as described in section 5.2.1., were 11·64 ± 0·092μm and 12·227 ± 0·056μm and the values of d corresponding to $P_N$ = 50% were 11·64 ± 0·186μm and 12·32 ± 0·26μm for the 'control' and 'fused' populations respectively. This $d_{av}$ of 11·64 ± 0·092μm for the 'control' sample corresponds to the mean cell diameter, of 11·6μm, determined for mononucleated myoblasts using a micrometer (Neff and Horwitz, 1982). Thus the increase in the values of $d_{av}$ and d for $P_N$ = 50 for the 'fused' sample relative to those of the 'control' probably reflected the fusion of predominantly mononucleated myoblast to form larger multinucleated cells. This was characterised by a net loss of cells with a diameter below $d_{av}$ (11·64μm) and a gain of cells with a diameter greater than 11·64μm to result in a cell population of $d_{av}$ = 12·227μm. Using the above equation to calculate $d_f$, the $d_{av}$ value of the 'fused' sample corresponds, for example, to fusion between 2 myoblasts of diameter 9·7μm or between myoblasts of diameters 10·5μm and 8·75μm. This difference in size is relatively small despite the observation that the size distribution profile of the 'fused' sample was clearly shifted to the right with respect to the non-fused, control sample. This was probably due to the fact that myoblasts in primary culture represent a cell population of heterogenous cell size. In this regard, the Coulter® counter-Channelyser® had the advantage over using a Coulter® counter alone since it allowed a quantitative rather than an empirical determination of the changes in cell size distribution which result from myoblast fusion and as such provides a method to analyse and directly compare samples. The calculations for $d_{av}$ and d corresponding to $P_N$ values of, for example 50%, require the application of a series of mathematical formulae, which are the same for each sample and could thus be computerised to greatly facilitate the rapid analysis and comparison of samples. This method would thus be suitable for screening of possible FSFs especially if it could be used to determine fusion in short term assays (up to 6 hours). Further experiments are required to establish whether the method is sufficiently sensitive to detect early fusion events as well as long term i.e. multiple fusion events. Access to the Coulter® counter - Channelyser® was however only available for a short period of time which limited the number of experiments able

to be performed. Other possible approaches for a short term fusion assay include the the use of fluorescent molecules to monitor the development of membrane and/or cytoplasmic continuity which occurs upon myoblast fusion.

# Chapter 6
# Final Discussion

## Final discussion

The differentiation of chick embryonic skeletal myoblasts results in the formation of myotubes which are the precursors of muscle fibres. Myoblast fusion represents a key stage in this process since it results in the formation of multinucleated myotubes and is associated with synthesis of muscle specific proteins required for mature cell function. The aim of this project has been to examine the biochemical events which regulate this process of terminal differentiation. The following section discusses the results obtained in the context of possible models for both myoblast fusion and its co-ordination with the stimulation of muscle specific protein synthesis that is temporally associated with this event.

## 6.1. Possible models for myoblast fusion and associated muscle specific protein synthesis.

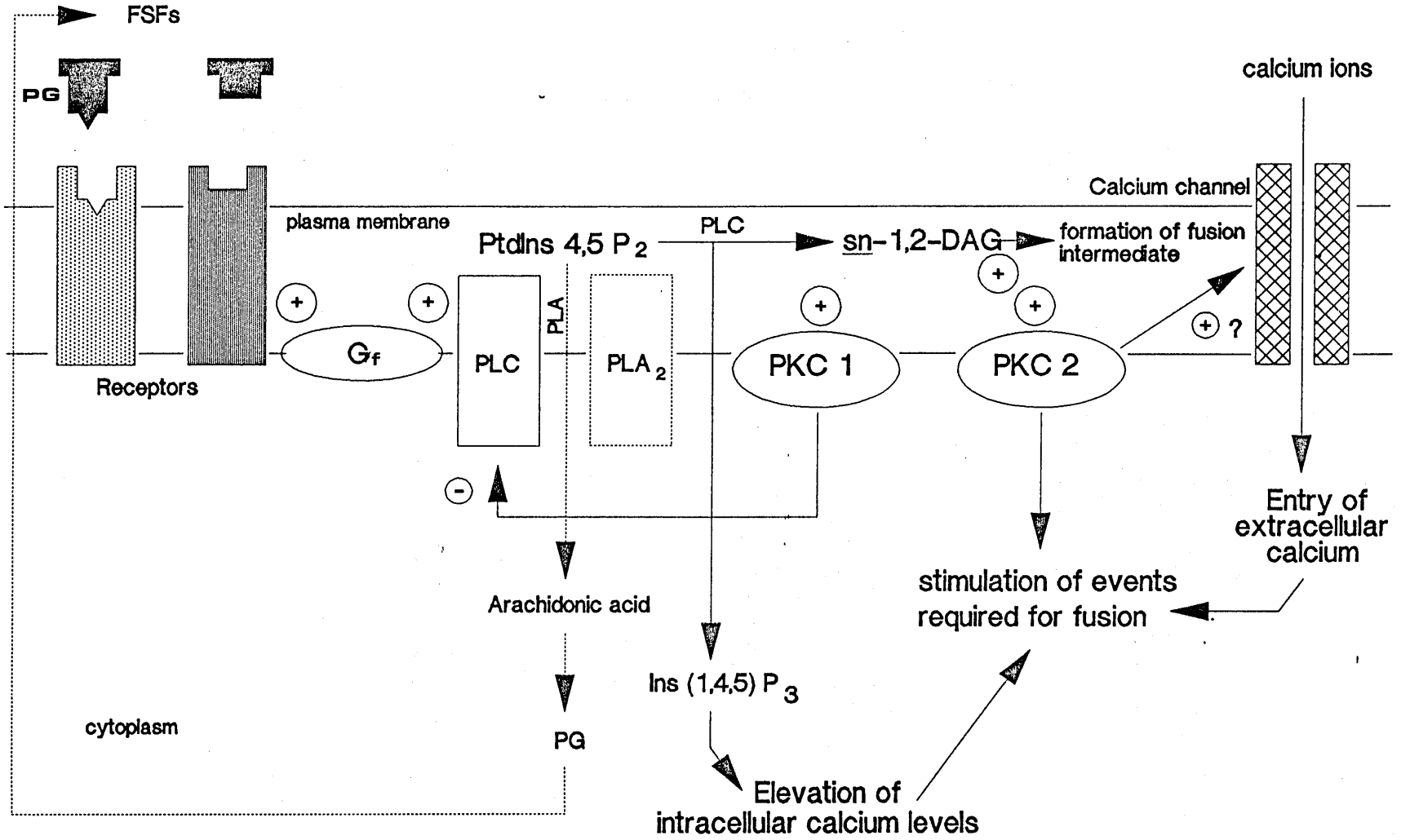
A possible scheme of the biochemical events underlying myoblast fusion is outlined in fig. 6.1.1. The fusion of myoblasts is a receptor mediated process which occurs upon agonist binding of a fusion stimulating factor(s) (FSF(s)) which are probably neuronal in origin. Several factors are capable of stimulating the fusion of myoblasts in primary culture, including ACh, $PGE_1$ (Entwistle *et al.*, 1988) and factors in chick embryo spinal cord extract (see Chapter 5). Entwistle *et al.*, (1988) propose that there is more than one factor to ensure that fusion is achieved *in vivo* since this is a critical stage in muscle fibre development. Fusion stimulation is apparently coupled to PI-PLC hydrolysis of inositol phospholipids (Wakelam and Pette, 1984a,b).which results in the loss of the hydrophilic headgroup and the consequent generation of *sn* - 1,2-DAG in the plasma membrane. This receptor - PI-PLC interaction in fusion-competent myoblasts may, in common with other signal transduction systems (Cockcroft and Gomperts, 1985), be G-protein mediated (section 4.3) by pertussis and cholera toxin insensitive G-protein designated $G_f$.

**Figure 6.1.1.**

**A model for myoblast fusion**

A possible scheme of the biochemical events involved in agonist stimulation of the myoblast fusion following agonist stimulation by FSF(s) (see text in section 6.1.).

Figure 6.1.1.


FSFs
PG
calcium ions
Calcium channel
plasma membrane
PtdIns 4,5 P2
PLC
sn-1,2-DAG
formation of fusion intermediate
PLA
Gf
PLC
PLA2
PKC 1
PKC 2
?
Receptors
Entry of extracellular calcium
Arachidonic acid
stimulation of events required for fusion
Ins (1,4,5) P3
cytoplasm
PG
Elevation of intracellular calcium levels

The concentration of *sn* -1,2-DAG in fusion-competent myoblasts has been demonstrated to increase 2-fold upon fusion stimulation, as determined using an assay specific for this stereoisomer of DAG (fig. 3.1.1.). This molecule can potentially play a dual role in the process of fusion because it exhibits fusogenic properties in model membrane systems and is a physiological activator of the $Ca^{2+}$/phospholipid protein kinase, PKC. DAG is a neutral hydrophobic molecule which tends to destabilise membrane bilayers by causing increased spreading of phospholipid headgroups (Das and Rand, 1986). This potentially leads to the formation of a non-bilayer, inverted hexagonal II ($H_{II}$) structure and subsequently to fusion of myoblasts via an inverted micellar fusion intermediate which allows mixing of membrane lipids at the point of contact (discussed in section 3.7).

The membrane destabilising effect of *sn* -1,2-DAG also results in the activation of PKC by allowing this enzyme to penetrate the plasma membrane. PKC is active when inserted in the membrane since this provides the appropriate lipid environment for activation. The role of PKC in myoblast fusion was investigated using compounds which modulate its activity and the results obtained suggest that its activation is required for fusion (section 3.2., 3.3. and 3.4.). Fusion mix stimulated myoblast fusion and total [$^3$H] inositol phosphate generation show different sensitivities to inhibition with TPA. Long or short term treatment of myoblasts with TPA results in the complete inhibition of fusion to control 'non-fused' levels (fig.3.2.1.1. and 3.4.3. respectively), but only partially inhibits total [$^3$H] inositol phosphate generation in response to fusion mix (fig. 3.5.3.2. and 3.5.3.1. respectively). A possible explanation for this may be that there is a threshold level of PtdIns 4,5 $P_2$ breakdown required for fusion, below which fusion is unable to occur. In this case, TPA could prevent fusion by inhibiting PtdIns 4,5 $P_2$ breakdown so that the required threshold level for fusion is not reached. Alternatively, there may be two or more isoforms of PKC present in fusion-competent myoblasts, one of which (PKC 1) regulates the breakdown of PtdIns 4,5 $P_2$ by negative control whilst another isoform (PKC 2) is involved in the fusion process itself. The role of PKC activation in fusion is unknown

but it may stimulate $Ca^{2+}$ entry by phosphorylation of the voltage dependent $Ca^{2+}$ channel in the plasma membrane. Such a mechansim has been proposed for D600 ~~sensitive~~ sensitive $Ca^{2+}$ channels of myotubes (Navarro, 1987) and Aplysia neurones (De Reimer *et al.*, 1985).

In addition to *sn* -1,2-DAG, inositol phospholipids also provide a potential source of arachidonic acid which is the precursor for prostaglandin (PG) synthesis via the cyclo-oxygenase pathway. The demonstration that $PGE_1$ can stimulate myoblast fusion (Entwistle *et al* , 1988; Farzeneh *et al.*, 1989) raises the possibility that prostaglandin(s) may act as agents to amplify and synchronise fusion *in vitro* (and possibly also *in vivo* ) via an autocrine mechanism resulting from initial agonist stimulation of PI-PLC activity.

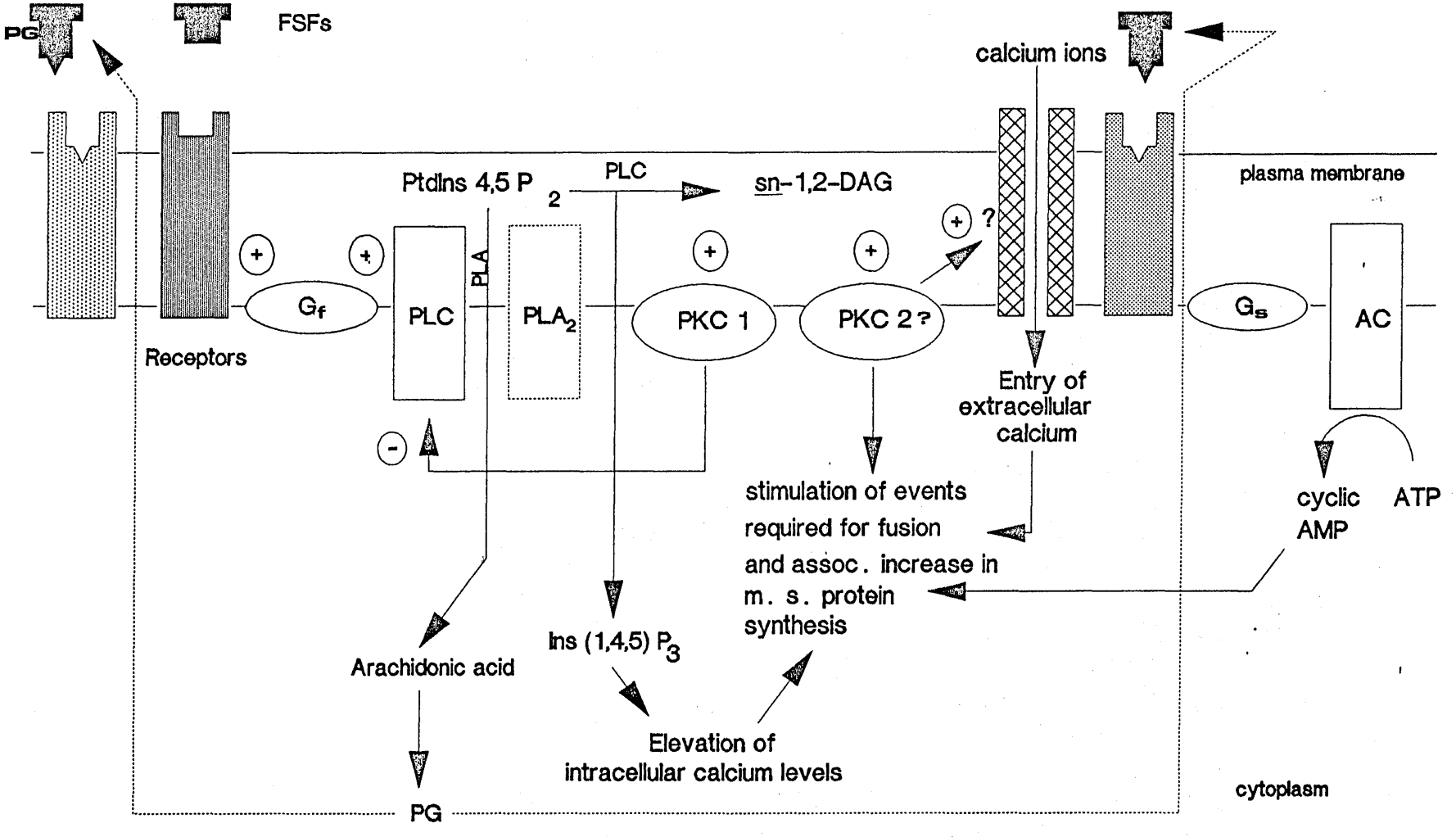
A possible scheme for the biochemical events involved in the apparent coupling of myoblast fusion with the synthesis of some muscle specific proteins is outlined in fig. 6.1.2. The levels of G-proteins that are cholera toxin and pertussis toxin substrates were found to increase following fusion. This increase in the amounts of G-proteins may reflect the enhanced synthesis of neurotransmitter receptors and the consequent responsiveness of myotubes to these receptor agonists. TPA, a direct activator of PKC was able to mimic these increases but they were prevented by pretreatment with the putative PKC inhibitor, polymyxin B. These results suggest that myoblast fusion and synthesis of these proteins may be co-ordinately regulated by the activation of PKC 2 or another isozyme of PKC. This provides a potential mechanism for the coupling of fusion with the synthesis of some muscle specific proteins via PKC activation. Evidence for PKC involvement in the synthesis of other muscle specific proteins comes from the demonstations that treatment of myoblasts with TPA causes an increase in the saturable component of the hexose transport system (Schimmel and Keldie, 1980) and it stimulates the synthesis of plasminogen activator in chick myotubes (Miskin *et al.*, 1978).

**Figure 6.1.2.**

**A model for the co-ordination of myoblast fusion and the stimulation of the synthesis of some muscle specific proteins.**

A possible scheme of the biochemical events involved in the process of FSF stimulated coupling of myoblast fusion with the differentiation linked increase in muscle specific protein synthesis (see text in section 6.1.).

Figure 6.1.2.


PG
FSFs
calcium ions
plasma membrane
Ptdlns 4,5 P 2
PLC
sn-1,2-DAG
?
Receptors
$G_f$
PLC
PLA
$PLA_2$
PKC 1
PKC 2?
$G_s$
AC
Entry of extracellular calcium
stimulation of events required for fusion and assoc. increase in m. s. protein synthesis
cyclic AMP
ATP
Arachidonic acid
Ins (1,4,5) $P_3$
Elevation of intracellular calcium levels
PG
cytoplasm

Treatment of fusion-competent myoblasts with forskolin to activate adenylate cyclase and thus elevate intracellular levels of cyclic AMP also resulted in an increase in the levels of the pertussis toxin substrates (fig. 4.4.2.). Activation of adenylate cyclase thus provides an additional mechanism for stimulating the synthesis of muscle specific proteins and evidence to support this comes from the demonstration that cyclic AMP may also be the second messenger involved in the calcitonin gene-related peptide (CGRP) stimulation of both adenylate cyclase activity (Laufer and Changeux, 1987) and the synthesis of surface nicotinic acetylcholine receptors (AChR) in primary cultures of chick myotubes (Fontaine *et al.*, 1986, New and Mudge, 1986). The rise in cyclic AMP which occurs following the onset of fusion has also been implicated in the stimulation of the synthesis of a muscle specific protein, creatine kinase, since it precedes the appearance of this enzyme (Schutzle *et al.*, 1984). This stimulation of creatine kinase synthesis is sensitive to treatment with the cyclo-oxygenase inhibitor, indomethacin, which suggests that a prostaglandin (PG) can stimulate synthesis of this, and perhaps other muscle specific proteins in addition to its ability to stimulate fusion.

## 6.2. Future work

The exact nature of the role(s) of PKC in fusion and the stimulation of muscle specific protein synthesis remain to be determined. Given that there appears to be more than one isozyme of PKC involved, they may each lead to the phosphorylation of a different set of as yet undefined substrates. One possible target is a $Ca^{2+}$ channel in the plasma membrane. The opening of such a channel would allow the entry of extracellular $Ca^{2+}$ into fusion-competent myoblasts and thus fulfil a pre-requisite for fusion. As discussed in Chapter 3, this could be addressed using a fluorescent $Ca^{2+}$ indicator, such as Quin 2 or Fura 2, to examine the effect of PKC modulators on extracellular $Ca^{2+}$ influx. The different isoforms of PKC and the changes in their relative levels during differentiation could be determined by resolution of the isoforms by hydroxyapatite chromatography.

The role of G-proteins in the event of fusion could be further investigated using an alternative to the non-hydrolysable GTP analog, GTPγS, as a means of direct activation. G-proteins can be directly activated by sodium fluoride (NaF) in the presence of $Al^{3+}$ (Sternweis and Gilman, 1982) due to the $[AlF_4]^-$ ion which interacts with the G protein in the GDP bound form. The combination of GDP and $[AlF_4]^-$ effectively mimicks GTP to promote subunit dissociation and thus activate the G proteins. Its effects on total [$^3$H] inositol phosphate generation and fusion could therefore be determined in order to gain further information about their role in fusion.

G-protein levels were found to increase upon differentiation. The increase in both the pertussis toxin and cholera toxin substrates was mimicked by TPA and inhibited by polymyxin B pretreatment (fig. 4.4.3. and 4.4.4), which suggests that these events are PKC dependent. The increase in the pertussis toxin substrates was also mimicked by forskolin, an agent which elevates intracellular levels of cyclic AMP, but this compound was without effect on the levels of the cholera toxin substrate. In the light of these results, it would be interesting to determine whether the increase in the cholera toxin substrate precedes that of the pertussis toxin substrates since it has been demonstrated TPA treatment increases in adenylate cyclase activity to raise the intracellular levels of cyclic AMP (Sulakhe *et al.*, 1985). If this is the case, then up-regulation of the cholera toxin substrate, $G_s$, by activation of PKC could account for the elevation of intracellular cyclic AMP levels by increasing receptor-adenylate cyclase coupling. This could, in turn, provide a mechanism for subsequently increasing the levels of the pertussis toxin substrates. The effect of TPA on adenylate cyclase activity should thus be further investigated to determine whether (i) it is sensitive to treatment with inhibitors of protein synthesis such cycloheximide and (ii) its time course is consistent with increased synthesis of $G_s$.

## 6.3. Physiological relevance

The study of myoblast differentiation may be of physiological importance to the repair of muscle following injury, a process which bears a striking resemblance to the differentiation of embryonic myoblasts. Regenerating muscle cells arise from satellite cells, an effectively dormant population of myoblasts which become reactivated in response to muscle injury and reassume the characteristics of embryonic myoblasts. Satellite cells possess similar rates of proliferation and fusion *in vitro* to embryonic myoblasts and can fusewith embryonic myoblasts to form mosaic myotubes (Jones, 1982. Thus a knowledge of the biochemical mechanisms underlying the regulation of myoblast differentiation could be of clinical use since it provides potential target sites for possible drugs to promote muscle repair.

# Appendices

## Appendix 1

## Composition of buffer and SDS-PAGE solutions

### 1.1 Buffer compositions

**Earles' Salts (x20)**

2.33M sodium chloride

107mM potassium chloride

33.2mM magnesium sulphate

23.33mM sodium dihydrogen phosphate

10mM glucose

The pH of the buffer was adjusted to between 7.2 - 7.4 with 1M sodium hydroxide.

**Hank's Buffered Saline**

1.26mM calcium chloride

0.5mM magnesium chloride

0.4mM magnesium sulphate

5.37mM potassium chloride

137mM sodium chloride

4.2mM sodium hydrogen carbonate

0.35mM sodium dihydrogen phosphate

The pH of the buffer was between pH 7.2 - 7.4 when freshly made.

**Phosphate Buffered Saline (PBS)**

146mM sodium chloride

5.36mM potassium chloride

9.6mM disodium hydrogen phosphate

1.46mM potassium dihydrogen phosphate

The pH of the buffer was adjusted to pH 7.2 - 7.4 with 0.1M hydrochloric acid.

**Phosphate buffer pH 7.0**

30.5% v/v 0.2M disodium hydrogen phosphate

19.5% v/v 0.2M sodium dihydrogen phosphate

50% v/v distilled water

## 1.2. SDS- PAGE solutions.

**Buffer 1**

1.5M Tris

0.4% w/v SDS

pH 8.8 with HCl

**Buffer 2**

0.5M Tris

0.4% w/v SDS

pH 6.8 with HCl

**SDS-PAGE slab gels 16cm x 18cm**

**(i) Resolving gel (10% acrylamide/ 0.27% bisacrylamide)**

6ml buffer 1

8.2ml distilled water

8ml 30% w/v acrylamide/ 0.8% w/v bisacrylamide

1.6ml 50% v/v glycerol

8µl N,N,N'N'-tetramethylenediamine (TEMED)

90µl 10% w/v ammonium persulphate.

**(ii) Stacking gel (3% w/v acrylamide/ 0.08% w/v bisacrylamide)**

9.75ml distilled water

3.75ml buffer 2

1.5ml 30% w/v acrylamide/ 0.8% w/v bisacrylamide

8µl TEMED

150µl 10% w/v ammonium persulphate

**SDS-PAGE slab gels 20cm x 20 cm**

**(i) Resolving gel (12.5% acrylamide/0.06% w/v bisacrylamide)**

11.6ml distilled water

12ml buffer1

20ml 30% w/v acrylamide/0.15% bisacrylamide

4ml 50% v/v glycerol

60µl 10% w/v ammonium persulphate

4µl TEMED

**(ii) Stacking gel (3% acrylamide/ 0.08 bisacrylamide)**

14.6ml distilled water

5.6ml buffer 2

2.3ml 30% w/v acrylamide/ 0.8% w/v bis acrylamide

12µl TEMED

225µl 10% w/v ammonium persulphate

**Laemmli buffer.**

5M urea

0.17M SDS

0.38M dithiothreitol

0.5M Tris/HCl

0.05% w/v bromophenol blue

## Appendix 2

### Sources of chemicals

The following is a list of sources for the chemicals and cell culture materials used. The various drugs culture materials and biochemical compounds used in addition to standard laboratory reagents are listed below together with their sources. All solutions were prepared using glass distilled water unless otherwise stated.

Amersham International PLC, Amersham, Buckinghamshire, England.

[$\gamma$–$^{32}$P] Adenosine triphosphate (specific activity =3Ci/ mmol)

myo [$^{3}$H] Inositol (specific activity = 22.8 Ci/ mmol)

[20 (n)$^{3}$H] Phorbol 12,13 dibutyrate (specific activity =10-20Ci/ mmol)

[methyl-$^{3}$H] Thymidine (specific activity = 1 Ci/ mmol)

Avanti Polar Lipids, Pelham, Alabama 35124 USA.

Cardiolipin

BDH Chemical Company, Poole, England.

Acrylamide, ammonium formate, bisacrylamide, dimethylsulphoxide, EDTA (Ethylenediaminetetra-acetic acid), lithium chloride, orthophosphoric acid, potassium hydrogen phosphate, potassium dihydrogen phosphate, perchloric acid, sodium hydroxide, sodium carbonate, trichloroacetic acid and universal indicator.

Boehringer (UK) Ltd., Lewes, England.

Adenosine 5' triphosphate (ATP), adenosine 5'($\beta$,$\gamma$–imino) triphosphate (App(NH)p), guanosine 5' triphosphate (GTP), guanosine 5'-0-[3-thiotriphosphate] (GTP$\gamma$S) Tris.

Calbiochem.

Forskolin.

Cambridge Research Biochemicals, Cambridge, England.

All peptides.

Formachem (Research International) PLC., Strathaven, Scotland.

Boric acid, D-glucose and sodium hydrogen carbonate.

FSA Laboratory Supplies, Loughborough, England.

HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid) Optiphase 'HiSafe' 3 and Propanol-2-ol (HPLC grade).

Flow Laboratories, Rickmansworth, England.

0.2μm sterile filter units and chick embryo extract.

Gibco, Paisley, Scotland.

MADE BY SPECIAL REQUEST

'$Ca^{2+}$ free' Dulbeccos Modified Eagle's Medium and glutamine (100x).

Koch-Light Ltd, Suffolk, England.

Calcium chloride, magnesium sulphate, potassium chloride and sodium potassium tartrate.

Lipidex, Westfield, New Jersey 07090, USA.

*sn* -1,2-DAG kinase from *Escherichia coli*.

May and Baker, Dagenham, England.

Ammonium persulphate, copper (II) sulphate, formic acid, hydrochloric acid and sodium tetraborate.

Molecular Probes Inc, Oregon 97448 USA.

5-N-(octadecanoyl) aminofluorescein.

National Diagnostics, Aylesbury, England.

Ecoscint.

NEN Research Products, Stevenage England.

[adenylate-$^{32}$P] Nicotinamide adenine dinucleotide (NAD)

di(triethylammonium) salt (specific activity 1000Ci/mmol)

Pierce and Warriner, London, England.

Micropor (Spectrapor 3) dialysis tubing.

Porton Products, Porton Down, England.

Pertussis toxin.

Riedel-DettaenAg Seelze-Hannover, Germany.

Magnesium chloride and sodium dihydrogen carbonate.

Ross Poultry, Aberdeenshire, Scotland.

Fertilised pathogen free chicken eggs.

Schleicher and Schuell, D-3354 Dassel, West Germany.

Nitrocellulose

Scottish Antibody Production Unit, Scotland.

Donkey anti-rabbit IgG coupled to horse radish peroxidase.

Whatman Limited, Maidstone, England.

0.45μm millipore filters.

All other chemicals were obtained from the Sigma Chemical Company, Poole, England.

# References

Adamo, S., Caporale, C., Nervi C., Ceci, R. and Molinaro, M. (1989) J. Cell Biol. **108** 153-158.
Adams, R.L.P. (1969) Exp. Cell Res. **56** 49-54.
Ahkong, Q.F., Fisher, D., Tampion, W. and Lucy, J.A. (1973) Biochem. J. **136** 147-155.
Akhtar, R.A. and Abdel-Latif, A.A. (1980) Biochem. J. **192** 783-791.
Allan, D., Low, M.G., Finean, J.B. and Michell, R.H. (1975) Biochim. Biophys. Acta **508** 277-286.
Antin, P.B., Forry-Schaudies, S., Friedman, T.M., Tapscott, S.J. and Holtzer, H. (1981) J. Cell Biol. **90** 300-308.
Ase, K., Berry, N., Kikkawa, U., Kishimoto, A. and Nishizuka, Y. (1988) FEBS Lett. **236** 396-400.
Aui, E.J., Holt, P.G. and Simons, P.J. (1973) Exp. Cell Res. **83** 436-438.
Avery, G., Chow, M. and Holtzer, H. (1956) J. Exp. Zool. **132** 409-423.
Bartlett, G.R. (1959) J. Biol. Chem. **234** 466-468.
Barton, C.H., Dickson, G., Gower, H.I., Rowett, L.H., Putt, W., Elsom, V., Moore, S.E., Goridis, C. and Walsh, F.S. (1988) Development **104** 165-173.
Bar-Sagi, D. and Prives, J. (1983) J. Cell Biol. **97** 1375-1380
Berridge, M.J. (1984) Biochem. J. **220** 345-360.
Berridge, M.J. and Irvine, R.F. (1984) Nature (Lond.) **312** 315-318.
Betz, H. and Changeux, J-P. (1979) Nature (Lond.) **278** 749-752.
Bischoff, R. (1978) Cell Surface Rev. **5** 127-179.
Bishop, W.R., Ganong, B.R. and Bell, R.M. (1986) J. Biol. Chem. **261** 6993-7000.
Bligh, E.A. and Dyer, W.J. (1959) Can. J. Biochem Physiol. **37** 911-937
Blosser and Appel (1980) J. Biol. Chem. **255** 1235-1238.
Bokoch, G.M., Katada, T., Northup, J.K., Ui, M. and Gilman, A.G. (1984) J. Biol. Chem. **259** 3560-3567.
Bonner, P.H. and Adams, T.R. (1982) Dev. Biol. **90** 175-184.
Boni, L.T. and Rando, R.R. (1985) J. Biol. Chem. **260** 10819-10825.
Bonincontro, A., Cametti, C., Hausman, R.E., Indovina, P.L. and Santini, M.T. (1987) Biochim. Biophys. Acta **903** 89-95.
Brandt, D.R. and Ross, E.M. (1986) J. Biol. Chem. **261** 1656-1664.
Brown, K.D., Blakeley, D.M., Hamon, M.H., Laurie, S.M. and Corps, A.N. (1987) Biochem. J. **245** 631-639.
Brumfeld, V. and Lester, D.S. (1990) Arch. Biochem. Biophys. **277** 318-323.
Buckingham, M.E. (1977) in Biochemistry of Cell Differentiation II (Paul, J.Ed) **15** 269-332, University Park Press, Baltimore, U.S.A.
Cassel, D. and Selinger, Z.V.I. (1976) Biochim. Biophys. Acta **452** 538-551.

Cassel, D. and Selinger, Z.V.I. (1977) PNAS (USA) **75** 4155-1459.
Castagna, M., Rochette, C., Rosenfeld, C. and Mishai, Z. (1979) FEBS Lett. **100** 62-66.
Castagna, M., Takai, Y., Kaibuchi, K., Sono, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. **257** 7847-7851.
Cates, G.A., Brickenden, A.M. and Sanwal, B.D. (1984) J. Biol. Chem. **259** 2646-2650.
Cerione, R.A., Codina, J., Kilpatrick, B.F., Staniszewski, C., Gierschik, P., Somers, R.L., Spiegel, A.M., Birnbaumer, L, Caron, M.G. and Lefkowitz, R.J. (1985) Biochemistry **24** 4499-4503.
Chen, L.B., Murray, A., Segal, R.A., Bushnell, A. and Walsh, M.L. (1978) Cell **14** 377-391.
Cheung, W.Y. (1980) Science **207** 19-27.
Chiquet, M., Eppenberger, H.M. amd Turner, D.C. (1981) Dev. Biol. **88** 220 235.
Cockcroft, S. and Gomperts, B.D. (1985) Nature (Lond.) **314** 534-536.
Cohen, R., Pacifici, M., Rubinstein, N., Biehl, S. and Holtzer, H. (1977) Nature **266** 538-540.
Collins, M.K.L. and Rozengurt. E. (1982) Biochem. Biophys. Res. Commun. **104** 1159-1166.
Correze, C., d'Alayer, J., Coussen, F., Berthiller, G., Deterre, P. and Monneron, A. (1987) J. Biol. Chem. **262** 15182-15187.
Corvera, S., Schwarz, K.R., Graham, R.M. and Garcia-Sainz J.A. (1986) J. Biol. Chem. **261** 520-526
Cossu, G., Adamo, S., Senni, M.I., Caporale, C., and Molinaro, M. (1986) Biochem. Biophys. Res. Commun. **137** 752-758.
Couch, C.B. and Strittmatter, W.J. (1984) J. Biol. Chem. **259** 5396-5399.
Cullis, P.R. and de Kruijff, B. (1979) Nature (Lond.) **271** 672-674.
Cullis, P.R., de Kruijff, B., Hope, M.J., Verkleij, A.J., Nayar, R., Farren, S.B., Tilcock, C., Madden, T.D. and Bally, M.B. (1983) Membrane fluidity in Biology **1** 39-81 Academic Press, New York.
David, J.D. and Higgenbotham C.A. (1981) Dev. Biol. **82** 308-316.
David, J.D., See, W.M. and Higgenbotham, C.A. (1981) Dev. Biol. **82** 297-307.
David, J.D., Faser, C.R. and Perrot, G.P. (1990) Dev. Biol. **139** 89-99.
Das, S. and Rand, R.P. (1984) Biochem. Biophys. Res. Commun. **124** 491-496.
Das, S. and Rand, R.P. (1986) Biochem. **24** 7092-7095.
De Reimer, S.A., Strong, J.A., Albert, K.A., Greengard, P and Kazmarek, L.K. (1985) Nature (Lond) **313** 313-316.
Den, H., Malnizak, D.A., Keating, H.J. and Rosenberg, A. (1975) J. Cell Biol. **67** 826-834.

Devlin, R.B. and Emerson, C.P. Jr (1978) Cell **13** 599-611.
Dickson, G., Gower, H.I., Barton, C.H., Prentice, H.M., Elsom, V.L., Moore, S.E., Cox, R.D., Quinn, C., Putt, W. and Walsh, F.S. (1988) Cell **50** 1119-1130.
Dlugosz, A.A., Tapscott, S.J. and Holtzer, H. (1983) Can. Res. **43** 2780-2789.
Downes, C.P. and Michell, R.H. (1985) Mol. Aspects Cell. Regul. **4** 3-56.
Edelman, G.M. (1983) Science **219** 450-457.
Ehrismann, R., Chiquet, M. and Turner, D.C. (1981) J. Biol. Chem. **256** 4056-4062.
Ellens, H., Bentz, J. and Szoka, F.C. (1986) Biochemistry **25** 4141-4147
Ellens, H., Siegel, D.P., Alford, D., Yeagle, P.L., Boni, L., Lis, L.J., Quinn, P.J. and Bentz, J. (1989) Biochemistry **28** 3692-3703.
Entwistle, A., Curtis, D.H. and Zalin, R.J. (1986) J. Cell Biol. **103** 857-866.
Entwistle,A., Zalin, R.J., Warner, A.E. and Bevan, S. (1988) J. Cell Biol. **106** 1693-1702.
Epand, R.M. (1985) Biochemistry **24** 7092-7095.
Epand, R.M. and Lester, D.S. (1990) Trends in Pharm. Sci. **11** 317-320.
Evans, T., Fawsi, A., Fraser, E.D., Brown, M.L. and Northup, J.K. (1987) J. Biol. Chem. **262** 176-181.
Farago, A. and Nishizuka, Y. (1990) Febs. Lett. **268** 350-354.
Farzeneh, F., Entwistle, A. and Zalin, R.J. (1989) Exp. Cell Res. **181** 298-304.
Fontaine, B., Klarsfeld, A., Hokfelt, T. and Changeux, J-P. (1986) Neurosci. Lett. **71** 59-65.
Foster, J.W. and Kinney, D.M. (1984) CRC Crit. Rev. Microbiol. **11** 263-298.
Fulton, A.R., Prives, J., Farmer, S.J. and Penman, S. (1981) J. Cell Biol. **91** 103-112.
Fung, B. K-K. (1983) J. Biol. Chem. **258** 10495-10503.
Ganong, B.R., Loomis, C.R., Hannun, Y.A. and Bell R.M. (1986) Proc. Natl. Acad. Sci. (U.S.A.) **87** 1184-1188.
Gao, B., Gilman, A.G. and Robishaw, J.D. (1987) Proc. Natl. Acad. Sci. (U.S.A.) **84** 6122-6125.
Gardner, S.D., Milligan, G., Rice, J.E. and Wakelam, M.J.O. (1989) Biochem. J. **259** 679-684.
Gat-Yablonski, G. and Sagi-Eisenberg, R. (1990) Biochem. J. **270** 679-684.
Geny, B., Stutchfield, J. and Cockcroft, S. (1989) Cell Signal. **1** 165-172.
Gibralter, D. and Turner, D.C. (1985) Dev Biol **112** 292-307.
Gierschik, P., Codina, J., Simons, C., Birnbaumer, L. and Spiegel, A. (1985) Proc. Natl. Acad. Sci. (U.S.A.) **82** 727-731.
Gilfix, B.M. and Sanwal, B.D. (1980) Biocem. Biophys. Res. Commun. **96** 1184-1191.
Gilman, A.G. (1987) Ann. Rev. Biochem. **56** 615-649.

Goldsmith, P., Gierschik, P., Milligan, G., Unson, C.G., Vinitsky, R., Malech, H. and Spiegel, A.G. (1987) J. Biol. Chem. **262** 14683-14688.
Gordon, J.F. (1989) PhD. thesis, Glasgow University.
Grumet, M., Rutishauser, U. and Edelman, G.M. (1982) Nature (Lond.) **295** 693-695.
Gruner, S. (1985) Proc. Natl. Acad. Sci. (U.S.A.) **82** 3665-3669.
Guillon, G., Gallopayes, N. Balestre, M.N. and Lombard, C. (1988) Biochem. J. **253** 765-775.
Guth, L. and Samaha, J. (1968) Physiol. Rev. **48** 645-687.
Haga, K., Haga, T., Ichiyama, A., Katada, T., Kurose, H. and Ui, M. (1985) Nature (Lond.) **316** 731-733.
Hannun, Y.A., Loomis, C.R. and Bell, R.M. (1986) J. Biol. Chem. **261** 7184-7190.
Hausman, R.E. and Velleman, S.G. (1981) Biochem. Biophys. Res. Commun. **103** 213-218.
Hausman, R.E., Dobi, E.T., Woodford, S., Petridis, S., Ernst, M. and Nichols, E.B. (1986) Dev. Biol. **113** 40-48.
Hawkins, P.T., Stephens, L.R. and Downes, C.P. (1986) Biochem. J.**238** 507-516.
He, H-T., Barbet, J., Chaix, J-C., and Goridis, C. (1986) EMBO J. **5** 2489-2494.
Hemperley, J.J., Edelman, G.M. and Cunningham, B.A. (1986) Proc. Natl. Acad. Sci. (U.S.A.) **83** 9822-9826.
Herman, B.A. and Fernandez, S.M. (1978) J.Cell Physiol. **94** 255-264.
Herman, B.A. and Fernandez, S.M. (1982) Biochemistry **21** 3275-3283.
Hildebrandt, J.D., Codina, J., Risinger, R. and Birnbaumer, L. (1984) J. Biol. Chem. **259** 2039-2042.
Holtzer, H., Abbot, J. and Lash, J. (1958) Anat. Rev. **131** 567.
Huang, F.L., Yoshida, Y., Cunha-Melo, J.R., Beaven, M.A. and Huang, K.P. (1989) J. Biol. Chem. **264** 4238-4243.
Haung, S.J., Monk, P.N., Downes, C.P. and Whetton, A.D. (1988) Biochem. J. **249** 839-845.
Hoffman, D.J. and Ramm, G.M. (1972) J. Exp. Zool. **182** 227.
Holtzer, H. and Bischoff, R. (1970) in 'The physiology and biochemistry of muscle as food' 29-31, University of Wisconsin Press, Madison.
Holtzer, H., Croop, J., Dienstman, S., Ishikawa, H. and Somlyo, A.P. (1975) PNAS (USA) **72** 513-517.
Hurley, J.B., Simon, M.I., Teplow, D.B., Robishaw, J.D. and Gilman, A.G. (1984) Science **226** 860-862.
Irvine, R.F. and Moor, R.M. (1986) Biochem. J.**240** 917-920.
Jones, P.H. (1982) Exp. Cell Res. **139** 401-404.
Kalderon, N. and Gilula, N.B. (1979) J. Cell Biol. 81 411-425.

Katada, T., Bokoch, G.M., Northup, J.K., Ui, M. and Gilman, A.G. (1984) J. Biol. Chem. **259** 3568-3577.
Katsuragi, T. and Furakawa, T. (1985) Trends in Pharmacol. Sci **6** 337-339.
Kaufman, S.J. and Foster, R.F. (1984) Dev. Biol. **110** 1-14.
Kaur, H. and Sanwal, B.D. (1981) Can. J. Biochem. **59** 743-747.
Kelvin, D.J., Simard, G., Tai, H.H., Yamaguchi, T.P. and Connolly, J.A. (1989) J. Cell Biol. **108** 159-167.
Kishimoto, A., Mikawa, K., Hashimoto, K., Yasuda, I., Tanaka, S-U., Tominaga, M., Kurada, T. and Nishizuka, Y. (1989) J. Biol. Chem. **264** 4088-4092.
Knight, D.E. and Scrutton, M.C. (1986) Biochem. J.**234** 497 506.
Knopf, J.L., Lee, M.H., Sultzman, L.A., Kriz, R.W., Loomis C.R., Hewick, R.M. and Bell, R.M. (1986) Cell **46** 491-503.
Knudsen, K.A. and Horwitz, A.F. (1977) Dev. Biol. **58** 328-338.
Knudsen, K.A. and Horwitz, A.F. (1978) Dev. Biol. **66** 294-307.
Knudsen, K.A. (1985) J. Cell Biol. **101** 891-895.
Knudsen, K.A., Smith, L. and McElwee, S. (1989) J. Cell Biol. **109** 1779-1786.
Konigsberg, I.R. (1963) Science **140** 1273-1284.
Konigsberg, I.R. (1971) Dev. Biol. **26** 133-152.
Kuo, J.F., Raynor, R.L., Mazzei, G.J., Schatzmann, R.C., Turner, R.S. and Kem, W.R. (1983) FEBS Lett. **153** 183-186.
Laemmli, U.K. (1970) Nature (Lond.) **227** 680-685.
Lass, Y., and Fischbach, G.D. (1976) Nature (Lond.) **263** 250-251.
Laufer, R. and Changeux, J-P. (1977) EMBO J. **6** 901-906.
Lee, K.S. and Tsien, R.W. (1983) Nature (Lond.) **302** 790-794.
Leeb-Lundberg, L.M.F., Cotecchi, S., Comasney, J.W., Lefkowitz, R.J. and Caron, M.G. (1985) PNAS (USA) **82** 5651-5655.
Lester, D.S., Doll, L., Brumfeld, V. and Miller, I.R. (1990) Biochim. Biophys. Acta **1039** 33-41.
Levin, R.M. and Weiss, B. (1978) Biochim. Biophys. Acta **540** 197-204.
Lipton, B.H. and Konigsberg, I.R. (1972) J. Cell Biol. **53** 348-364.
Litosch, I., Calista, C., Wallis, C. and Fain, J.N. (1986) J. Biol. Chem. **261** 638-643.
Lo, W.W.Y. and Hughes, J. (1987) FEBS Lett.**220** 327-331.
Lodhi, S., Weiner, N.D., Shacht, J. (1979) Biochim. Biophys. Acta **557** 1-8.
Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. **193** 265-275.
Lucy, J.A. (1970) Nature **227** 814-817.
Lucy, J.A. (1974) in 'Biomembranes, lipids proteins and receptors' (eds. Burton, R.M. and Packers, L.) 95-116 (BI Science Publishing Divison).

Macdougall, S.L., Grinstein, S. and Gelfand, E.W. (1988) Cell **54** 229-234.
Maggio, B., Cumar, F.A. and Caputto, R. (1978) FEBS Lett. **90** 149-152.
May, W.S., Sayhoun, N., Wolf, M. and Cautrecasus, P. (1985) Nature (Lond.) **317** 549-551.
McCaffrey, P.G. and Rosner, M.R. (1987) Cancer Res. **47** 1081-1086.
Means, A.R., Lagace, L., Guerrier, V., Chafoule, J.G. (1982) J.Cell Biol. 20 317-330.
Milligan, G. (1988) Biochem. J. **255** 1-13.
Miskin, R., Easton, T.G. and Reich, E. (1978) Cell **15** 1301-1312.
Mitchell, F.M., Griffiths, S.L., Saggerson, E.D., Houslay, M.D., Knowler, J.T. and Milligan, G. (1989) Biochem. J. **262** 403-408.
Moore, S.E., Thompson, J., Kirkness, V., Dickson, J.G. and Walsh, F.S. (1987) J. Cell Biol. **105** 1377-1386.
Moriyama, Y. and Murayama, K. (1977) Cell Struct. Funct. **2** 339-345.
Morris, H.R., Etienne, A.T. and Dell, A. and Alberquerque, R. (1980) J. Neurochem. **34** 574-582.
Moss, M., Norris, J.S., Peck, E.J. and Schwartz, R.J. Exp. Cell Res. **113** 445-450.
Mullaney, I., Magee, A.I., Unson, C.G. and Milligan, G. (1988) Biochem. J. **256** 649-656.
Murray, M.R. (1965) in 'Cells and tissue culture' **2** 311-372 (Academic Press, New York).
Nakadate, T., Jeng, A.Y. and Blumberg, P.M. (1988) Biochem. Pharmacol. **37** 1541-1545.
Nakornchai, S.N., Falconer, A.R., Fisher, D., Goodall, A.H., Hallinan, T. and Lucy, J.A. (1981) Biochim. Biophys. Acta **643** 152-160.
Nameroff, M., Trotter, J.A., Keller, J.M. and Munar, E. (1973) J. Cell Biol. **58** 107-118.
Navarro, J. (1987) J. Biol. Chem. **262** 4649-4651.
Neff, N.T. and Horwitz, A.F. (1982) Exp. Cell Res. **153** 25-31.
Neff, N.T., Decker, C. and Horwitz, A.F. (1984) Exp. Cell Res. **142** 479-483.
New, H.V. and Mudge, A.W. (1986) Nature (Lond.) **323** 809-811.
Nishizuka, Y. (1984) Nature (Lond.) **308** 693-698.
Nishizuka, Y. (1986) Science **233** 305-309.
Nishizuka, Y. (1988) Nature (Lond.) **334** 661-665.
Nishizuka, Y. (1986) Cancer **63** 1892-1903.
Northup, J.K., Sternweis, P.C. and Gilman, A.G. (1983) J. Biol. Chem. **258** 11361-11368.
Oh, T.H. and Johnson, D.D. (1972) Exp. Neurol. **37** 360-362.
Oh, T.H. (1975) Exp. Neurol. **46** 432-438.
Oh, T.H. and Markelonis, G.J. (1980) PNAS (USA) **77** 6922-6925.

Ohta, H., Okajima, F. and Ui, M. (1985) J.Biol. Chem. **260** 5771-5780.
Olden, K., Lay, J., Hunter, V.A., Romain, R. and Parent, J.B. (1981) J. Cell Biol. **88** 199-204.
Parfett, C.L.J., Jamieson, J.C. and Wright, J.A. (1981) Exp. Cell Res. **136** 1-13.
Parent, J.B., Tallman, J.F., Henneberry, R.C. and Fishman, P.H. (1980) J. Biol. Chem. **255** 7782-7786.
Paris, S. amd Pouyssegur, J. (1986) EMBO J. **5** 55-60.
Parker, P.J., Coussens, l., Totty, N., Rhee, L., Young, J., Chen, E., Stabel, S., Waterfield, M.D. and Ullrich, A. (1986) Science **233** 853-859.
Parsegian, V.A., Fuller, N.L. and Rand, R.P. (1979) Proc. Natl. Acad. Sci. (U.S.A.) **76** 2750-2754.
Parsegian, V.A., Rand, R. and Gingell, D. (1984) in Cell fusion. CIBA Found. Synposium. **103** 9-27.
Paterson, B. and Strohman, R.C. (1972) Dev. Biol. **29** 113-138.
Pauw, P.G. and David, J.D. (1979) Dev. Biol. **70** 27-38.
Plevin, R., Palmer, S., Gardner, S.D. and Wakelam, M.J.O. (1990) Biochem. J.**268** 605-610.
Podleski, T.R., Greenberg, I., Schlessinger, J. amd Yamada, K.M. (1979) Exp. Cell Res. **122** 317-326.
Poste, G. and Allison, A.C. (1973) Biochim. Biophys. Acta **300** 421-466.
Preiss, J.E., Loomis, C.R., Bishop, W.R., Stein, R., Neidel, J.E. and Bell, R.M. (1986) J. Biol. Chem. **261** 8597-8600.
Prives, J. and Shinitsky, M. (1977) Nature (Lond.) **268** 761-763.
Puri, E.C., Chiquet, M. and Turner, D.C. (1979) Biochem. Biophys. Res. Commun. **90** 883-889.
Puri, E.C., Caravatti, M., Perriard, J.C., Turner, D.C. and Eppenberger, H.M. (1980) Proc. Natl. Acad. Sci. (U.S.A.) **77** 5297-5301.
Rapuano, M., Ross, A.F. and Prives, J. (1990) Dev. Biol. **134** 271-278.
Rash, J.E. and Fambrough, D. (1972) Dev. Biol. **30** 166-186.
Reiger, F., Grumet, M. and Edelman, G.M. (1985) J. Cell Biol. **101** 285-293.
Reinhart, P.H., Taylor, W.M. and Bygrave, F.L. (1984) Biochem. J.**223** 1-13.
Rittenhouse, S.E. and Sassan, J.P. (1985) J. Biol. Chem. **260** 8657-8600.
Rodriguez-Pena, A. and Rozengurt, E. (1984) Biochem. Biophys. Res. Commun. **120** 1053-1059.
Roof, D.J., Applebury, M.L. and Sternweis, P.C. (1985) J. Biol. Chem. **260** 16242-16249.
Ruegg, U.T. and Burgess, G.M. (1989) Trends in Pharm. Sci. **10** 218-220.
Rutishauser, U., Grumet, M. and Edelman, G.M. (1983) J. Cell Biol. **97** 145-152

Santini, M.T., Indovina, P.L. and Hausman, R.E. (1987) Biochim. Biophys. Acta **896** 19-25.
Santini, M.T., Indovina, P.L. and Hausman, R.E. (1988) Biochim. Biophys. Acta **938** 489-492.
Schimmel, S.D. and Keldie, B. (1980) Fed. Proc. **37** 1817.
Schudt, C., van der Bosch, J. and Pette, D. (1973) FEBS Lett. **32** 296-298.
Schudt, C., Gaertner, U., Dolken, G. and Pette, D. (1975) Eur. J. Biochem. **60** 579-586.
Schudt, C. and Pette, D. (1976) Cytobiology **13** 74-84.
Schutzle, U.B., Wakelam, M.J.O. and Pette, D. (1984) Biochim. Biophys. Acta **805** 204-210.
Session, A. and Horwitz, A.F. (1981) Biochim. Biophys. Acta **728** 103-111.
Shainberg, A., Yagil, G. and Yaffe, D. (1969) Exp. Cell Res. **58** 163-167.
Shainberg, A., Yagil, G. and Yaffe, D. (1971) Dev. Biol. **25** 1-29.
Shears, S.B. (1989) Biochem. J.**260** 313-324
Siegel, D.P. (1982) Biophys. J. **45** 399-420.
Siegel, D.P. (1986) Biophys. J. **49** 1155-1170.
Siegel, D.P., Bansbach, J., Alford, D., Ellens, H., Lis, L.J., Quinn, P.J., Yeagle, P.L. and Bentz, J. (1989) Biochemistry 28 3703-3709.
Smith, C.D., Uhing, R.J. and Snyderman, R. (1987) J. Biol Chem. **262** 6121-6127.
Stabel, S., Rodriguez, A., Young, S., Rozengurt, E. and Parker, P. (1987) J. Cell Phys. **130** 111-117.
Sternweis, P.C. and Gilman, A.G. (1982) Proc. Natl. Acad. Sci. (U.S.A.) **79** 4888-4891.
Sternweis, P.C. and Robishaw, J.D. (1984) J. Biol. Chem. **259** 13806-13813.
Sternweis, P.C. (1986) J. Biol. Chem. **261** 631-637.
Sulakhe, P.R., Johnson, D.D., Phan, N.T. and Wilcox, R. (1985) FEBS Lett **186** 281-285.
Sundler, R. and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta **649** 743-750.
Takamori, M. and Yoshikawa, H. (1989) J. Neur. Sci **90** 99-109.
Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. **135** 397-402.
Toutant, M., Barhanin, J., Bockaert, J. and Rouot, B. (1988) Biochem. J. **254** 405-409.
Van der Bosch, J., Schudt, C. and Pette, D. (1972) Biochem. Biophys. Res. Commun. **48** 326-332.
Van Dop, C., Tsubokawa, M., Bourne, H., and Ramachandran, J. (1984) J. Biol. Chem. **259** 696-698.
Verkleij, A.J., Levnissen-Bijvelt, B., de Kruijff, B., Hope, M. and Cullis, P.R. (1984) CIBA Found. Symp. **103** 45-59.

Vogt, W. (1978) Adv, Prostaglandin Thromboxane Res. **3** 89-95.
Wahrmann, J.P., Winand, R. and Luzatti, D. (1973) Nature New Biol. **245** 112-113.
Wakelam, M.J.O. and Pette, D. (1982) Biochem. J.**202** 723-729.
Wakelam M.J.O. (1983) Biochem. J.**214** 77-82.
Wakelam, M.J.O. and Pette, D. (1984a) CIBA Found. Symp. **103** 100-118.
Wakelam, M.J.O. and Pette, D. (1984b) Exp. Biol. Med. **9** 68-71.
Wakelam, M.J.O. (1985) Biochem. J.**228** 1-12.
Wakelam, M.J.O. (1986) Biochem. Soc. Trans. **14** 253-256.
Wakelam, M.J.O., Patterson, S. and Hanley, M.R. (1987) FEBS Lett. **210** 181-184.
Watson, S.P. and Lapetina, E.G. (1985) PNAS (USA) **82** 2623-2626.
Watson, S.P., McNally, J., Shipman, L.J. and Godfrey, P.P. (1988) Biochem. J. **249** 345-350.
Weidekamm, E., Schudt, C. and Brdiczka (1976) Biochim. Biophys. Acta **443** 169-180.
Weinstein, I.B., Lee, L.S., Fisher, P.B., Mufson, A. and Yamasaki, H. (1979) J. Supramol. Struct. **12** 195-208.
West, R.E.J., Moss, J., Vaughn, M., Liu, T., Liu, T-Y. (1985) J. Biol. Chem. **260** 14428-14430.
Whatley, R., Ng, S. K-C, Rogers, J., McMurray, W.C. and Sanwal, B.D. (1976) Biochem. Biophys. Res. Commun. **70** 180-185.
Wilschut, J., Holsappe, M., Jansen, R. (1982) Biochim. Biophys. Acta **690** 297-301.
Wise, B.C., Glass, D.B., Chou, C-H.J., Raynor, R.L., Katoh, N., Schatzmann, R.C., Turner, R.S., Kibler, R.F. and Kuo, J.F. J. Biol. Chem. **257** 8489-8495.
Yaffe, D. (1968) Proc. Natl. Acad. Sci. (U.S.A.) **61** 477-483.
Yaffe, D. (1969) Curr. Top. Dev. Biol. **4** 37-77.
Yaffe, D. (1971) Exp. Cell Res. **66** 33-48.
Yaffe, D. and Saxel, O. (1977) Differentiation **7** 159-166.
Yamanishi, J., Takai, Y., Kaibuchi, K., Sano, K., Castagna, M. and Nishizuka, Y. (1983) Biochem. Biophys. Res. Commun. **112** 778-786.
Young, S., Parker, P.J., Ullrich, A. and Stabel, S. (1987) Biochem. J. **244** 775-779.
Zalin R.J. and Montague W. (1974) Cell **2** 103-108.
Zalin, R.J. and Leaver, R. (1975) FEBS Lett. **53** 33-36.