TRANSDUCER FUNCTION OF G PROTEIN IN RECEPTOR MEDIATED EC COUPLING IN THE GASTROCNEMIUS MUSCLE OF THE FROG
Rana hexadactyla

Dissertation submitted to the Jawaharlal Nehru University
in partial fulfilment of the requirements
for the award of the Degree of
MASTER OF PHILOSOPHY

JITENDER

SCHOOL OF LIFE SCIENCES
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI - 110067
INDIA

1989

जवाहरलाल नेहरु विश्वविद्यालय
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI-110067

SCHOOL OF LIFE SCIENCES

CERTIFICATE

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. The work is original and has not been submitted in part or full for any other degree or diploma of any University.

JITENDER
Candidate

PROF. M. HABIBULLA
Dean

PROF. M. HABIBULLA
Supervisor

JULY 1989

Gram : JAYENU Tel. : 667676, 667557 Telex : 031-4967 JNU IN

ACKNOWLEDGMENTS

I express my deep sense of gratitude to Professor M. Habibulla and Dr C. Kumar for their inspiring guidance, encouragement, valuable suggestions and thoughtful discussion throughout this investigation.

I am also grateful once again to Professor M. Habibulla, Dean, School of Life Sciences, for providing me necessary facilities. I am thankful to Dr. R.K. Kale for extending their laboratory facilities to me.

I sincerely acknowledge the co-operation and help received from Dr. B.K. Shamkumar, Miss Anita Jagota, Mr. P. Karunakar, Mr. A.P. Singh, Mr. Rajeev Varshney, Mr. P.K. Garg, Mr. Ranjan Chaturvedi, Miss Sandhya, Mr. C.S. Reddy and Mr. Sunil Sudhakar. I am also thankful to Mr Mange Ram and Mr Prem Singh for their technical assistance.

Thanks are also due to Mr. A. Ahmad, Mr. Khan, Mr Alexander for their help in CIF, Mr. R.N. Saini for his photography work and Mr. G. Hegde for Wordprocessing of this dissertation.

Financial assistance provided by the University Grants Commission in the form of Junior Research Fellowship is also gratefully acknowledged.

NEW DELHI JITENDER

ABBREVIATIONS

A.A.	:	Arachidonic Acid
Ach	:	Acetylcholine
ADP	:	Adenosine diphosphate
ATP	:	Adenosine triphosphate
BF_3-MeOH	:	Boron trifluoride-methanol complex
BHT	:	Butyl-hydroxy-toluene
^oC	:	Degree celcius
$CaCl_2$	:	Calcium chloride
Ca^{2+}	:	Calcium Ions
cm	:	Centimeter
C.T.	:	Cholera Toxin
DAG, DG	:	Diacylglycerol
ER	:	Endoplasmic reticulum
Gi	:	Inhibitory G protein
GLC	:	Gas-liquid-chromatography
GNM-SN	:	Gastrocnemius muscle-Sciatic nerve
G protein	:	Guanosine nucleotide binding protein
Gs	:	Stimulatory G protein
GTP	:	Guanosine triphosphate
HCl	:	Hydrochloric Acid
H_2O	:	Water
hrs	:	Hours
IP_3	:	Inositol triphosphate

Irr.	:	Irreversible
KCl	:	Potassium chloride
M	:	Molar
$MgCl_2$	:	Magnesium chloride
min	:	Minute
mM	:	Millimolar
mS	:	Millisecond
µM	:	Micromolar
µS	:	Microsecond
PDE	:	Phosphodiesterase
PIP	:	Phosphatidylinositol monophosphate
PIP_2	:	Phosphatidylinositol biphosphate
PM	:	Plasma membrane
PPS	:	Pulses per second
S.D.	:	Standard Deviation
SR	:	Sarcoplasmic reticulum
TLC	:	Thin-layer-chromatography
v/v	:	volume/volume
$\tilde{\tilde{}}$	:	nearly equal

CONTENTS

INTRODUCTION

I. MUSCLE-SKELETAL MUSCLE

Muscle is composed of contractile cells that actively develop tension and shortens. As a result, muscle is important in the movement of body as a whole and movements of its parts. Body has three types of muscles - cardiac muscles, smooth muscles and skeletal muscles. Cardiac type forms wall of the heart and is specialized for that. It is an involuntary muscle and cells are of striated type. Smooth muscle lacks striations, is found in walls of hollow organs and tubes such as stomach, intestine and blood vessels. As cardiac muscles, smooth muscle is also of involuntary type.

As the name implies, most skeletal muscles attach to the bones of skeleton. Activities like walking and manipulating objects in the environment are performed by them. Different from other two types, skeletal muscle are of voluntary type. Gross dissection of skeletal muscle shows that it is composed of many muscle cells called muscle fibres. Muscle fibers are held together by thin sheets of fibrous connective tissue called **fascia**. The fascia that invests entire muscle is called **epimysium**. Muscle fibers are separated into bundles called **fasciculi**. Skeletal muscles are anchored to the skeleton by **tendons**.

Microscopically muscle fibers have regular subcellular structure. Skeletal muscle fibers are multinucleate cells, frequently many centimeters long. Each fiber contains several hundreds to thousands regularly arranged, thread-like **myofibrils**, that extends lengthwise throughout the cell. Myofibrils exhibit alternating light and dark bands, responsible for **striated** appearance of skeletal muscles. Light bands are named **isotropic** or **I bands.** Crossing the light bands is a dense, fibrous **Z line**, which divide the myofibrils into a series of repeating units called **sarcomeres.** In the center of sarcomere is a somewhat less dense region, the **H zone.** A thin dark **M line** crosses the center of H zone.

A sarcomere contains two distinct types of longitudinally oriented myofilaments, thick filaments and thin filaments. Thick filaments occupy A band and, H zone contains only thick filaments. The M line is formed by linkages between thick filaments that hold them in parallel arrangement. Their filaments occupy of I band and part of the A band. Their filaments attach to the Z line (Fig. 1A).

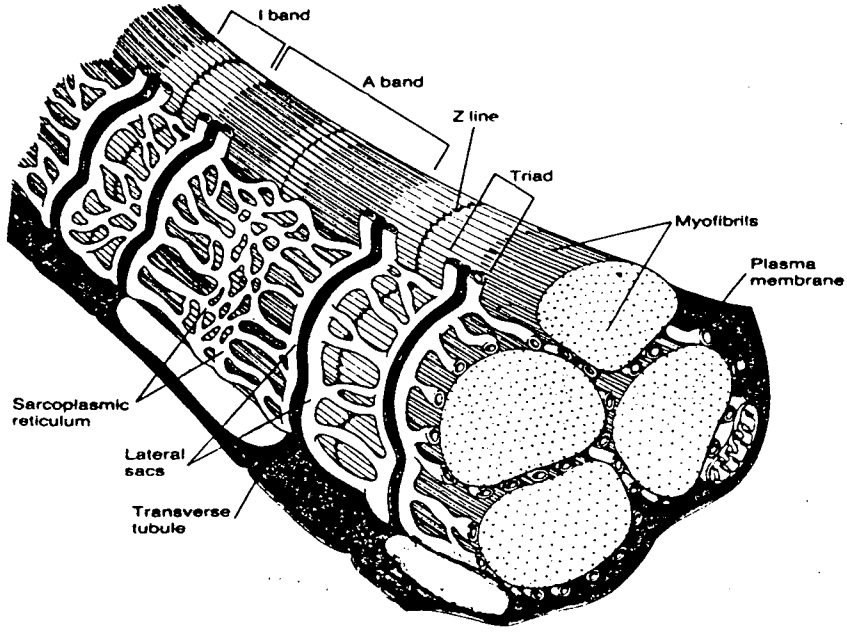
The thick filaments consist mainly of a protein **myosin** (Szent-Gyorgi, 1951). A myosin molecule is made up of 2 subunits, each shaped something like a golf-club and tightly wound around each other so that each molecule has two

bulbous, protruding heads. A thick filament contains approximately 200 myosin molecules arranged in such a way that shafts of the molecules are bundled together with heads of the molecules called, cross bridges, facing outwards (Warrick and Spendich, 1987).

Their filaments consist mainly of the proteins, **actin, tropomyosin,** and **troponin** (Szent-Gyorgy, 1951). The actin portion of thin filaments consist of spherical subunits of globular (G) actin that are organized into a double chain of fibrous (F) actin. Associated with each chain of G-actin subunits are thread like molecules of tropomyosin, which lie end to end along the surface of actin chains. Attached to each tropomysin molecule is a small molecule of troponin (Milligou and Flicker, 1987).

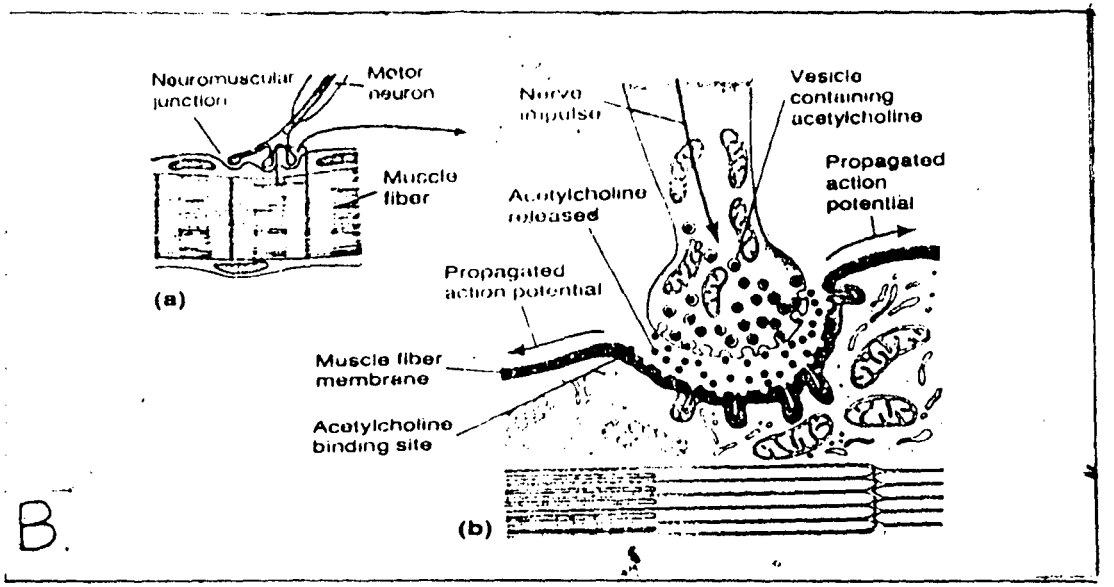
Transverse tubules (t-tubules) pass deep into a skeletal muscle fiber from plasma membrane (PM). In addition, a membranous network, the sarcoplasmic reticulum (SR) extends throughout the fibers and surrounds the myofibrils (Fig. 1A).

Neurons forms specialized junctions neuromuscular junctions, with skeletal muscle fibers (Fig.1B). Motor neurons transmit never impulse (Hill 1951; Aidley, 1971) towards neuromuscular junctions (Homma et al., 1986),



From Spence and Mason.

Fig 1 A.



(from Alexander S and Mason EB).

Fig. 1B

depolarization occurs and acetylcholine (Ach) is released from presynaptic neurons which diffuse to muscle fiber plasma membrane and bind to its receptors. This causes a stimulatory action potential which travels along the muscle cells (Huxley 1974; Squire 1981; Iwasha **et al.**, 1982; Augustine, 1987). The series of events by which a propagated action potential in PM causes interaction between thick and thin filaments is said excitation-contraction coupling (EC coupling). During EC coupling phenomenon, a time lag of 100 ms occurs between the arrival of nerve impulse at synaptic junction and muscle contraction. ACh is immediately hydrolyzed by an enzyme acetycholine esterase (Thesleff **et al.**, 1974; Betz **et al.**, 1984; Held **et al.**, 1987).

Huxley and Niedergerke (1954) and Huxley and Hanson (1954) on the basis of X-ray, light microscope and electron microscope studies proposed sliding-filament model for muscle contraction. The essential features of which are:

1. The length of thick and thin filaments does not change during muscle contraction.

2. Instead, the length of sarcomeres decreases during contraction because the two types of filaments overlap more. The thick and thin filaments slide past each other in contraction.

3. The force of contraction is generated by a process that actively moves one type of filament past the neighbouring filaments of the other types.

Sequence of events involved in the EC coupling of skeletal muscle fibers are:

1. Nerve impulse, following a stimulus, arrives at a neuromuscular junction, acetylcholine (agonist) is released from motor neurons and binds to the receptors on the muscle fiber PM (Homma **et al.**, 1986) [Fig. 1B].

2. A propagated action potential travels across the plasma membrane of muscle fiber and along two tubules into the interior of the cell.

3. The propagated action potential trigers release of Ca^{2+} from lateral sacs of SR (Huxley and Taylor, 1955 and 1958).

4. Ca^{2+} binds to the troponin (Ebashi, 1960).

5. Tropomyosin moves away from its blocking position, permitting actin and myosin to interact.

6. High-energy myosin binds with actin subunits of the thin filaments (Huxley, 1957; Gollinick **et al.**, 1985; Pette, 1985).

7. Energy stored in high-energy myosin is discharged and myosin head swivel, pulling on the thin filaments (Huxley, 1957; Huxley, 1974; Obinata **et al.**, 1981; Cooke 1986; Small, 1988).

8. ATP binds with myosin head, which is released from actin subunits.

9. ATP splits into ADP and Pi, again producing high energy myosins, and step 6 and 7 are repeated as long as Ca^{2+} is bound to troponin (Barany 1967; Eisenberg and Hill, 1980; Nosek **et al.**, 1987).

10. When Ca^{2+} recycles again to SR through an ATP-driven Ca^{2+} pump, tropomyosin moves back into its blocking position (Ebashi, 1976).

11. Contraction ceases and muscle fiber relaxes.

II. SECOND MESSENGERS

The events which mobilize Ca^{2+} from SR and its regulation is still obscure. There is no sound evidence for mechanism of Ca^{2+} mobilization in skeletal muscles during EC Coupling.

Inositol triphosphate (IP_3) and diacylglycerol (DAG) are attracting enormous interest as second messengers in

regulating various physiological processes like secretion, metabolism, contraction in smooth muscles and various short-term and long-term processes at the cellular level (Berridge and Irvine, 1984; Nishizuka, 1986). It forms a bifurcating signalling system. The primary function of IP_3 is mobilization of Ca^{2+} and forms IP_3/Ca^{2+} pathway and DAG activates protein kinase C, an enzyme, to form DAG/C kinase pathway (Fig. 2).

There are essentially 3 components involved in the transmembrane signalling. 1. Receptor for agonist; 2. a GTP binding protein, G protein; 3. effector, an enzyme.

When agonist binds to the receptor an enzyme phosphodiesterase (PDE), also called phosphoinositidase, is activated through G protein (Gilman 1984). Phosphatidyl-inosital pool (generally PIP_2) in the membrane acts as substrate and IP_3 and DAG are produced as a result of its hydrolysis (Berridge and Irvine 1984; Nishizuka, 1984; Nishizuka, 1986). Two phosphoinositide pools, one agonist sensitive and another insensitive to agonist seem to be confined to PM (Rana **et. al.**, 1986).

Increases in levels of IP_3 by PIP_2 hydrolysis has been measured in many different cell types in response to many agonists like neurotransmitters, releasing factors, hormones, growth factors, fertilization and even light (Berridge, 1984;

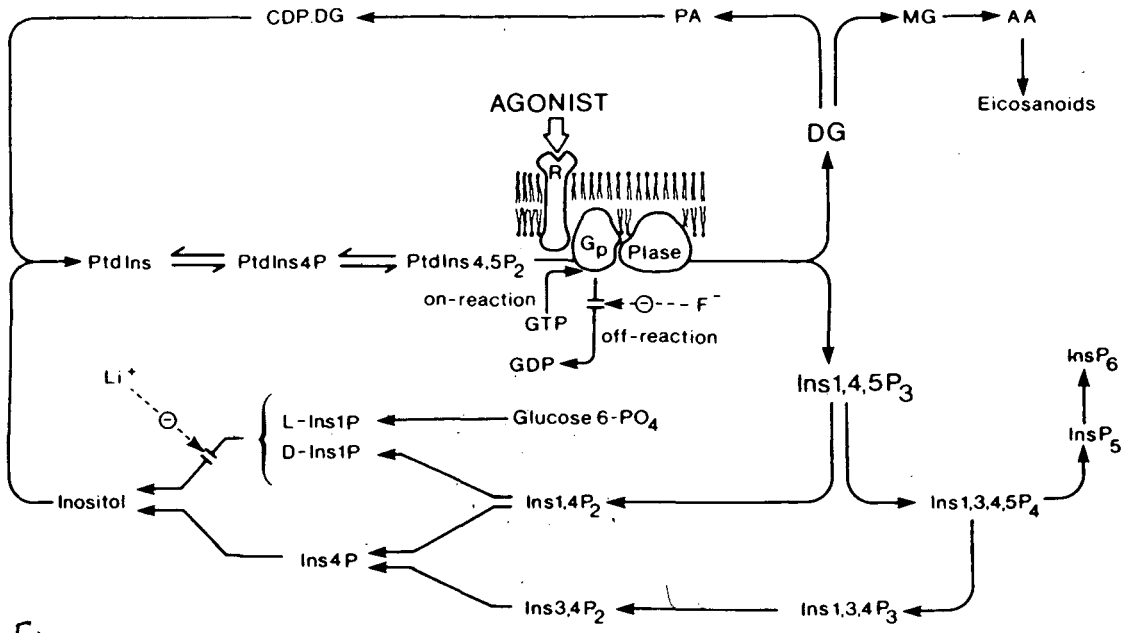


Fig 2. The bifurcating signal pathway begins with the hydrolysis of PtdIns4,5P_2 to give DG and Ins1,4,5P_3. When an agonist occupies its receptor (R), it activates a G-protein (G_p), which then binds GTP as part of an on-reaction leading to the stimulation of phosphoinositidase (PIase). Both second messengers (DG and Ins1,4,5P_3) can be metabolized via two separate pathways.

(Berridge, 1987).

Berridge and Irvine, 1984; Hokin, 1985). The increase in the level of IP_3 was found to preced the onset of Ca^{2+}-dependent events in blowfly salivary gland and neutrophils (Dougherty **et al.**, 1984). Measurements of intracellular Ca^{2+} in different cell types have revealed that the increase in IP_3 either preceded (Wollheim and Biden, 1986) or coincided with the onset of Ca^{2+} signal (Thomas **et al.**, 1984; Reynolds and Dubyak 1985; Ramsdell and Tashijian 1986). The evidence shows that external signals act through a receptor mechanism to produce IP_3, which then mobilizes Ca^{2+}. IP_3 involved in the process is Inositol 1,4,5 triphosphate (Biden and Wollheim, 1983; Turk **et al.**, 1986; Gouri and Habibulla, 1988).

Source of Ca^{2+} is intracellular stores, endoplasmic reticulum (ER) (Streb **et al.**, 1983; Prentki **et al.**, 1985). The initial response to stimulation by Ca^{2+} mobilizing agonist is release of internal calcium (Phase 1), which is soon followed by entry of Ca^{2+} across the PM (Phase 2). Much of the Ca^{2+} mobilized from ER is pumped out of the cell (Altin and Bygrane, 1985), resulting in the Ca^{2+} content of cell declining by as much as 50% (Brown **et al.**, 1984). Adding or removing of external Ca^{2+} has no effect on phase I, but severely curtails phase II (Kojima **et al.**, 1985; Reynolds and Dubyak, 1985).

In order to release Ca^{2+}, IP_3 acts through a specific receptor. Specificity for receptor is phosphates at position 4 and 5 in inositol phosphates (Irvine, **et al.**, 1982; Burgees **et al.**, 1984; Hirata **et al.**, 1985).

IP_3 released from ER can be metabolized by two pathways. One involves sequential dephosphorylation by enzyme phosphatases (Downes **et al.**, 1982; Storey **et al.**, 1984 and Sasaguri **et al.**, 1985). In another pathway IP_3 is transformed first into polyphosphates and then dephosphorylation occurs afterwards (Irvine **et al.**, 1986; Heslop **el al.**, 1985).

Li^+ has been used earlier in the cases of **Mania** and **Depressions** (Johnson, 1979). Now stdies by Berridge **et al.**, 1982; Sherman **et al.**, 1985 and Sherman **et al.**, 1986, shows that Li^+ administration has effect on phosphoinositide metabolism in different organs and different systems and even brain. Inositol 1-phosphate was shown to accumulate. Inositol levels in different systems decreased appreciably. The effect was mainly on stopping of resynthesis of inositol lipids. Most of the inositol 1-P comes from phosphoinositols by action of enzyme phospholipase C. But Li^+ had no effect on the ability of hormones to stimulate smooth muscle contraction. There are no evidences in the effects of Li^+ on IP_3 and DAG.

DAG

DAG that stays in the membrane (PM) functions as the second messenger by activating protein kinase C (Nishizuka 1984; Nishizuka 1986). Phosphatidylserine (PS) and Ca^{2+} are also required in addition to DAG (Kojima **et al.**, 1985; Elliot **et al.**, 1988). The increase in Ca^{2+} perhaps increases the binding affinity of enzyme, protein kinase C, for DAG (Kojima **et al.**, 1985; Dougherty and Niedel, 1986).

DAG (1-oleoyl-2-acetylglycerol) and phorbol esters couldalso activate protein kinase C in intact cells (Nishizuka, 1984; Ashendel **et al.**, 1985; Bazzi and Nelsestuen, 1989). In adrenal medulla cells, protein kinase C may be activated solely through Ca^{2+} entering via voltage-dependent Ca^{2+} channels (Brocklehurst **et al.**, 1985). Physical translocation of enzyme from the cytosol into the membrane is the important aspect of activation of process, which might be the role of Ca^{2+} (Wolf **et al.**, 1985; May **et al.**, 1985).

Protein kinase C is inhibited by a variety of compounds like flavonoids, adriamycin, staurosporine, acridine orange, calphostine C (Kobayashi **et al.**, 1989; Smal, 1989; Ferriola, 1989).

The dual signal hypothesis concerns the way in which the IP_3/Ca^{2+} pathway and DAG/C kinase pathway cooperate with each other to control a wide range of processes. In many cells two pathways acts synergistically (Nishizuka 1984; Nishizuka, 1986).

On protein kinase C activation by DAG and Ca^{2+}, it begins to phosphorylate specific proteins that are thought to contribute to the final response. Some of the identified substrates include viculin, epidermal growth factor (EGP) receptor, glycogen synthase, lipocortin and Na^{+}-K^{+}-ATPase (Williamson **et al.**, 1985; Bertorella and Aperia, 1989).

At the time of stimulation, the fall in level of PIP_2 is soon restored as a consequence of a positive feedback effect operated through protein kinase C because similar increase can be obtained on stimulation of cells with phorbol esters (Taylor **et al.**, 1984; Boon **et al.**, 1985). DAG/C kinase pathway has an important effect on phosphatidyl inositol metabolism by having both positive and negative feedback mechanisms (Fabbro **et al.**, 1988; Dreher, 1988).

Protein kinase C, the major phorbol ester receptor, has been purified from bovine brain and through the use of oligonucleotide probes based on partial amino acid sequence, cDNA clones were derived from bovine brain cDNA libraries

(Parker **et al.**, 1986). Protein kinase C can undergo proteolysis to generate catalyticaly active fragment that is no longer dependent upon Ca^{2+} and phospholipid (Fry **et al.**, 1985). The fragment of protein kinase C generated after proteclysis has kinase activity and is not dependent on Ca^{2+} and phospholipid (PS) (Fry **et al.**, 1985). It would appear that the regulatory domain by proteolysis leads to activation. Presumably the binding of Ca^{2+} and PS provokes a confirmational change with consequent activation of protein kinase C (Parker **et al.**, 1986). A model for structure of protein kinase C has been proposed upon the basis of above considerations by Parker **et al.**, 1986 (Fig. 3).

There is a family of protein kinase C enzymes in the cells. These enzymes work differently. Amino acid sequences of these indicates homologies in all known protein kinases at the catylytic site, thus, belongs to a related family of protein that has evolved in past from a common ancestral origin (Coussens **et al.**, 1986; Taylor 1987; Dreher and Hanley, 1988).

INVOLVEMENT OF G PROTEINS

In early experiments, the role of G-proteins in transmembrane signalling reactions was given. Now many experiments are being continued frequently in other systems

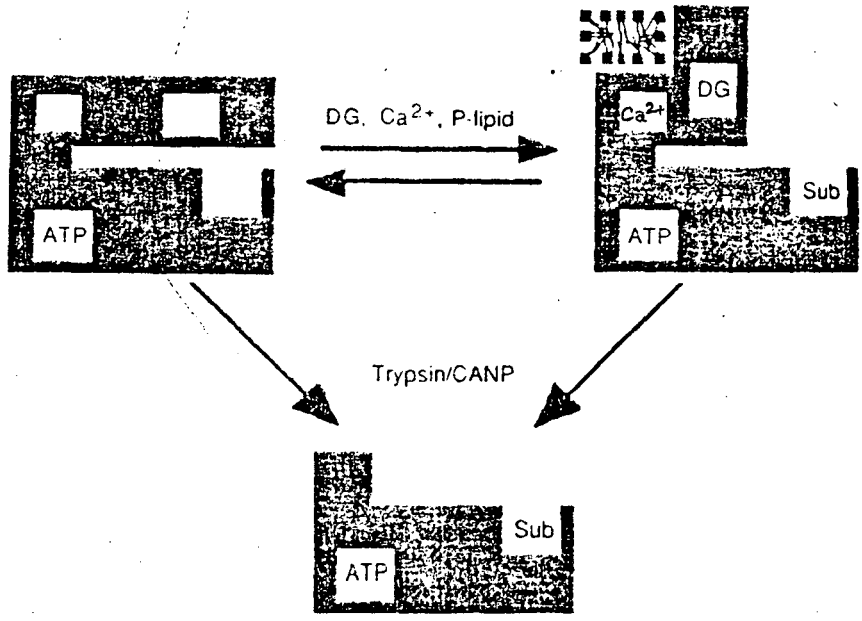


Fig. 3. Model for activation of protein kinase C. Abbreviations: DG, diacylglycerol; P-lipid, phospholipid; CANP, calpain; Sub, substrate-binding site.

including smooth muscle cells. It has become possible to define criteria for involvement of G proteins in these cases. These are listed as:

1. An appropriate agonist for the receptor and GTP is required to initiate the response in question (Rodbell **et al.**, 1971; Cassel and Selinger, 1976).

2. The response can be provoked independently of receptor by inclusion of nonhydrolyzable analogues of GTP (GTPrS by $G_{pp}(NH)_p$ or F^- & Al^{3+}). It has been possible to introduce nucleotide analogues into intact cells by injection or perfusion (Praffinger **et al.**, 1985 and Breitwieser and Szabo, 1985) or after permeabilization (Gompert, 1983).

3. There is a negative heterotropic interaction between the binding of guanine nuclecotide to a G protein and binding of agonist to a G protein-linked receptor (Orly and Schramm, 1976; Pfeuffer, 1977).

4. Cholera toxin and/or pertussis toxin have chracteristic effects on the functions of known G proteins, and they can be utilized with either intact cells or purified components (Cassel and Pfeuffer, 1978; Katada and Ui, 1987).

5. Certain mutants have been extraordinarily useful in the definition of some G proteins-regulated functions. It is hoped that novel mutants, deficient in the activities of various G proteins, can be developed.

6. Antibodies with different reactivities for individual G proteins have recently become avaiable (Spicher **et al.**, 1988).

7. Purification and reconstitution have been achieved with adenylyl cyclase complex and the retinal phosphodiesterase system (Orly and Schramm 1976; Ross and Gilman, 1977).

Various Functions Regulated by G Proteins

Activation of adenylyl cyclase (Pfeuffer, 1977; Northup **et al.**, 1980; Ross **et al.**, 1980) is by Gs, stimulating G protein, under physiological conditions.

Inhibition of adenylylcyclase and action of pertussis toxin (Katada and Ui, 1982) suggests involvement of a distinct G protein, Gi, inhibitory G protein (Manning and Gilman, 1983; Bokoch **et al.**, 1984). Subsequent reconstitution studies also showed involvement of Gi (Katada **et al.**, 1984).

Light activates a cyclic GMP-specific phosphodiesterase in retinal rod outer segments. The observations of light-activated GTPase activity in retina and guanine nucleotide requirement for activation of the phosphodiesterase led to purification of G_t, another member of G protein family, transducin (Liebmann, 1978; and Kuhn, 1980; Fung **et al.**, 1981).

Many hormones mobilize Ca^{2+} from intracellular stores by virtue of their ability to stimulate cleavage of PIP_2 to IP_3 and DAG. The relevant phosphodiesterase is influenced by guanine nucleotides (Berridge, 1984; Cockcroft and Gomperts, 1985).

A few reports suggest the possibility of G protein in direct control over the ion channels (Brestwieser and Szabo, 1983; Pfaffinger **et al.**, 1985).

Some structural details regarding G proteins are beginning to emerge. Molecular cloning has revealed the primary structures of nearly all types of G proteins that have been purified (Northup **et al.**, 1980; Sternweis **et al.**, 1981). The G protein are shown to be heterotrimers with subunits designated as α, β and γ in the order of decreasing mass. The subunit differs among the members of the family and at least for the moment define the

individual G protein. Common ß and γ subunits are probably shared among some subunits to form specific oligomers (Kuhn, 1980; Fung, 1981; Bokoch 1984; Kauaho, 1984; Katada, 1984; Yatsunami **et al.**, 1985).

Pederson and Ross (1982), and Asano and Ross (1984) developed reconstitution studies with the help of G proteins and receptors in phospholipid vesicles and same basic approach has been applied by others. Properties of these interaction showed that:

1. Hormone-receptor (H.R.) stimulates dissociation of G. GDP.

2. H-R. stimulates Guanine nucleotide binding.

3. H-R. stimulates steady state of GTPase activity of G protein.

4. H-R. Functions catalytically.

5. Requirement of Mg^{2+} for activity of G_{α}-GTP.

6. G_{α}-GTP activates the effector for its action. Scheme for interaction of receptor, G protein, GTP and effector has been provided by Gilman (1987) [Fig. 4].

7. Kig **et al.** (1989) and Kurachi **et al.** (1989) have shown that ß γ subunit formed after dissociation of G protein can activate K^{+} channels via phospholipase A_2.

Studies of interactions of G protein α-subunits with nucleotides have focussed particularly from studies with non-hydrolyzable analogues of GTP [GTPγS or Gpp(NH)p]. The binding is influenced by Mg^{2+} ions and proteins that interact with Gα (particularly receptors and G$\beta\alpha$). There is negative cooperativity of binding of GTP γs and $\beta\gamma$ to Gα, thus, GTPγS promotes G protein subunit dissociation (Sternweis **et al.**, 1981; Codina **et al.**, 1983; Northup **et al.**, 1983).

$$G\alpha\beta\gamma + GTP\gamma S \underset{<-----}{-----> } G\alpha.GTP\gamma S + G\beta\gamma$$

Mg^{2+} shifts the equilibrium for this reaction far right. The effect of Mg^{2+} on the binding of GTP S is striking: the rate of dissociation of the nucleotide from Go or Gi is reduced to near zero (Higashijima **et al.**, 1987). At low concentration of Mg^{2+} the dissociation is slow but at high Mg^{2+} concentration Gα.GTPγS.Mg^{2+} is highly stable.

F^-, Al^{3+} causes a similar effect. Curiously, Al^{3+} (or Be^{2+}) was found to be required for activation of G_s by F^-, and it was suggested that activating ligand was AlF_4^- (Sternweis **et al.**, 1982; Higashijima **et al.**, 1987).

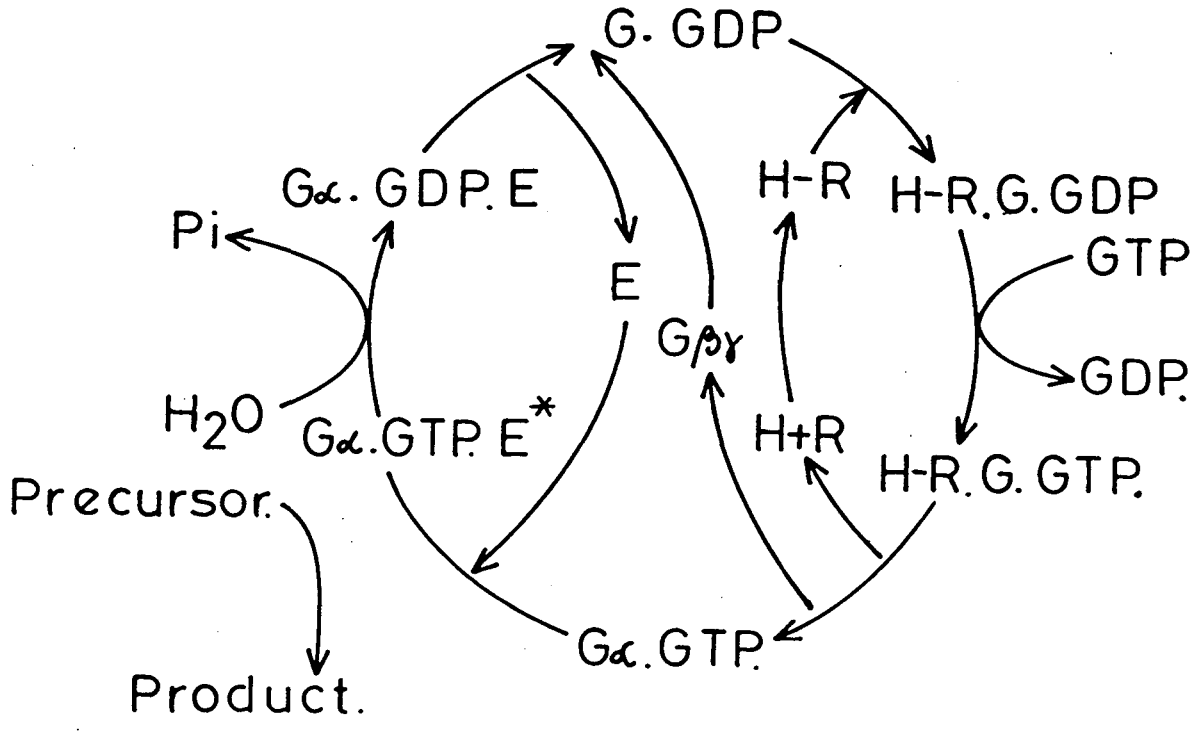


FIG4. INTERACTION OF RECEPTOR: G PROTEIN, GTP, AND EFFECTOR.
(From Gilman AG, 1987)

III. PURPOSE, PLAN OF STUDY AND CHOICE OF MATERIAL

Purpose of Present Study

In skeletal muscles, when stimulation occurs through nerve impulse, depolarization at presynaptic neurons occurs and acetylcholine is released. This Ach binds to the receptor and action potential develops across the transverse tubule (t tubule), Ca^{2+} is released from terminal cisternae of SR, and muscle contraction ensues (Somlyo **et al.**, 1981). Three different hypotheses have been proposed to explain EC coupling during twitch, however, no conclusion has been drawn.

Electrical hypothesis states occurrence of a transient electrical pathway that allows a small current flow across the triadic junction (Mathias **et al.**, 1980). However, it seems clear that t tubule action potential does not propagate along SR (Volpe **et al.**, 1986) so the electrical phenomenon cannot mobilize Ca^{2+} from SR and hence EC coupling by electrical phenomenon.

Mechanical hypothesis given by Schneider and Chandler (1973) says that the charge movements at the transverse tubule membrane level controls the Ca^{2+} channels in the junctional SR by alternating long-connecting molecules in the feet. Such a mechanical linkage might open one SR channel

per charge site. But no proof regarding this has been given uptil now.

Chemical hypothesis postulates that specific chemical transmitters are released in response to an action potential. Simple diffusion of which at junctions requires less than 1 uS, whereas the time between the upswing of the t tubule action potential and the rise of myoplasmic free Ca^{2+} is about 2-5 mS (Vergara **et al.**, 1983). EC coupling therefore is not very fast for chemical transmission. Regarding the studies in chemical messengers in skeletal muscles is very less. Only recently a very few reports regarding IP_3 as second messenger in EC coupling has come up (Vergara **et al.**, 1985; Volpe **et al.**, 1985; Lee **et al.**, 1986). But its role as second messenger in smooth muscle fibers and other types of cells is clear (Burgess **et al.**, 1984; Streb **et al.**, 1989). However, the role of chemical messengers in skeletal muscles remains still unclear.

Since phosphoinositide forms membrane lipids in skeletal muscles also, this pool in membrane can also act as substrate for the synthesis of messengers like IP_3 and DAG as in other types of cells. Here we feel that when neurotransmitter (ACh) binds to the receptor in skeletal muscles, PIP_2 is hydrolyzed to generate IP_3 and DAG through Hormone-Receptor-G Protein-Phosphodiesterase interaction. Evidence is indicated

from the previous experiments of Vergara **et al.** (1985) and Lee **et al.** (1986). Like in other cells IP_3 and DAG must be mobilizing Ca^{2+} from SR and must also be regulating the EC coupling phenomenon, through IP_3/Ca^{2+} and DAG/C-kinase pathways. In skeletal muscles, role of DAG/C-kinase pathway becomes more important as it is regulatory in function by feedback mechanisms. This model is proposed for this study (Fig.5).

Present study is intended to study the role of G protein in transduction of agonist-receptor interaction for the production of DAG in EC coupling phenomenon in gastrocnemius muscle of frog (**Rana hexadactyla**).

Following Studies are aimed at in the present work:

(i) To see production of DAG with electrical stimulus at different levels of stimulation - subthreshold stimulus, threshold stimulus, tetanization and complete exhaustion of muscles.

(ii) to establish involvement of GTP in the phenomenon.

(iii) to study the requirements of the nucleotide ATP and its effects;

(iv) effects of cholera toxin and snake venom will be studied in relation to levels of DAG produced.

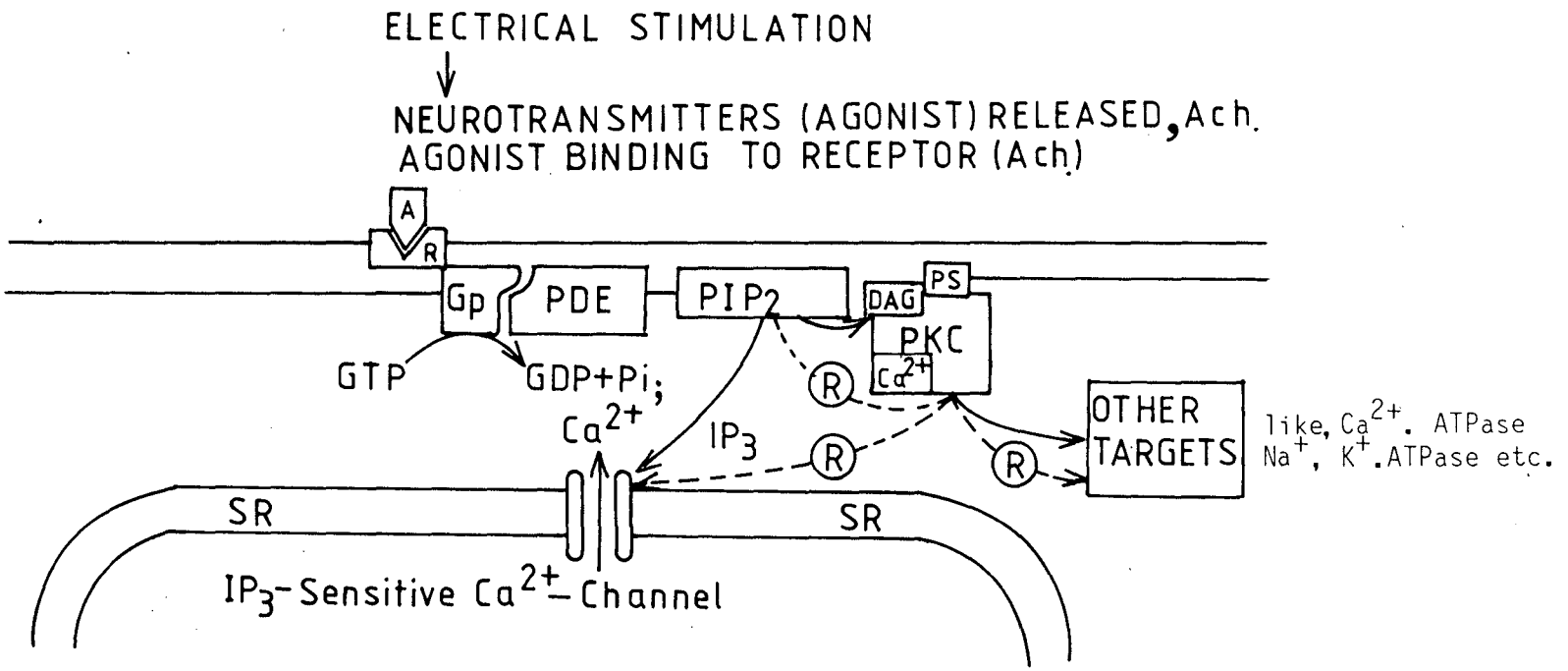


FIG 5. PROPOSED SCHEME SHOWING IP_3 AND DAG AS THE MESSENGERS FOR EC COUPLING IN SKELETAL MUSCLE (ABB. AS GIVEN ALREADY), ------ R ------, REGULATORY PATHWAYS; ——— STIMULATING PATHWAYS.

(v) Mg^{2+} involvement in EC coupling phenomenon would be studied, as it is required in other cells for G protein activity.

(vi) involvement of Al^{3+} and F^- would also be studied.

(vii) effects of Li^+ on DAG production would also be worked out.

Such studies would establish the involvement of G protein as transducer for signalling in the PM and DAG production as chemical messenger in EC coupling phenomenon. With the help of these studies we will be able to fill the gap which exists in understanding of mechanism of EC coupling. The effects will be studied in terms of production of DAG levels. Estimations of DAG will be done by the method of gas-liquid-chromatography (GLC).

Choice of Material

Frog as an experimental animal in research has been a choice in this century. Extensive information is available on the morphological and functional properties of frog's nervous system (Steinback and Precht, 1976). Much knowledge in neurobiology has been derived from studies of this vertebrate (Edman **et al.**, 1985; Kawata, 1985; Konishi **et al.**, 1985; Barry, 1987). Muscular and neuromuscular aspects of

the frog have been well documented (Linasa Precht 1976). Easy handling and maintenance in laboratory conditions, size and poikilothermous nature of animal become other points for choice of frog as an experimental animal. Neuromuscular junctions are easy to isolate and its stability were among other important factors for its choice.

MATERIALS AND METHOI

EXPERIMENTAL ANIMALS

Frogs (Rana hexadactyla) were acquired from local suppliers of animals. They were maintained at room temperature in laboratory in specially made small glass troughs (1 ft x 3 ft x 2 ft, in about 1 cm water inside). They were not fed any special food. Weight range was from 100-150 gms. Both males and females were used without any distinction.

CHEMICALS

1,2-sn-Dioctanoyl glycerol (DiC_8), arachidonic acid, phosphatidic acid, 1-oleoyl 2-acetylglycerol (OAG), GTP, ATP, Cholera toxin, snake venom were obtained from Sigma Chemical Company, USA. Boron trifluoride-methanol (BF_3-MeOH) complex, silica gel G were acquired from E-Merck, Germany, n-Pentane (GC grade), Chloroform, methanol were obtained from Spectrochem, India. Other solvents and chemicals were of highest purity available from good Indian Chemical Companies.

PREPARATION OF GASTROCNEMIUS MUSCLE-SCIATIC NERVE (GNM-SN):

Stunned frogs were taken and the gastrocnemius muscle (GNM) - sciatic nerve (SN) preparation was made immediately after removal of the whole skin from the body to avoid any secretion to enter into the GNM-SN preparation. Preparation

was carefully removed into Ringer's solution, containing 111.3 mM NlCl,11.9 mM KCl, 1.1 mM $CaCl_2$, 2.4 mM $NaHCO_3$ and 1.6 mM $MgSO_4$, pH kept at 7.4 (Edstron and Mattison, 1972) (Fig II).

EXPERIMENTAL SET UP

a) The experimental muscles were stimulated through nerve for necessary period, using a SD9 Grass Stimulator (Fig. I).

b) The unstimulated contralateral preparation was employed as control (Fig. II).

ELECTRICAL STIMULATION

(i) Sub-threshold stimulus: Different GNM-SN preparations were isolated in frog's Ringer's. Experimental sets were stimulated with .1 volt stimulus of duration 0.1 mS, and for 2 minutes at a frequency of 10 pulses per second (PPS).

(ii) Threshold Stimulus: GNM-SN preparations were stimulatd through nerve by increasing the voltage to 1.1 V, while frequency, duration remains the same as in sub-threshold stimulus.

(iii) Tetanization: A set of GNM-SN preparations were tetanized at the stimulus of 1.1 V, 100 PPS frequency, .2 mS duration, for 8-10 minutes.

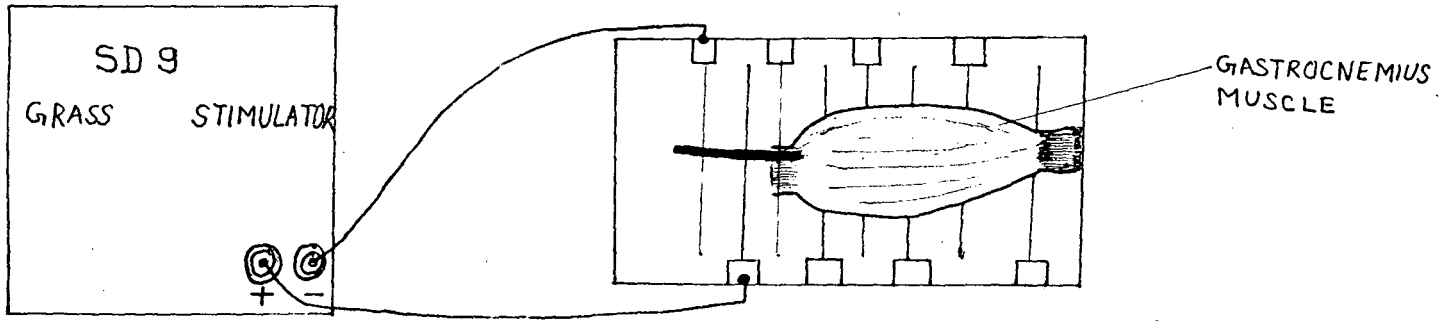
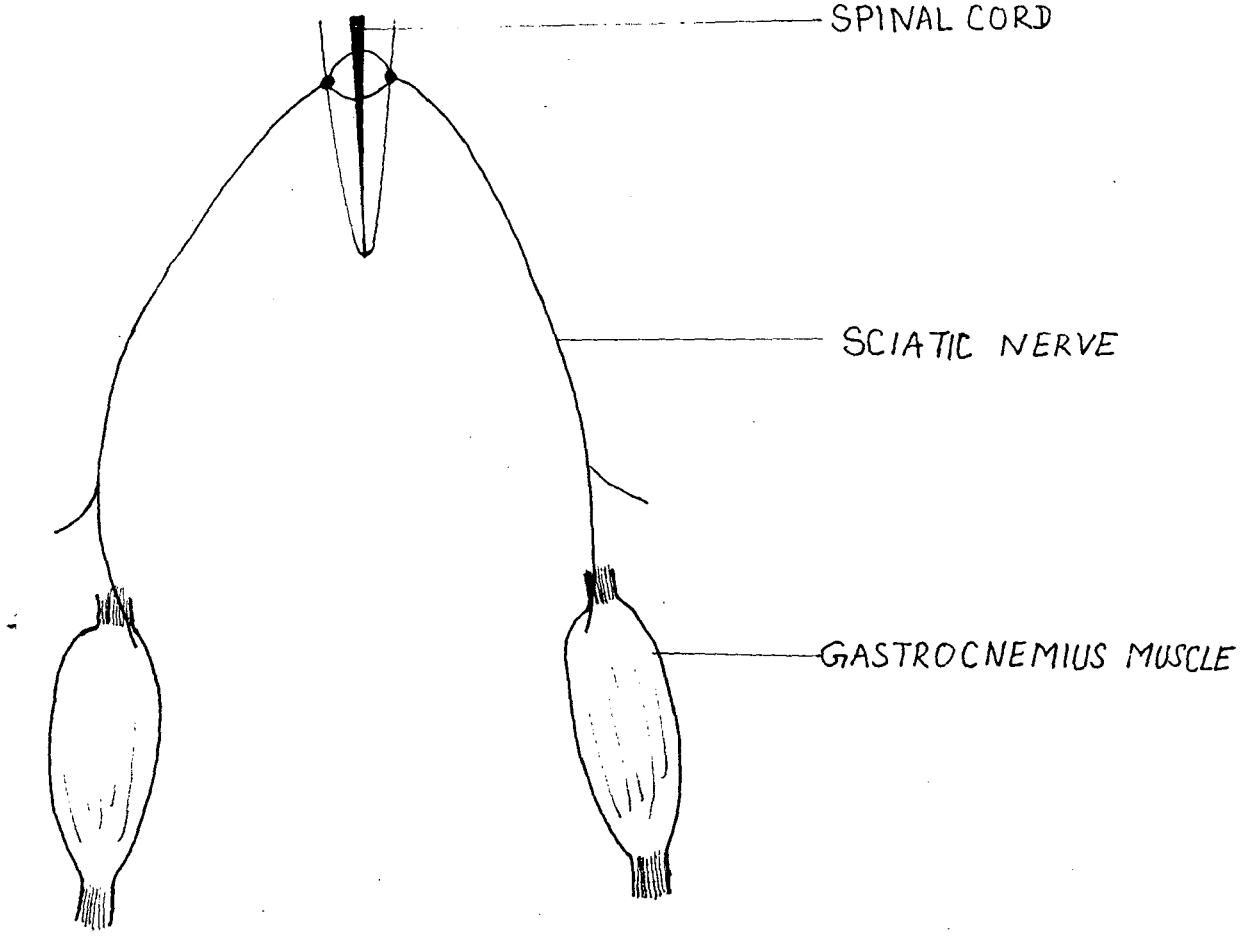


Fig. I



CONTROL EXPERIMENTAL

Fig. II

(iv) Irreversible exhaustion: A set of GNM-SN preparation was stimulated for 30 minutes, rest of the conditions were same as during tetanization. In this condition muscles were completely exhausted and did not recover at all when kept in Ringer's solution.

PREPARATION OF HOMOGENATE OF MUSCLE TISSUE

Electrically stimulated and control preparations of GNM were homogenized immediately after stimulation without loss of time separately in 0.25 M sucrose. Homogenization was done for 30-40 seconds at medium speed in REMI homogenizer. Any contamination with ions was taken care of by washing thoroughly and homogenizing in the pure sucrose solution. All operations were performed in cold over melting ice.

PLASMA MEMBRANE ISOLATION

Plasma membranes (PM) were isolated by the method of Kidwai (1974). Homogenized GNM preparation (in 0.25 M surcose) was passed through a cheese cloth and then the residue was rehomogenized for 5-10 seconds. To avoid this step and losses involved centrifugation could be done at 10,000 x g for 20 minutes. The filterates were pooled and ultracentrifuged at 100,000 x g for 1 hour. The sediment was resuspended in 0.25 M surcose. The gradient of sucrose of

0.25 M to 2.0 M range was made and was left in refrigeration for equilibrium for 12 hrs (continuous gradient). The resuspended sediment was layered on the top of density gradient and centrifuged for 2 hrs at 111,688 x g at 4°C. PM was collected at the interphase of loading sample and sucrose density gradient. The band of PM was collected with a pasteur pipette, diluted to reduce the concentration of sucrose. PM Recentrifuged for 30 minutes to get the PM pelleted. Pellet was resuspended in Tris-HCl if required. Estimations of membranes were done in terms of membrane protein by the method of Lowry et al. (1951). All operations for PM isolation were done at 4°C or under ice.

PM from control and experimental preparations were made separately but in similar conditions.

EXTRACTION OF LIPIDS FROM PM

From the PM isolated, lipids were extracted with chloroform and methanol (1:1 v/v) containing 5 mg BHT/100 ml of solvent. BHT acts as an antioxidant. Washing was done with 0.9% NaCl to remove nonlipid contaminants. The lower organic phase was separated and dried under nitrogen (N_2) gas under reduced temperature and pressure. The lipid was resuspended in chloroform and methanol and stored at -10°C or was used immediately for estimation.

TLC PURIFICATION OF DAG

Thin-layer-chromatography of lipid samples was done by the method as described by Spiski et al. (1968). TLC glass plates 20x20 cm size were coated by silica gel G evenly to about 0.25 mm in thickness with the help of gel spreader. They were dried under room temperature. Activation of plates was done in oven at 80°C for about 6 hrs. The chromatography chamber was saturated for 15 minutes with solvent prior to the experiment. Two solvent systems, system I and II were used for purification. Solvent I contained dichloroethane and methanol in the ratio of 196:4 (v/v). Solvent system II was made up of benzene, diethylether, ethanol and ammonia in the ratio of 100:80:40:0.2 (v/v/v/v). The separation was done at constant temperature less than 10°C to avoid any lipid from hydrolysis. Loading (spotting) of samples on the plates was done 1 inch above the base. TLC plates were removed after the solvent system has moved at about 1 inch below the top of the plate. Spots were detected by iodine vapours after the plates have been dried.

Standard lipids were run in different lanes. Quantifications were done with gravimetry.

FORMATION OF METHYL ESTERS OF LIPIDS

Various catalysts for methyl esters formation were tried for different times of heatings. BF_3-MeOH complex, 140 gm per litre of method, was best suited for fatty acids and DAG. Method was simple, fast and yield was high in comparison to other catalysts. Lipids dried under N_2 were treated in BF_3-MeOH complex for 10 minutes at 100°C in closed tubes (1 ml of BF_3MeOH was used for 10 mg lipid). Reaction mixture was cooled and methyl esters were extracted in n-pentane. Before extraction with pentane small amount of water (approx. half of volume of Pentane) was also added and shaking was done well to remove water soluble impurities. Phase separation was acquired by centrifugation at 2000 x g for 15 minutes. Methyl esters were collectd by pasteur pipette and were stored in airtight ependroff tubes at -10°C. Sample was found to be stable for one week.

For control and experimental samples same quantity of GNM was taken in both, one stimulated and other unstimulated. Both were subjected to similar conditions but separately for extraction, purification of DAG, and also for methyl ester formation. Same value of pentane was used for extraction of methyl esters in both samples.

Same procedure was adopted for the formation of methyl esters of standard lipids.

STANDARDIZATION OF GLC ASSAY AND ASSAY OF STANDARD DAGs AND FATTY ACIDS

1,2-sn-Dioctanoyl glycerol (DiC_8), 1,2-sn-dipalmitoyl glycerol, 1,2-sn-dimyristoyl glycerol, 1,2-sn-distearoyl glycerol, palmitic acid, stearic acid, arachidonic acid were used as standard lipids for the experiment. Methyl esters were prepared as given above.

Standardization of assay was done with stearic and palmitic acid. Following conditions were found to be suitable to our experiment.

Column : DEGS (Diethylene glycol succinate), 10% CW and mesh size 60~80.

Column temperature	:	$200^{o}C$ (Isothermal conditions)
Injection temperature	:	$240^{o}C$.
Carrier gas	:	N_2
Flow rate of N_2	:	40 ml/min
Stop time	:	10 minutes
Detector	:	Flame ionization detector (FID)
Range	:	10^2 or 10^3
Attenuation	:	Varied time to time.

GC of Model GC-9A from Shimadzu, Japan, was used for assay. Printer of model C-R2A was employed fpr experiment.

Lipids were detected by the retention times of respective peaks. The area of the peaks gave the quantity of lipid in the sample. Peaks with retention time less than 1.2 minutes, were locked as these were of no use and were of solvent.

GLC ASSAY OF DAG

DAG was assayed from all types of samples in same conditions in the similar way as given above. But range and attenuation factor was varied in some. Equal volume of samples were injected both for control and experimental conditions. Variable conditions were written against respective samples or conditions of stimulation.

DIFFERENT LEVELS OF STIMULUS AND DAG PRODUCTION

Methyl esters of extracted DAG were prepared by the the method given above. GLC assay was done under similar conditions as given in standardization procedure. Range was kept at 10^2 and attenuation at zero. Similar type of conditions were employed for assay to all four smaples types.

1. Subthreshold stimulus
2. Threshold stimulus
3. Tetanization
4. Irreversible or complete exhaustion

Same volume of sample (2 µl) of extracted esters was used in all four conditions during injection.

DAG ASSAY AT DIFFERENT GTP CONCENTRATIONS

PM were isolated by the same method as given from unstimulated GNM. Stimulation was done with acetylcholine (Ach). Different levels of Ach were tested (0,2,4,6,8,10 µM) for stimulation. Later in other experiments 10 µM Ach levels were employed, and this was not far from physioloical levels. The physiological level is 7-12 µm.

Different concenterations of GTP were employed for experiment - zero, 0. 2 µM, 0.4 µM, 0.6 µM, 0.8 µM and 1 µM. Reaction mixture for the experiment contained besides GTP, 50 mM Tris-HCl (pH 7.4), 1 µM ATP, 10 µM $MgCl_2$; 10 µM $AlCl_3$- NaF and membranes (in term of membrane proteins). Incubation at 30^{o}C was done for 5 minutes. Reaction was stopped by adding chloroform, methanol into it. Lipids were extracted with chloroform: methanol 1:1 (v/v) containing BHT and washing was made. TLC as given above was done to purify DAG. DAG was scrapped and methyl esters of it were prepared by method

given above. GLC assay for DAG was done under similar conditions, as in electrical stimulation. Same volume (2 µl) was injected from each sample for assay.

EFFECT OF DIFFERENT ATP CONCENTRATIONS

Method used in this experiment was same as given in the case of GTP. Here all the factors remained constant and only ATP concentrations used were zero (control), 0.2 µM, 0.4 µM; 0.6 µM; 0.8 µM and 1 µM. Reaction mixture contained 50 mM Tris-HCl, 1 µM GTP, 10 µM $MgCl_2$; 10 µM $AlCl_3$-NaF; 10 µM Ach and membranes (membrane proteins) besides different concentrations of ATP.

EFFECT OF Mg^{2+} CONCENTRATIONS

In this experiment Mg^{2+} concentration in the reaction mixture was varied and were kept as zero (control), 2 µM, 4 µM; 6 µM, 10 µM and 20 µM. Besides Mg^{2+} reaction mixture contained same 50 µM Tris-HCl (pH 7.4), 1 µM ATP, 1 µM GTP, 10 µM $AlCl_3$-NaF, 10 µM Ach and membranes. After same time of incubation under similar conditions as above reaction was stopped with chloroform, methanol. All steps of lipid extraction, DAG purification , methyl esters formation and GLC assay were repeated in the same way. Attenuation was kept to zero and range to 10^2.

EFFECT OF Al^{3+} AND F^- ON DAG PRODUCTION

Here in this experiment, Al^{3+} and F^- concentration employed were - zero (control) 2 μM, 4 μM, 6μM, 8 μM, and 10 μM. Reaction mixture contained the same ingradients as in Mg^{2+} and rest of the experiments were repeated in the same way under similar conditions of assay.

EFFECT OF TREATMENT WITH CHOLERA TOXIN

In this experiment, membranes were treated with 1.0 ugm Cholera toxin (from Vibrio cholerae) with Ach as stimulating substance (no toxin in control). Reaction mixture contained 25 mM phosphate buffer (pH 7.4), .1 μM thymidine, .1 μM NAD^+, 1 μM ATP, 1 μM GTP, 10 μM $MgCl_2$, 10 μM $AlCl_3$NaF, 10 μM Ach and membranes as in other experiments. Incubation at 30°C was done for 5 and 10 minutes both. Reaction was stopped by adding chloroform, methanol containing BHT. DAG purification by TLC and then methyl ester formation and GLC assay were done in same way as in other experiments given above.

EFFECT OF SNAKE VENOM TREATMENT

Membranes isolated again by same way were treated with snake venom 1 μgm from Naja naja alongwith acetylcholine. Three sets were formed in this experiment one contained no

snake venom and no acetylcholine. Second set contained both and in third set only 10 μM acetylcholine was employed. Common elements in reaction mixture were 50 mM Tris-HCl (pH 7.4), 1 μM GTP, 1 μM ATP, 10 μM Mg^{2+}, 10 μM $AlCl_3$-NaF, and membranes as in other experiments. Rest of the pocedure was same as in others except range was kept at 10^3 and attenuation at 2.

EFFECT OF Li^+ ON DAG PRODUCTION

GNM membranes were treated with different Li^+ concentration ranging from zero to 0.1 M, - zero (control), 1 μM, 10 μM, 1 mM and 0.1 M during Ach stimulation. Reaction mixture contained besides Li^+, 50 mM Tris-HCl (pH 7.4), 1 μM GTP, 1 μM ATP, 10 μM $MgCl_2$: 10 μM $AlCl_3$NaF, 10 μM Ach and membranes (GNM) as in other experiments. Rest of the procedure of GLC assay of DAG was same except range was kept to 10^3 and attenuation to 2.

RESULTS

Signal transduction from PM surface to SR and mechanism of Ca^{2+} release is relatively less studied phenomenon in EC coupling phenomenon.

Chemical messengers can also be involved in EC coupling phenomenon. The changes in lipids of PM, especially DAG, were investigated quantitatively in GNM in response to various levels of stimuli - subtreshold, threshold, tetanic and complete exhaustion. Effects of various cellular ingradients and ions were investigated in the study. GNM preparationwas was made from frog Rana hexadactyla. Complete method has been given in previous chapter of this work. Effects of different stimuli were seen in terms of total DAG production. Since we are interested more in quantity of DAG produced in response to stimulus, different peaks of fatty acids were not identified individually, but the total increase of DAG production was quantified with reference to basal levels.

After the extraction of lipids from the PM of GNM their separation was done with TLC. DAG was identified at Rf value of 0.31 in solvent I (dichloroethne, methanol 196:4 (v/v) (Fig. 1, method has been described earlier).

On separation of lipids extracted from PM of GNM in solvent II (benzene, diethylether, ethanol, ammonia 100 : 80 : 4 : 0.2 v/v/v/v), DAG was identified at Rf value of 0.66 (Fig. 2, method has been described earlier).

Experiments show the presence of DAG in PM of GNM muscles.

ELECTRICAL STIMULATION OF GNM THROUGH SCIATIC NERVE

GNM-SN preparation was stimulated by electrical method at various levels of stimulus - subthreshold, threshold, tetanization and complete exhaustion. GLC assay for purified DAG was done by the method as given earlier. The unstimulated contralateral preparation of GNM was employed as control. Chromatogram shows 3 peaks for different acyl groups in DAGs.

On stimulation at sub-threshold stimulus increase in DAG production was of the order of 10% (Fig. 3, method is described earlier).

At threshold stimulus increase in DAG production was of the order of $\simeq$ 300% to the basal levels (Fig. 4, method is described earlier).

On tetanization increase in DAG production was of the order of $\simeq$ 750% to the basal level (Fig.5, method is described earlier).

When muscles were completely exhausted so that it does not relaxes in Ringers, the increase in DAG production was $\simeq$50% to the basal (Fig. 6, method is described earlier).

This shows the relationship between the level of excitation of GNM and the DAG production.

STUDY OF INVOLVEMENT OF ACETYLCHOLINE IN EC COUPLING PROCESS

PM from GNM were treated with Ach, it is neurotransmitter which brings about stimulation of the muscles to contract. During stimulation of of the muscles to contract, PIP_2 pool stimulation by Ach, involvement of GTP, ATP, NaF-$AlCl_3$, Mg^{2+} was studied. In all the other studies PM were treatd with 10 uM of Ach (physiological levels).

In present experiment the involvement of Ach in stimulation was studied. PM were treated with different levels of Ach - O (control), 2 µM, 4 µM, 6 µM, 8 μM_3 and 10 µM. Besides Ach 1 µM GTP, 1 µM ATP, 10 µM $MgCl_2$, 10 µM NaF-$AlCl_3$ in Tris-HCl (pH 7.4) were added. The increase in levels of DAG after stimulation was studied by GLC assay.

DAG levels increase to basal levels was ≃ 100% with 2 μM Ach, ≃ 160% with 4 μM Ach, ≃ 210% with 6 μM Ach, ≃ 280% with 8 μM Ach and ≃ 360 % with 10 μM Ach (Fig. 7 (a) and (b), method is described earlier).

Experiment showed the gradual increase of DAG production in response to increasing levels of Ach.

INVOLVEMENT OF SNAKE VENOM IN EC COUPLING PROCESS

Snake venom from Naja naja competes with Ach for binding to Ach receptor. When PM were treated with 10 μM Ach in presence of 1 μgm snake venom, there was seen a decrease of stimulated DAG levels. Other materials i.e. GTP, ATP, $MgCl_2$, $AlCl_3$-NaF were used as indicated in the method given earlier.

A decrease of ≃ 30% stimulation was observed with 1 μgm snake venom. (Fig. 8, method is described earlier).

This shows the competition between snake venom and Ach for binding to Ach receptor and inhibition of DAG production.

INVOLVEMENT OF GTP IN EC COUPLING PROCESS

PM were treated with different GTP levels - O(control), 0.2 μm, 0.4 μM, 0.6 μM, 0.8 μM and 1.0 μM - and other

conditions for ATP, $MgCl_2$, $AlCl_3$-NaF, Ach were same as given earlier. At zero GTP concentration no DAG production was observed. As the levels GTP was increased DAG production increased. There was an increase of ≃60%, ≃150%. ≃250%, ≃280% and≃400% with 0.2 μM, 0.4 μM, 0.6μM, 0.8 μM and 1.0 μM GTP respectively. [Fig. 9 (a) and (b), method is given earlier.] Thus experiment showed the involvement of GTP in EC coupling phenomenon.

INVOLVEMENT OF $AlCl_3$-NaF IN EC COUPLING PHENOMENON

PM were treated with 10 μM Ach and different concentration of $AlCl_3$-NaF -- zero (control), 2 μM, 4 μM, 6 μM, 8 μM and 10 μM -- along with ATP, GTP, Mg^{2+} in Tris-HCl (pH 7.4) as given earlier. DAG extracted was assayed by the method of GLC as given earlier. In absence of $AlCl_3$ the increase of DAG in response to stimulus was ≃ 40% and as $AlCl_3$-NaF concentration increased the DAG levels also increased. Increase in levels of DAG was ≃80%. ≃150%, ≃175%, ≃250% and ≃300% with 2 μM, 4 μM, 6 μM, 8 μM and 10 μM. $AlCl_3$-NaF concentrations respectively (Fig. 11(a) and (b), method is described earlier).

Thus experiment shows the involvement of $AlCl_3$-NaF in EC coupling phenomenon.

EFFECT OF CHOLERA TOXIN IN EC COUPLING

PM were treated with 10 μM Ach and 1 μgm of cholera toxin (from Vibrio cholerae) in one sample and no cholera toxin in other sample alongwith ATP, GTP, Mg^{2+}, $AlCl_3$-NaF in phosphate buffer at pH 7.4 as given earlier. DAG production in response to stimulus was measured by the method as given earlier. No change in stimulated (by Ach) levels of DAG was observed with cholera toxin. DAG levels of samples with cholera toxin and without cholera toxin remained the same (Fig. 12, method is given earlier).

Experiment showed no change in DAG production with cholera toxin during stimulation with Ach.

INVOLVEMENT OF Mg^{2+} IN EC COUPLING PHENOMENON

PM were treated with 10 μM Ach and different concentrations of Mg^{2+} -- O(control), 2 μM, 4 μM, 6 μM, 8 μM, 10μM -- along with ATP, $AlCl_3$-NaF in Tris-HCl (pH-7.4) as given earlier. After stimulation DAG was assayed by GLC method. At zero Mg^{2+} no DAG increase was observed. Increase of DAG levels was ≃ 100%. ~150%, ~360%, ~490% at 4 μM, 6 μM, 10 μM and 20 μM respectively. After 6 μM Mg^{2+} upto 10 μM Mg^{2+} increase is rapid as seen from ≃ 150% — ≃360%.

Thus experiment shows the requirement of Mg^{2+} for EC coupling phenomenon is compulsory. As the levels of Mg^{2+} increases the DAG levels also increases. (Fig. 10 (a) and (b), method is described earlier).

REQUIREMENT OF ATP FOR EC COUPLING PHENOMENON

PM from GNM were incubated with 10 μM Ach along with ATP and other materials, GTP, Mg^{3+}, $AlCl_3$-NaF in Tris-HCl buffer (pH 7.4) as given earlier. Different levels of ATP - 0(control), 0.2 μM, o.4 μM, 0.6 μM, 0.8 μM and 1.0 μM - were employed for the experiments and other materials remained same as given in the method with Ach [Fig. 7(a) and (b)]. At zero ATP concentration no increase in DAG production was observed and as the concentration of ATP increased from 0.2 μM — 1.0 μM, DAG levels gradually increased from ≃ 60% - ≃ 250%. (Fig. 13(a) and (b), method has been described earlier).

Experiment showed the requirement of ATP for the production of DAG and EC coupling phenomenon.

EFFECT OF LITHIUM ON DAG PRODUCTION

PM from gastrocnemius muscles were incubated with 10 μM Ach, different concentrations of Li^{+} - 0(control), 1 μM, 10

μM, 1mM, 0.1M -- alongwith ATP, GTP, Mg^{2+}, $AlCl_3$-NaF in Tris-HCl (pH 7.4) as given earlier. ≃ 60% inhibition in levels of DAG production was seen with 1 μM Li^+. Li^+ is important in therapeutic value in case of mania and depression. Therapeutic range is 0.8 - 1.2 mM.

Thus experiments show the inhibition of DAG production with Li^+.

Fig 1: Thin-layer-chromatographic separation of membrane lipids of GNM.

Chromatogram is shown, solvent system I [dichloroethane, methanol 196:4(v/v)] was used. Method is given earlier. Experiment was done 5 times. Arrow indicates the spots for DAG.

Experiment showed the presence of DAG in PM.

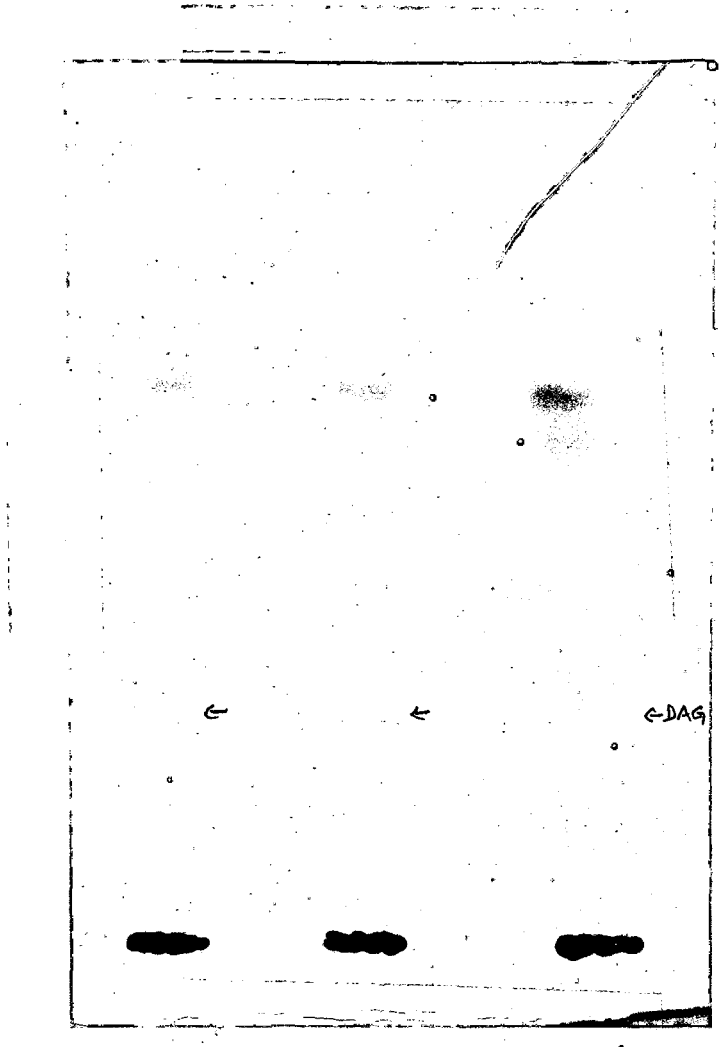

DAG

Fig. 2: Thin-layer-Chromatographic separation of membrane lipids of GNM

Solvent system II was used (benzene, diethylether, ethanol, NH_3 100:80:4:0.2 (v/v/v/v). Method has been described earlier. Experiment was done 5 times. Arrow indicates the DAG spots.

Experiment shows the presence of DAG in PM of GNM.

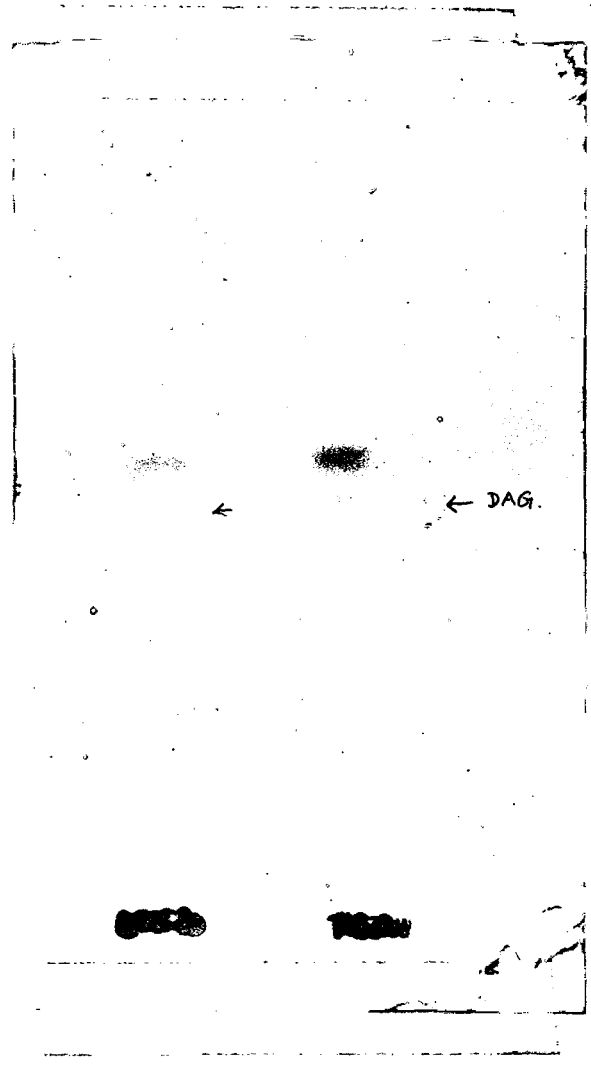

← DAG.

Fig.3 : Effect of subthreshold stimulus on GNM.

GNM were given subthreshold stimulus. Immediately after stimulation, DAG was estimated by the method given earlier. Experiment was done 5 times. Increase in DAG is shown by the area of the peaks after stimulation. Chromatogram is shown with control and after stimulation values of DAG. 3 peaks show 3 different fatty acids of DAGs.

Negligible production of DAGs in response to subthreshold stimulus is shown in chromatogram by the area of the peaks.

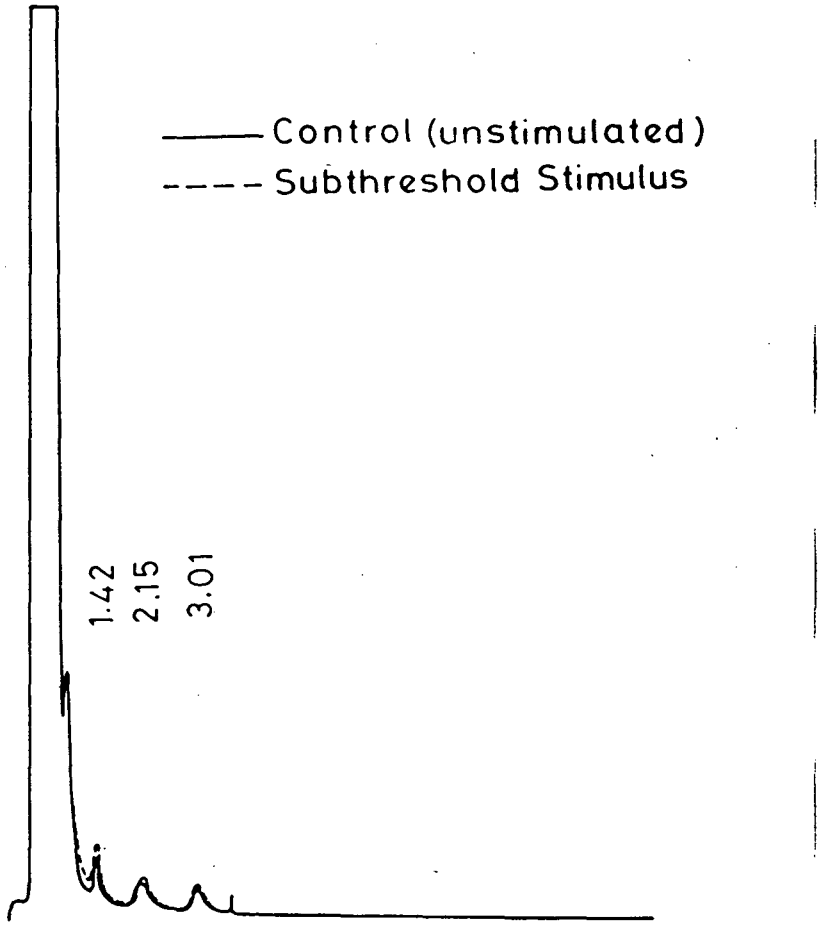


FIG. 3. EFFECT OF SUBTHRESHOLD STIMULUS ON LEVELS OF DAG (—— UNSTIMULATED, ----- SUBTHRESHOLD STIMULUS).

Fig. 4 : Effect of threshold stimulus on GNM.

GNM-SN preparation was given threshold stimulus for 2 min. After stimulation DAG was estimated by the GLC method, given earlier in this work. Change in DAG levels is shown by area of the peaks.

Experiment was done 5 times. Chromatogram is shown giving control and experimental peaks of DAG in terms of fatty acid composition of DAGs.
An increase upto 4 times to the basic levels is indicated.

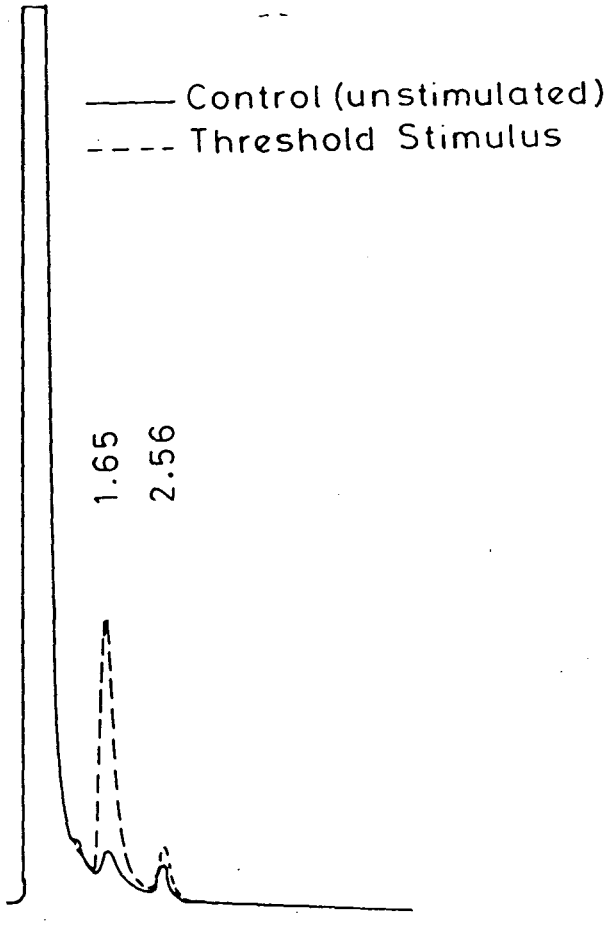


FIG. 4. EFFECT OF THRESHOLD STIMULUS ON LEVELS OF DAG (—— UNSTIMULATED, ----- THRESHOLD STIMULUS).

Fig. 5 : Effect of tetanization on GNM.

GNM-SN preparation was given tetanic stimulus for 8-10 min. After stimulation DAG was assayed by GLC as given earlier. Change in DAG levels after stimulus is given by areas of the peaks. Experiment was done five times. Chromatogram is shown giving peaks for control DAG levels and tetanized DAG levels in terms of the fatty acid compositions.

Increase in levels of DAG is indicated by increase in area of the peaks to 8.5 times the basic levels.

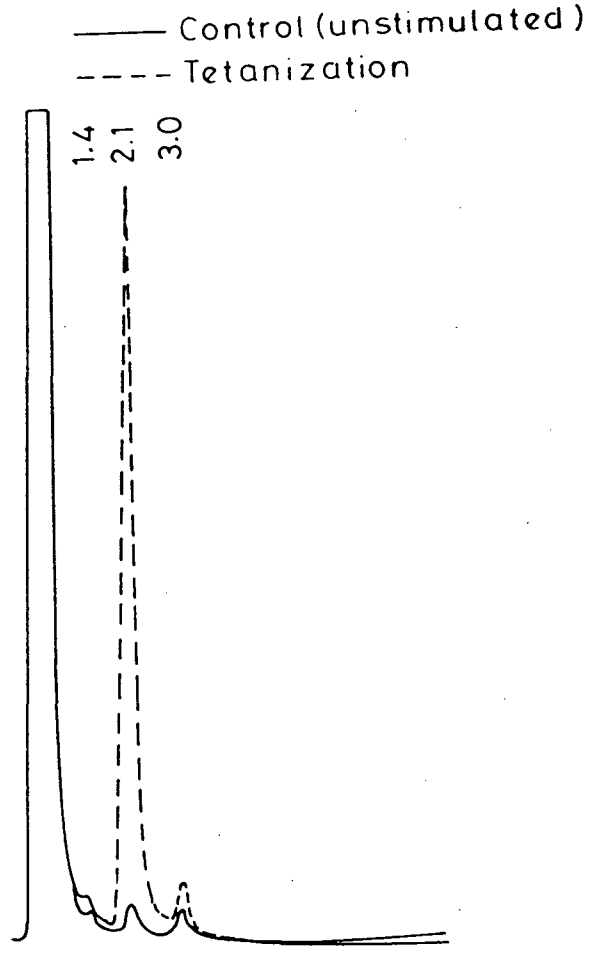


FIG. 5. EFFECT OF TETANIZATION ON LEVELS OF DAG (—— UNSTIMULATED, ----- TENTANIZED).

Fig. 6 : Effect of irreversibly exhausitive stimulus on GNM.

GNM-SN preparation was given exhausitve stimulus for 30 minutes so that it does not become normal in Ringer's. After stimulation DAG was assayed by GLC. Chromatogram is shown. Experiment was done 5 times. Chromatogram is shown before and after exhaustive stimulation. Peaks are showing levels of DAG in terms of fatty acids. Increase upto 50% to the basal levels is indicated by increase in area of peaks.

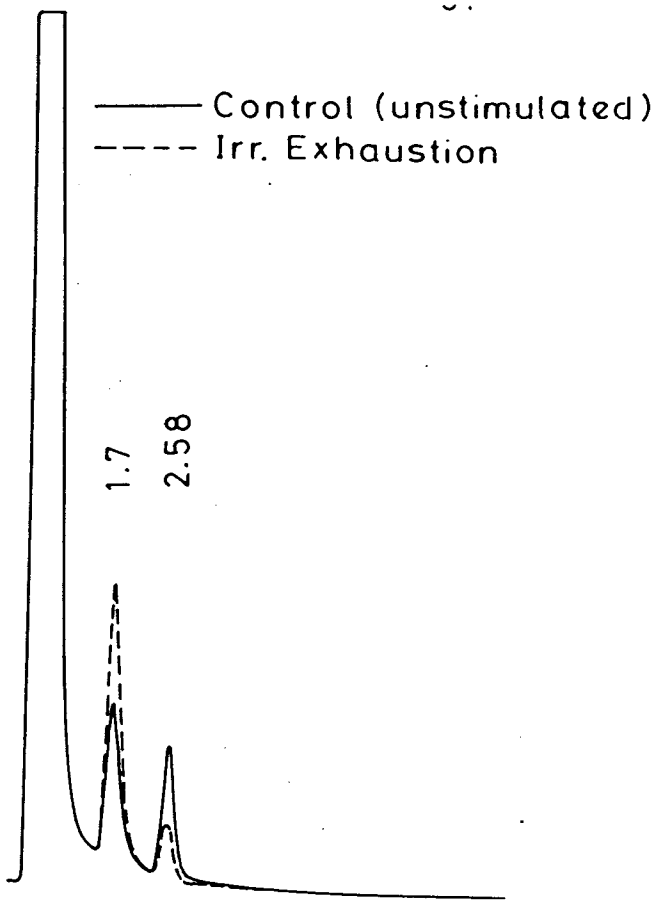


FIG. 6. EFFECT OF IRREVERSIBLE EXHAUSTION ON LEVELS OF DAG (—— UNSTIMULATED, ----- IRREVERSIBLE EXHAUTION).

Fig. 7 (a) and (b) Effect of acetylcholine on DAG production. PM were treatd with 0(control), 2, 4, 6, 8, 10 μM Ach levels. DAG produced was assayed by GLC method, as described earlier. Reaction mixture contained GTP, ATP, Mg^{2+}, $AlCl_3$-NaF as given earlier. Experiment was done 5 times. Fig. 7 (a) shows chromatogram of DAG on stimulation with different Ach levels. Fig. 7(b) graph showing the relationships between levels of stimulus and % increase in DAG levels. Graph is made by means of 5 values and standard deviation (S.D.) is shown. DAG is shown in terms of its fatty acid compositions.

Gradual increase in levels of DAG production is shown in relation to increase in Ach levels.

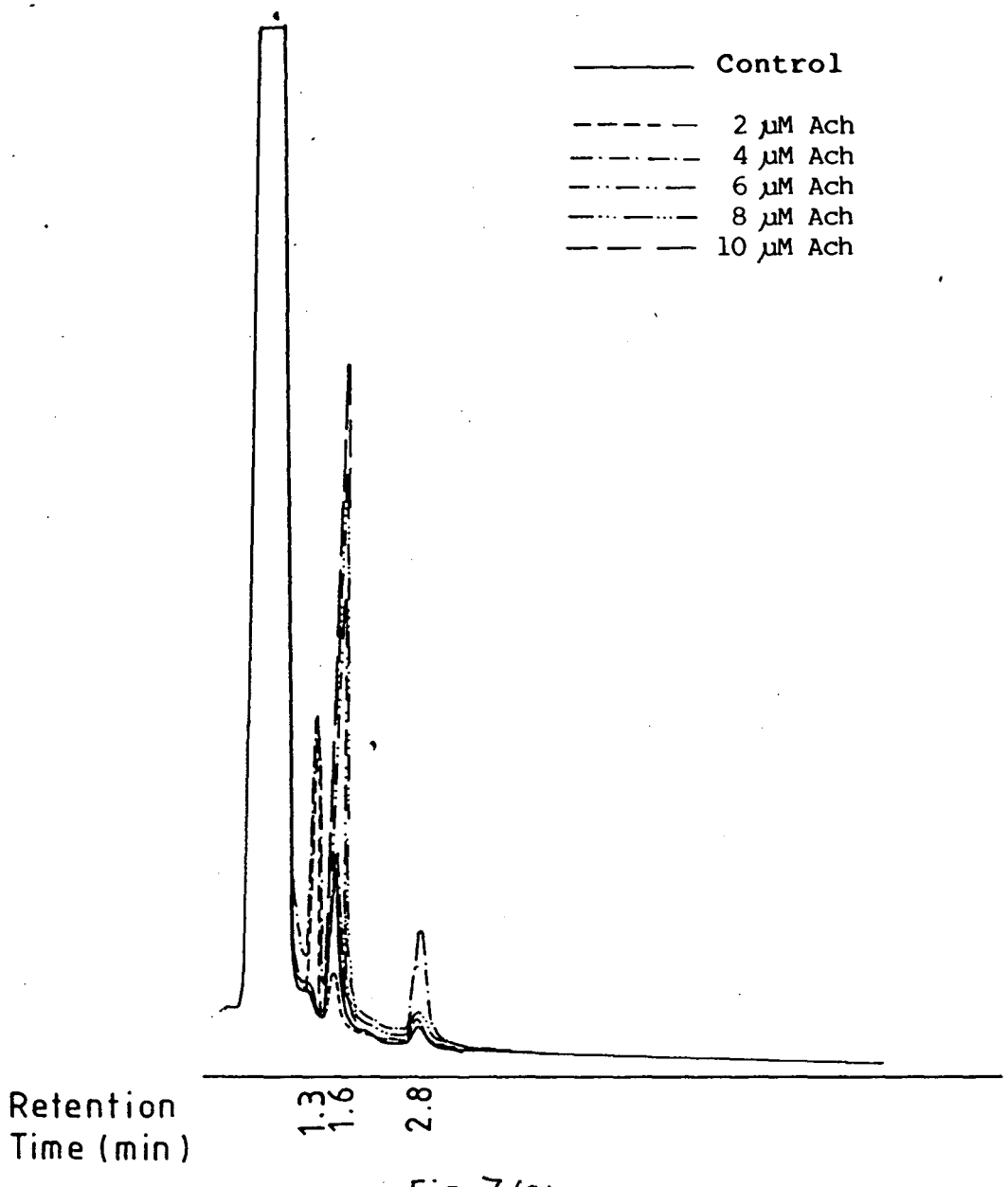

Control
2 µM Ach
4 µM Ach
6 µM Ach
8 µM Ach
10 µM Ach
Retention Time (min)
1.3
1.6
2.8

Fig. 7(a)

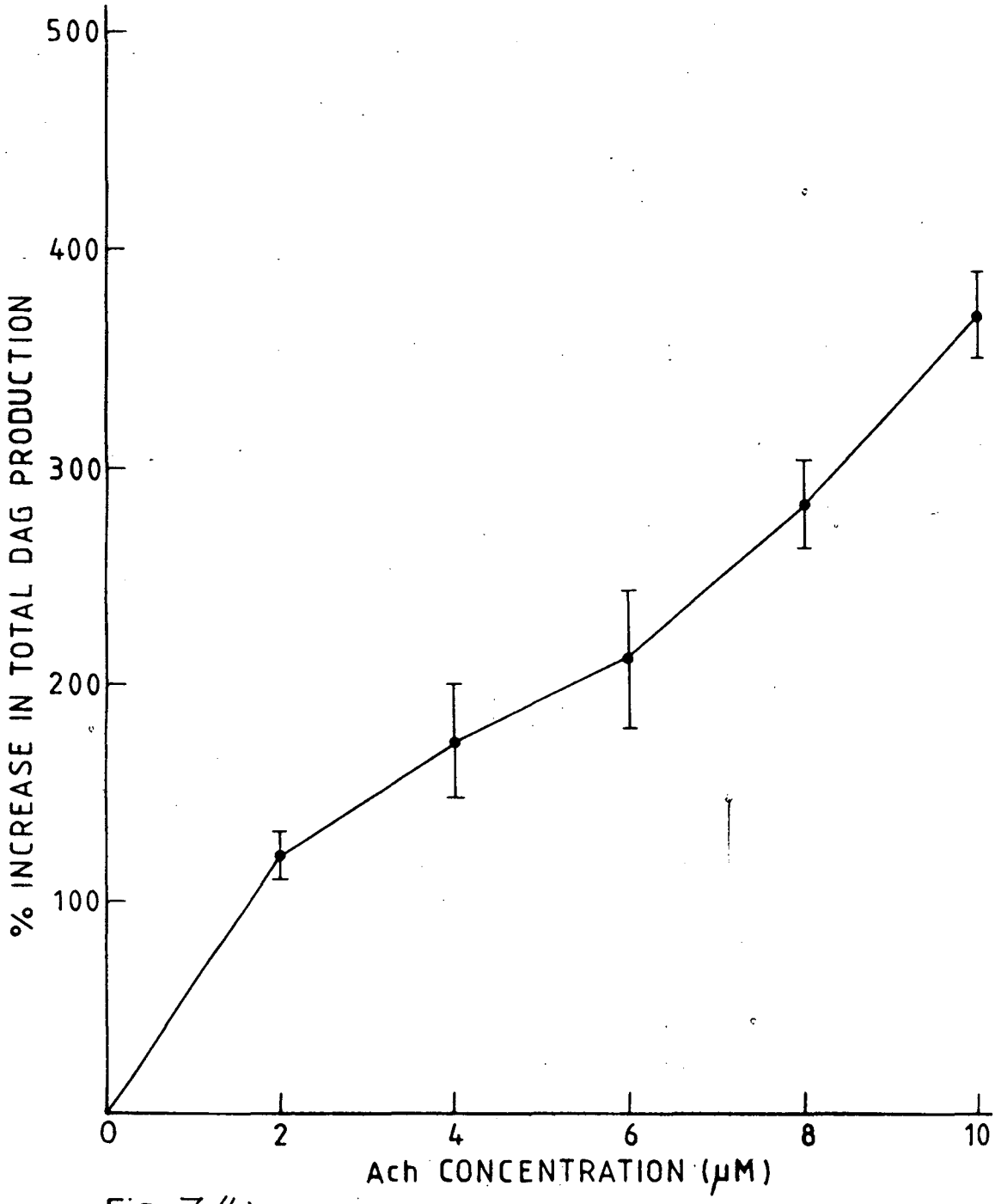

500
400
300
200
100
% INCREASE IN TOTAL DAG PRODUCTION
0
2
4
6
8
10
Ach CONCENTRATION (μM)

Fig. 7 (6).

Fig. 8 : Effect of snake venom on DAG production. PM was treated with 1 µgm of snake venom (from Naja naja) alongwith 10 µM Ach and GTP, ATP, Mg^{2+}, $AlCl_3$-NaF as given earlier. Method for GLC assay has also been described earlier. Experiment was done 5 times. Chromatogram for DAG levels is shown before and after treatment of snake venom. Control value of DAG is also shown. Peaks show DAG in terms of its fatty acid compositions and its levels in terms of area.

Decrease in levels of DAG production in response to snake venom is indicated.

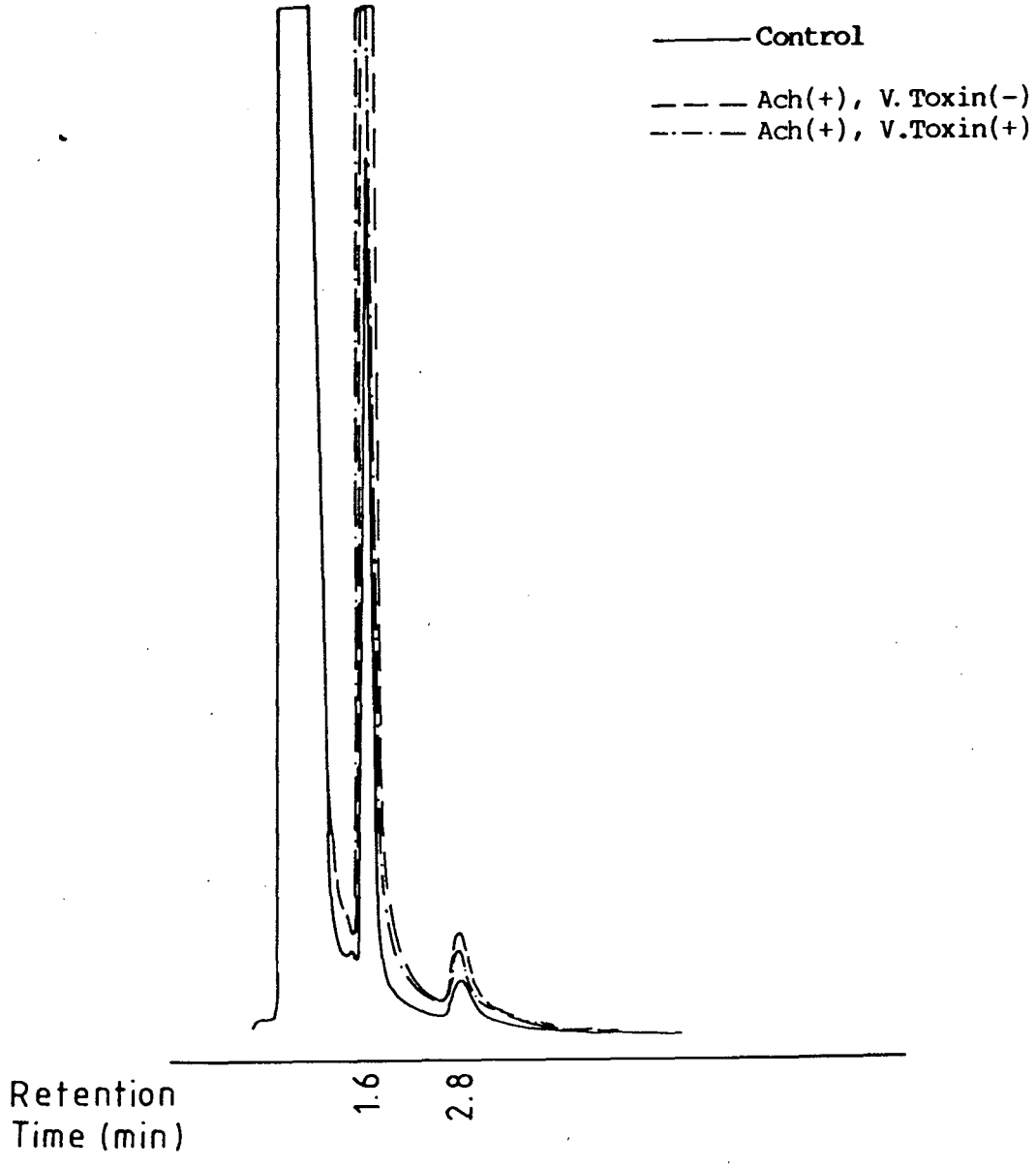


Fig. 8.

Fig. 9(a) and (b) Effect of GTP on DAG production.

PM treated and with 10 µM Ach were incubated in different levels of GTP - Zero (control), 0.2, 0.4, 0.6, 0.8 and 1.0 µM alongwith ATP, Mg^{2+}, $AlCl_3$-NaF as given earlier.

Method for GLC assay of DAG is given and described earlier. Experimet was done 4 times. Fig. 9(a) chromatogram for DAG at different GTP levels.

Fig. 9(b) curve showing the effect of different GTP levels on DAG. Curve is drawn with % increase in values of mean of 4 experiments and S.D. is shown.

An increase in DAG with increase in GTP is shown. At zero GTP no DAG production is shown.

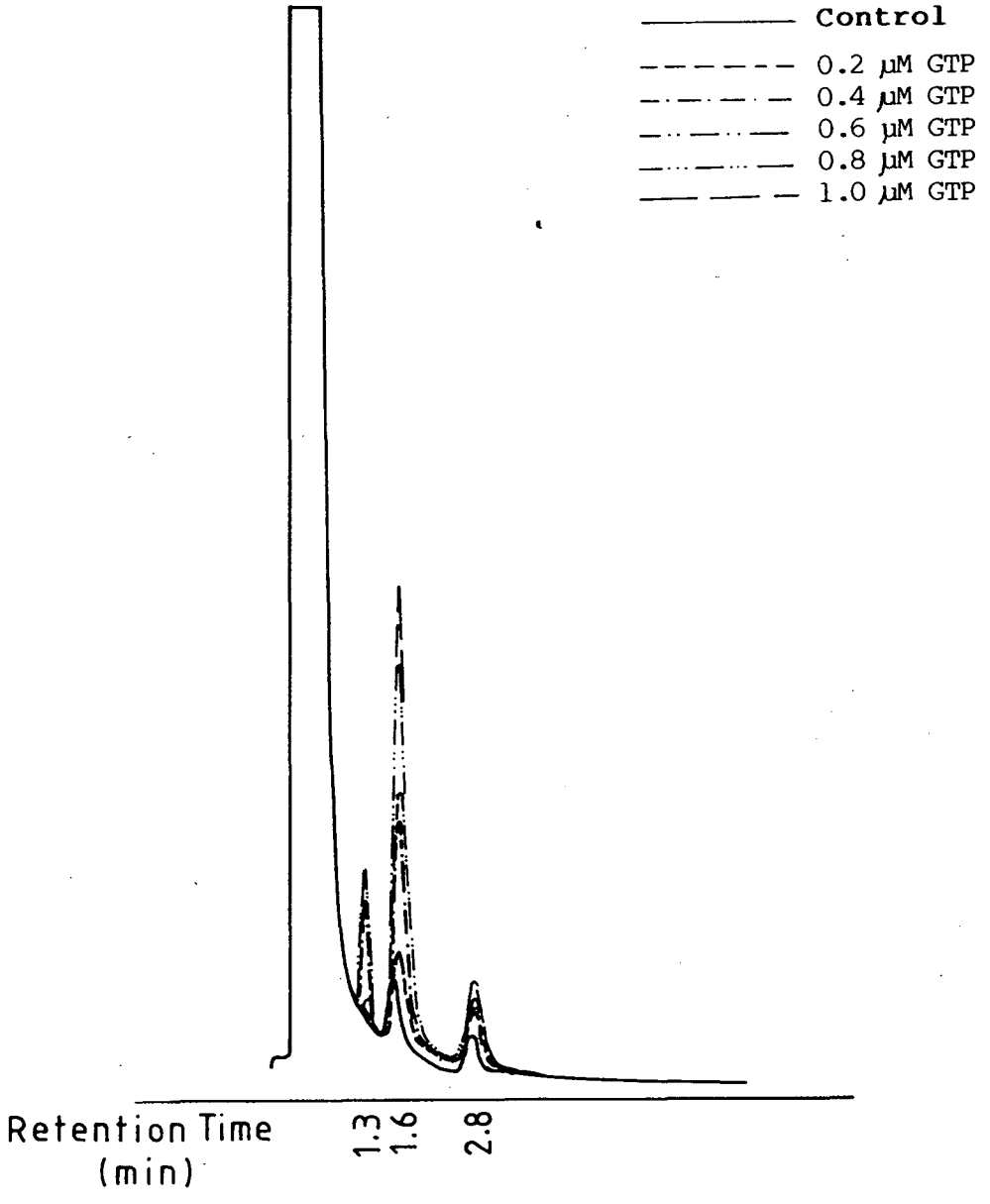

Control
0.2 μM GTP
0.4 μM GTP
0.6 μM GTP
0.8 μM GTP
1.0 μM GTP
Retention Time
(min)
1.3
1.6
2.8

Fig. 9 (a).

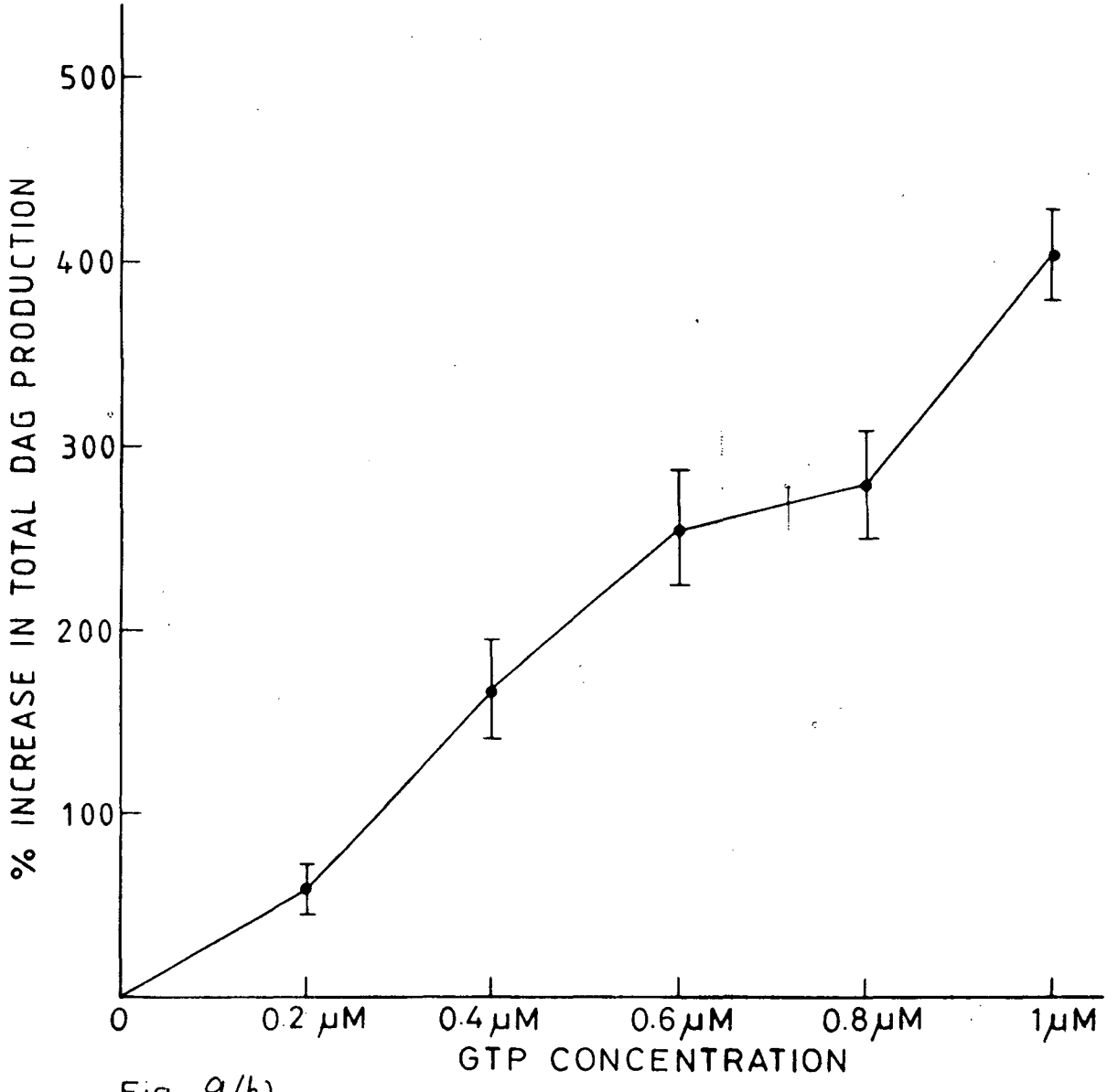

% INCREASE IN TOTAL DAG PRODUCTION
500
400
300
200
100
0
0.2 μM
0.4 μM
0.6 μM
0.8 μM
1 μM
GTP CONCENTRATION

Fig. 9(b).

Fig. 10 (a): Effect of Mg^{2+} on DAG production.
and (b)

PM isolated were treated with different concentrations of Mg^{2+} -- O(control), 2,4,6, 10 and 20 µM alongwith 10 µM Ach, GTP, ATP, $AlCl_3$-NaF as given earlier. GLC assay for DAG was done in the same way as discussed earlier. Experiment was done 5 times. Fig. 10(a) chromatogram for DAG at different Mg^{2+} levels.

Fig. 10 (b) graph showing the effects of different Mg^{2+} levels on DAG increase (% increase in total DAG levels). Curve is taken after mean of 5 experiments and S.D. is shown. DAG interms of its fatty acid composition is shown.

Increase in levels of DAG is shown in response to Mg^{2+} increase.

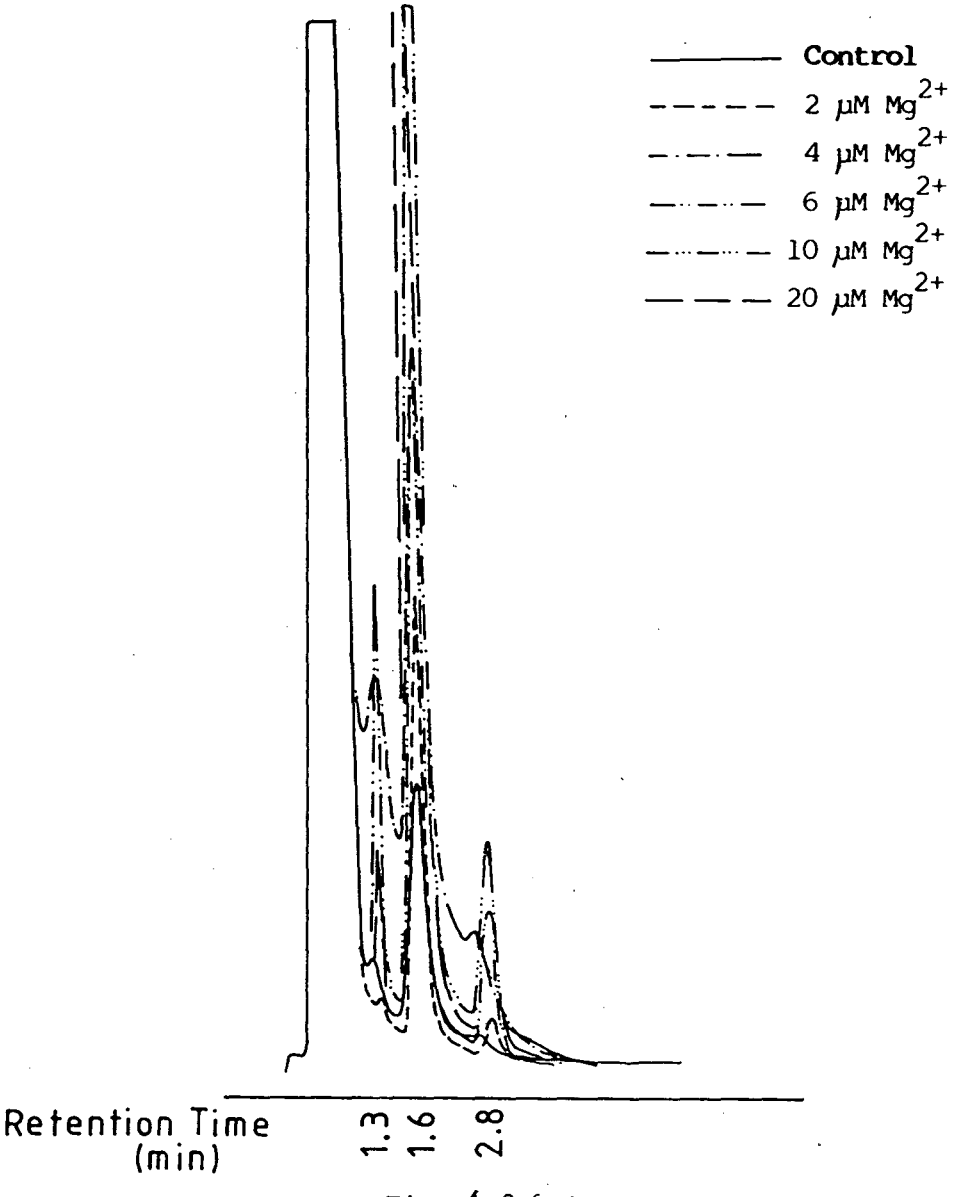

Control
2 μM Mg^{2+}
4 μM Mg^{2+}
6 μM Mg^{2+}
10 μM Mg^{2+}
20 μM Mg^{2+}
Retention Time (min)
1.3
1.6
2.8

Fig. 10(a).

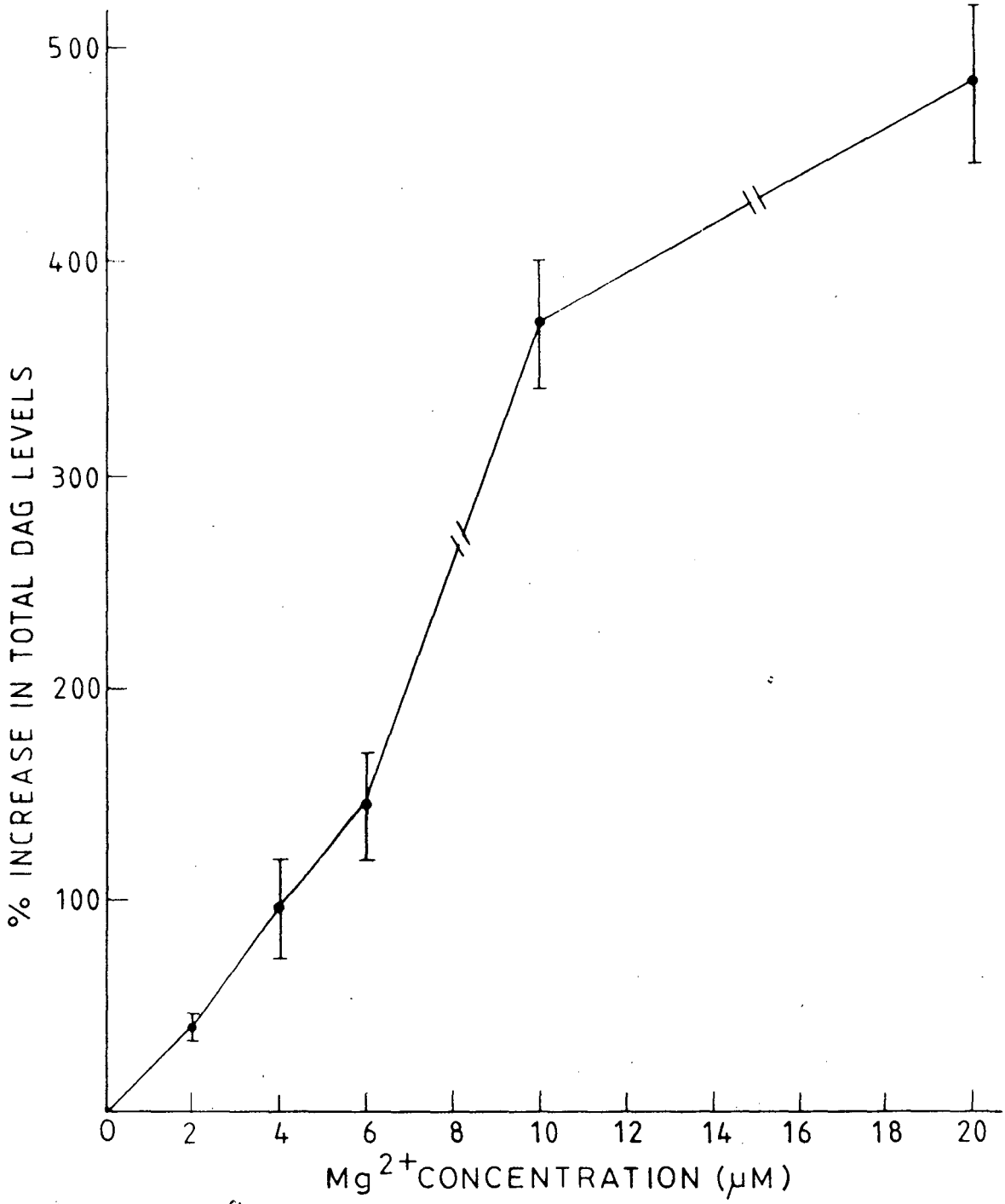

500
400
300
200
100
% INCREASE IN TOTAL DAG LEVELS
0
2
4
6
8
10
12
14
16
18
20
Mg 2+ CONCENTRATION (μM)

Fig. 10 (b).

Fig. 11 (a) and (b) Effect of $AlCl_3$-NaF on DAG production.

PM were treated with different $AlCl_3$-NaF levels - 0 (control), 2, 4, 6, 8, 10 μM alongwith 10 μM Ach and GTP, ATP, Mg^{2+} as given earlier.

GLC assay for DAG was performed by the method given earlier in this work. Experiment was done 5 times. Fig. 11(a) chromatogram for DAG at different $AlCl_3$-NaF levels. Fig 11(b) curve showing the effects of different levels of $AlCl_3$-NaF on DAG levels of production (% increase in total DAG). Curve is plotted at mean value of 5 experiments and S.D. is shown.

DAG is shown in terms of its fatty acid composition.

Increase in levels of DAG in response to increase in $AlCl_3$-NaF levels is indicated.

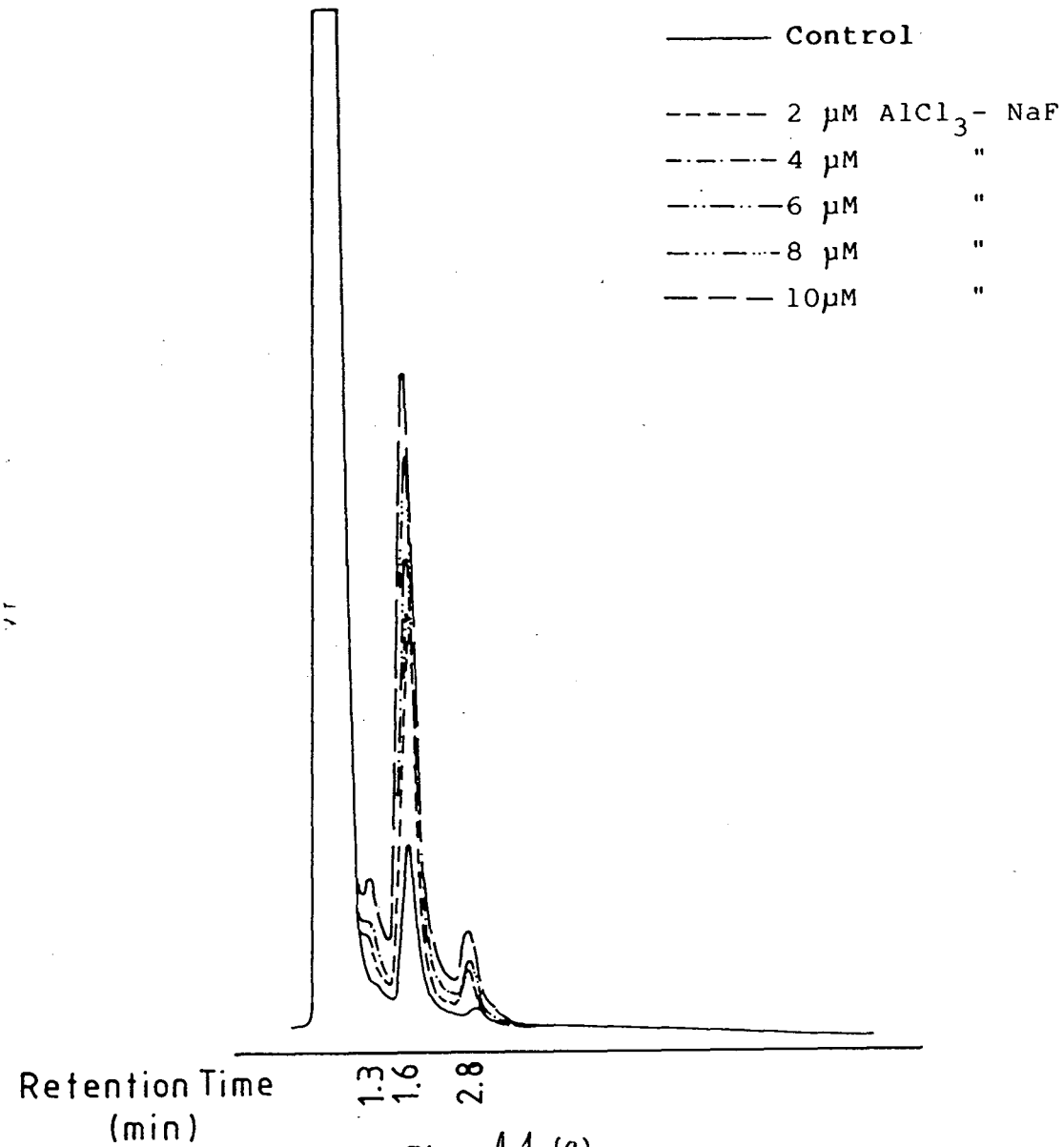

Control
2 µM AlCl3 - NaF
4 µM "
6 µM "
8 µM "
10µM "
Retention Time
(min)
1.3
1.6
2.8

Fig. 11 (a).

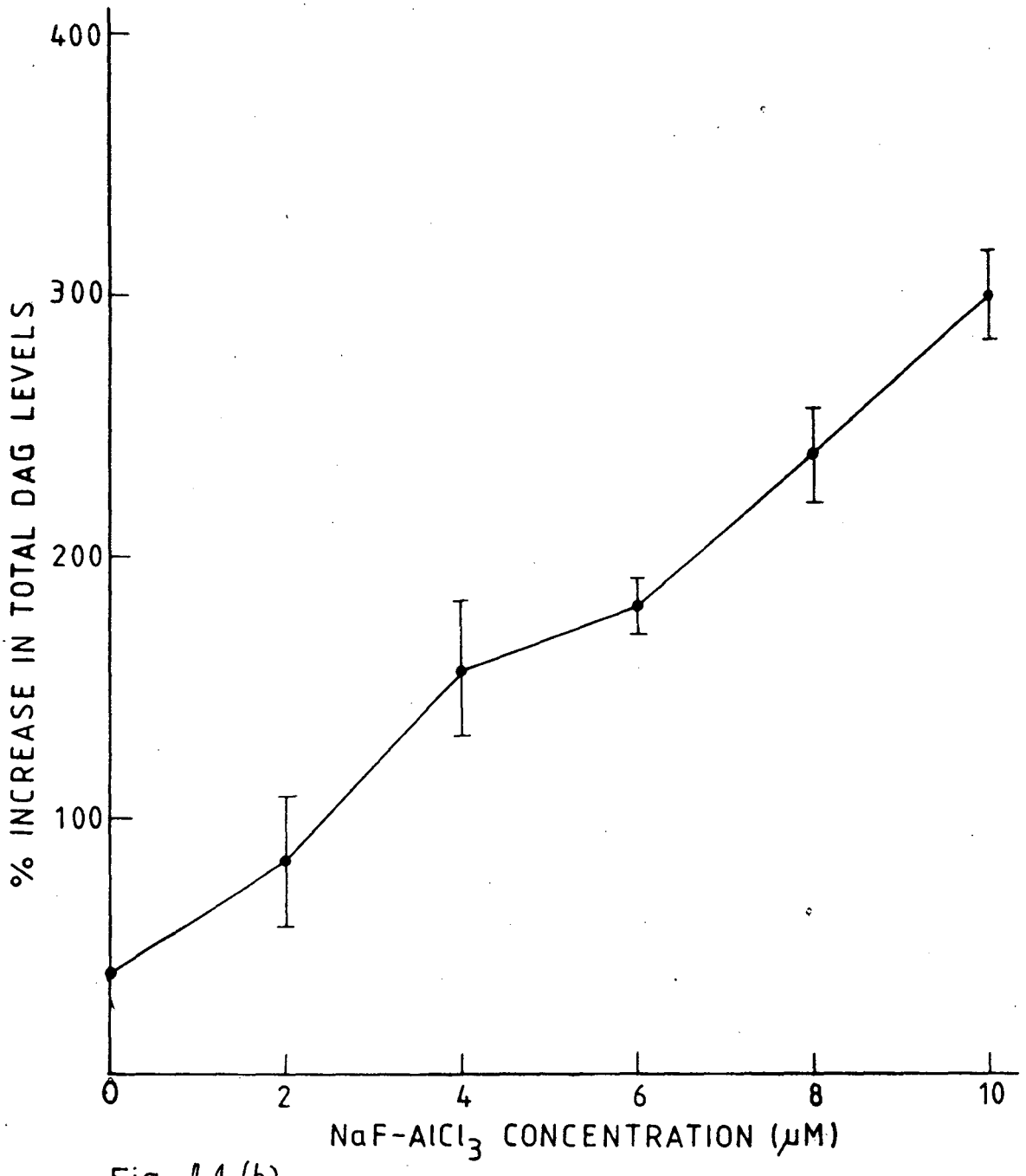

400
300
200
100
% INCREASE IN TOTAL DAG LEVELS
0
2
4
6
8
10
NaF-AlCl3 CONCENTRATION (μM)

Fig. 11 (b).

Fig.12 : Effect of cholera toxin on DAG production.

PM were treated with 1 µgm of cholera toxin alongwith 10 µM Ach in presence of NAD^+ and other materials, GTP, ATP, Mg^{2+}, $AlCl_3$-NaF as given earlier in this work. GLC assay for DAG has also been described earlier.

Experiment was done 5 times.

Chromatogram shows 3 sets.

1. Control [Ach (-), Cholera toxin (-)]

2. I [Ach (+), Cholera toxin (-)]

3. II [Ach (+), Cholera toxin (+)]

DAG levels is shown by the area of the peaks.

DAG levels was measured in terms of its fatty acid composition.

No change in DAG levels is shown in the chromatogram.

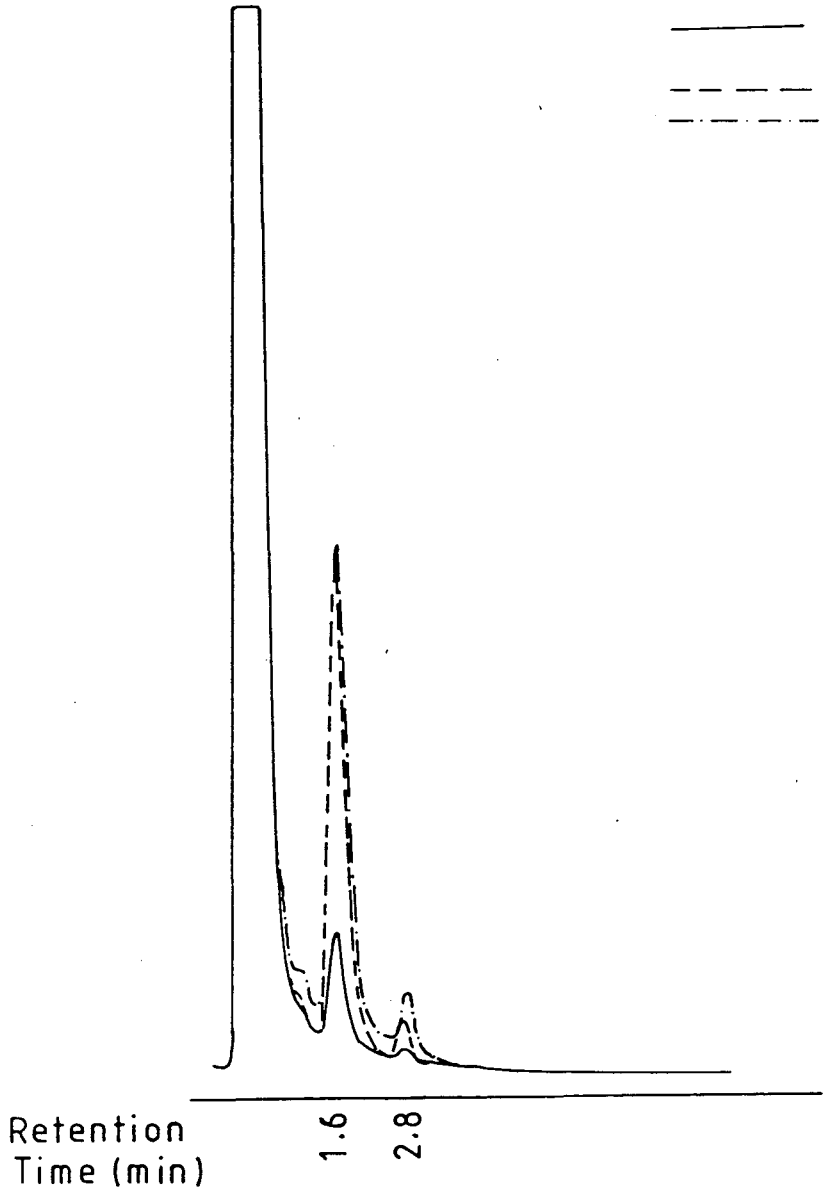


Fig. 12.

Fig.13 (a) and (b) Effect of ATP on DAG production.

PM were isolated and treated with different ATP levels - zero (control), 0.2, 0.4, 0.6, 0.8, and 1 μM alongwith 10 μM Ach and GTP, Mg^{2+}, $AlCl_3$-NaF as given earlier in this work. Method for GLC assay of DAG has also been described earlier. Experiment was done 5 times.

Fig. 13(a) chromatogram for DAG at different ATP levels.

Fig. 13(b) curve showing % increase of DAG after different ATP levels of responses. Curve is shown with mean value of 5 experiments and S.D. is shown. DAG in terms of fatty acid composition is shown.

Increase in levels of DAG in response to ATP increase is indicated.

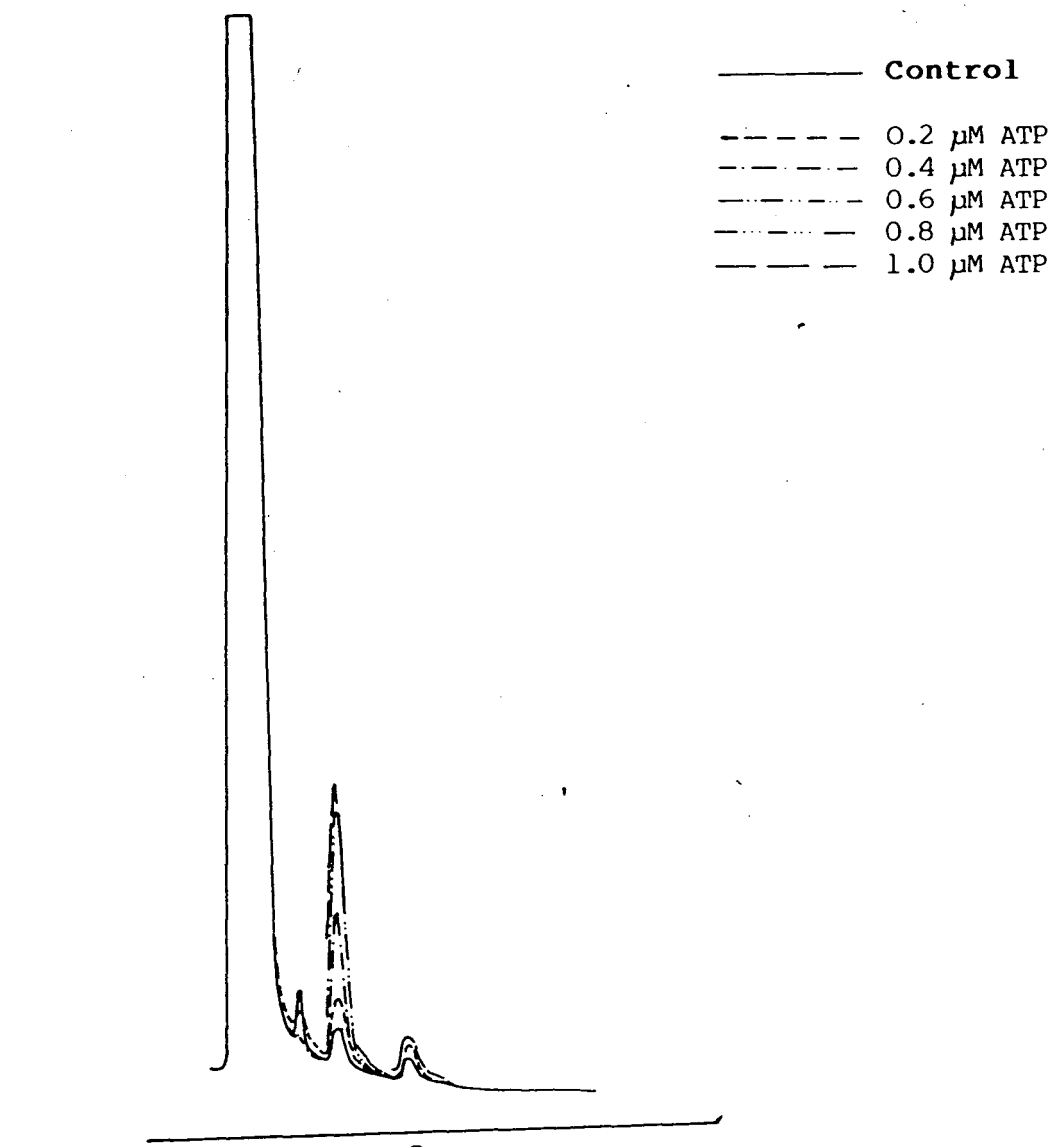

Control
0.2 μM ATP
0.4 μM ATP
0.6 μM ATP
0.8 μM ATP
1.0 μM ATP
Retention Time (min)
1.3
1.6
2.8

Fig. 13.(a).

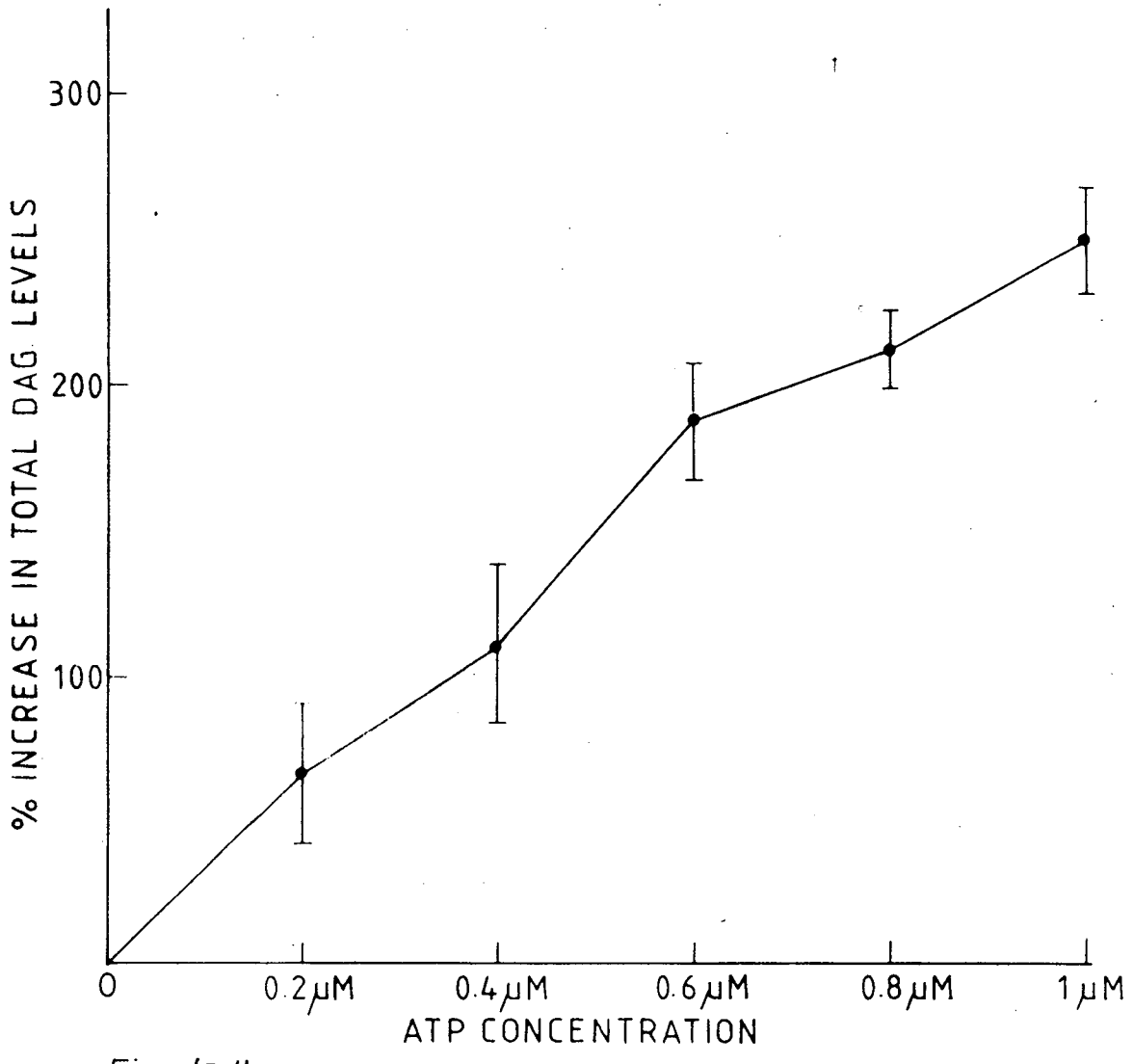

300
200
100
% INCREASE IN TOTAL DAG LEVELS
0
0.2μM
0.4μM
0.6μM
0.8μM
1μM
ATP CONCENTRATION

Fig. 13(b).

Fig.14 (a) and (b): Effect of Li^+ on DAG production.

PM were treated with different levels of Li^+ - zero (control) 1 μM, 10 μM, 1 mM and 0.1 M - along with 10 μM Ach and other materials GTP, ATP, Mg^{2+}, $AlCl_3$-NaF as given earlier. GLC assay for DAG was done as given earlier. Experiment was done 5 times.

Fig. 14(a) chromatogram for DAG at different levels of Li^+ treatment.

Fig. 14(b) curve showing the effects of different Li^+ levels on DAG production. Each value shown in the curve is a mean of experiments. S.D. is also indicated.

DAG is indicated in terms of its fatty acid composition.

Decrease in levels of DAG production in response to Li^+ is shown in the Fig.

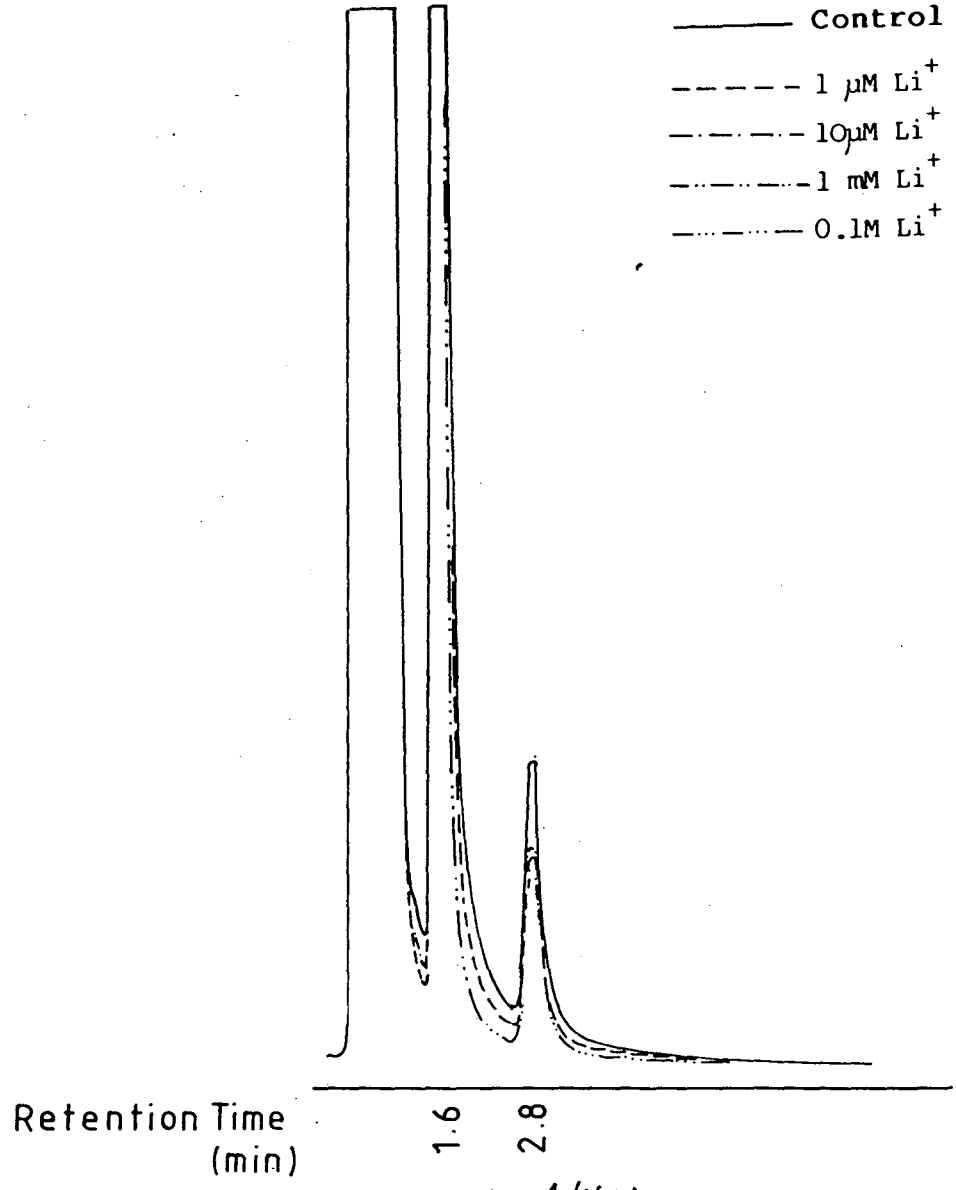


Fig. 14(a).

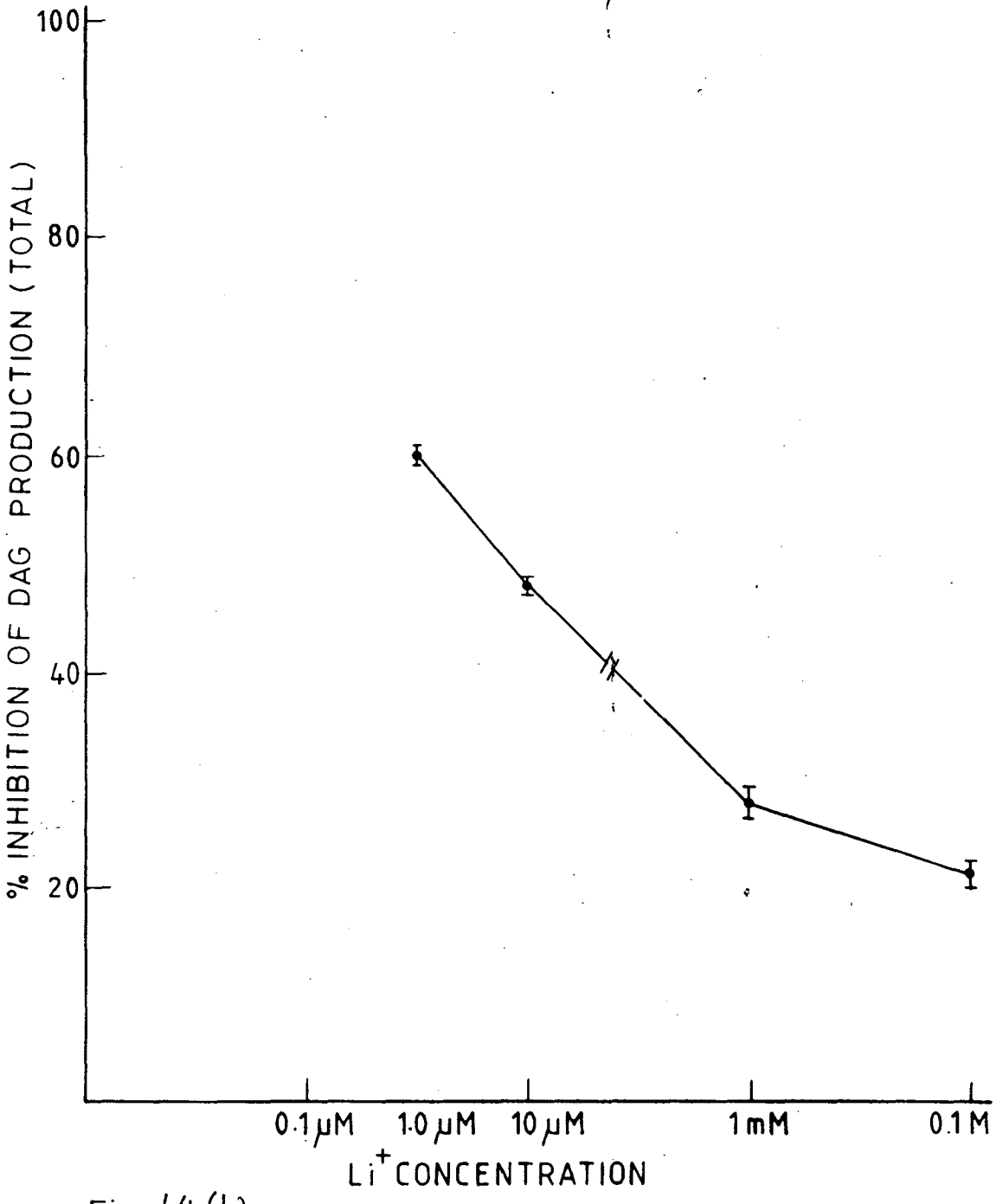

100
80
60
40
20
% INHIBITION OF DAG PRODUCTION (TOTAL)
0.1μM
1.0μM
10μM
1mM
0.1M
Li+ CONCENTRATION

Fig. 14 (b).

DISCUSSION

DAG AS CHEMICAL MESSENGER

From the forementioned experiments, DAG production during EC coupling could be demonstrated. This suggests the involvement of DAG in EC coupling phenomenon. Involvement of IP_3 and DAG as second messengers is a well studied phenomenon in various cellular processes like secretion, metabolism, smooth muscle contraction and various short-term and long-term responses at the cellular level. (Berridge and Irvine, 1984; Nishizuka, 1984; Nishizuka, 1986; Wolfman and Macare, 1987; Reddy and Bazan, 1987; Obson et al, 1989). DAG has been shown to bind protein kinase C in various cells and activates it. By activating the enzyme, protein kinase C, DAG can play a crucial role in EC coupling phenomenon.

Firstly, it can regulate the Ca^{2+} channels by the activation of Ca^{2+}ATPase (i.e. Ca^{2+} homeostasis). Secondly, protein kinase C activation through DAG can regulate the ionic concentration as per required by the cell through pathways like $Na^{+}.K^{+}$ATPase activation or deactivation and similar pathways for ionic channels, pumps and ion exchanging proteins by phosphorylation. Phosphorylation of such proteins is known in many systems(Nishizuka, 1986; Reibman et al., 1988).

Protein kinase C can also act in EC coupling by feedback regulation pathways. This seems clear from the experiment. During sub-threshold stimulus very less production of DAG indicates its non-involvement under these conditions. During threshold stimulus level at 300% increase and ≃750% to the basal level in tetanization, a stressed condition. Such a drastic increase in levels of DAG can only be attributed to the regulation of cellular metabolism by feedback mechanisms through protein kinase C. Feedback pathway for protein kinase C has been shown by many workers to be operating in various cellular processes (Rink et al., 1983; Kojima et al., 1985; Nishizuka, 1986; Fabro et al., 1988).

DAG has been known to stimulate the increase of Ca^{2+} efflux but not its uptake (Brass and Laposta, 1987; Restrep et al., 1989). So it becomes clear in the experiment on skeletal muscles that such a drastic increase of DAG (750% to basal levels) during tetanization can work in both ways, one is to stimulate the excess of Ca^{2+} efflux through protein kinase C-independent process and in other way can work for feedback pathway through protein kinase C involvement. Ultrastructural morphometry of the membrane can be altered by the DAG. Evidence comes by very small change of DAG levels on subthreshold stimulus for preparation of muscles for EC coupling phenomenon. This small change of DAG

can also be due to preparation of phosphoinositide pool for EC coupling phenomenon. Morphometric changes by DAG in the membranes have also been reported by Reibman et al. (1988). Radiolabelling of DAG was done by some workers for seeing at the changes. There is a family of protein kinase C, which is known to be activated by DAG, but have specificity for acyl groups in the individual DAGs, and even for their substrates (Taylor, 1987; Breher and Hanley, 1988).

In condition of complete exhaustion of muscles, stimulus by which the muscle could not relax at all in Ringers, DAG level increase was only of the order of≈50%. This shows inactiveness of the complete system of both the formation of DAG and it seems from this study that the DAG formation by the PIP_2 hydrolysis becomes not possible in the GNM during exhaustive condition. So the condition again shows the involvement of DAG in EC coupling. This small change can be attributed to morphometric changes in the membranes. Thus the demonstration of DAG as a chemical messenger in the signal transmission in skeletal muscles appears to be a significant contribution.

STIMULATION OF PM WITH ACETYLCHOLINE

Increased level of DAG showed the role of Ach in stimulating the DAG production during EC coupling Phenonmenon of the frog skeletal muscle.

Binding of acetylcholine on muscle PM after a stimulus is a well studied phenomenon (Huxley, 1974; Squire, 1981; Iwasha et. al., 1982; Augustine, 1987). After this binding EC coupling occurs. This phenomenon is fast and is completed within a period of 100 ms. Possibility of DAG acting as second messenger even in a fast reaction like muscle contraction is indicated in the experiment.

During muscle contraction DAG released causes Ca^{2+} release from intracellular stores i.e. SR. Moreover, DAG can control the levels of Ca^{2+} inside the cell through activation of protein kinase C. Release of Ca^{2+} by DAG independent of protein kinase C has been shown in other systems also (Brass and Lapsata, 1987; Restropo et. al., 1989). By this experiment the nature of chemical messenger in EC coupling becomes clear. In adition IP_3 the second messenger of the same bifurcating pathway also mobilizes Ca^{2+} from intracellular stores by receptor mechanism (Volpe et al., 1985; Vergara et al., 1985; Gouri and Habibullah, 1988).

EFFECT OF SNAKE VENOM

Inhibition of DAG production by snake venom clearly indicates that increase in production of DAG in PM during stimulation was in response to Ach binding to the receptor. Competition for the same receptor by cobratoxin (Naja naja)

has already been reported by Stryer (1988). When toxin competes for the same receptor, inhibition of DAG production is brought about thereby effecting the EC coupling phenomeon. With 1µgm of toxin ≃ 30% inhibition of DAG production could be demonstrated. Such inhibition by cobratoxin again highlights the involvement of DAG in EC coupling phenomenon in the skeletal muscle of frog.

INVOLVEMENT OF G PROTEINS AS TRANSDUCERS IN THE SKELETAL MUSCLE CONTRACTION

The EC coupling phenomenon is dependent upon several factors as seen in our present experiments.

GTP dependence: In the absence of GTP, the muscle does not contract. The reason for this probably is that GTP is a prerequisite for the action of receptor bound Ach and subsequent production of DAG.

Requirement of GTP for hormonal activation of adenylyl cyclase was described by the involvement of a protein, G protein (Rodbell et al., 1971). The function ascribed to it was in transmembrane signalling. This transmembrane signalling was only possible when any agonist binded to its receptors. Crucial were the studies then made by Cassel and

Selinger (1976), who first assayed catecholamine-stimulated GTPase activity in turkey erythrocyte membranes. Observations made by him and other later on proved the activation of GTP-linked systems on binding of GTP, hydrolysis of which leading to its deactivation. Later studies involving the bacterial toxins (from Bordetella pertussis, Pertussis toxin and Virbrio cholerae, cholera toxin) also proved useful in understanding the mechanism of function of G proteins as transducers in transmembrane signalling (Cassel and Pfeuffer, 1978; Katada and Ui, 1982; Gilman, 1984; Brandt and Ross, 1986; Spicher et al., 1988).

Mg^{2+} involvement:

Requirement of Mg^{2+} for the production of DAG clearly indicates its role in EC coupling phenomenon. These results of involvement of Mg^{2+} in skeletal muscle is comparable to other processes like secretion, metabolism, smooth muscle contraction in response to various stimuli as studied earlier (Pederson and Ross, 1982; Asano and Ross, 1984; Gilman, 1984; Brandt and Ross, 1986; and Gierschik et al., 1988). Their studies shows that there are binding sites for Mg^{2+} on the G protein which stimulates the GTP binding to G proteins in response to agonist and hence the G protein activation and transduction of the signal to phospholipase C to produce IP_3

and DAG. Involvement of Mg^{2+} in EC coupling phenomenon and production of DAG in GNM clearly indicates the presence of G protein in the PM and transmembrane signalling to phospholipase C for the production of DAG and which may further activate and regulate the EC coupling phenomenon.

Cholera toxin treatment

No inhibition of stimulated levels of DAG by cholra toxin in presence of NAD^{+} clearly indicated the involvement of other G proteins in addition to Gs, stimulatory G protein. Cholera toxin (from Vibrio cholerae) is known to ADP-ribosylate a class of G proteins in many types of cells and their membranes (Cassel and Pfeuffer, 1978;Ohta et al., 1985). Gi and Go types of G proteins are known to be the substrates for cholera toxin and pertussis toxins. Observations of having no effect on the IP_3 and DAG production has been reported in cardiac myocytes and fibroblasts (Murayama et al.,1985). Our experiments with cholera toxin indicated the involvement of Gi, inhibitory G protein and G_o, Other G proteins in resemblance with experiments by others. It can be derived that on cholera toxin treatment Gi is inhibited and stimulated levels of DAG are not inhibited through Gi. GTP involvement for Gi action has also already been reported by Cassel and Pfeuffer (1978).

$\underline{AlCl_3\text{-}NaF}$

Increase of DAG production with addition of $AlCl_3$-NaF indicated involvement of G protein in the EC coupling phenomenon. Only small increase in DAG production in its absence showed that it has something to do with the deactivation of G protein. In absence of Al^{3+} no action of NaF showed the involvement of GTP hydrolysis after it was bound to the G protein. Similar observations were made by Sternweis and Gilman (1983), Blackmore et al., (1985), Paris and Poyssegur (1987). It was shown by these, AlF_4^- was the ion which inhibited the GTP hydrolysis in the system. Observation again confirms the involvement of Ach - Receptor - G protein - phospholipase C pathway for the chemical messenger formation in EC coupling phenomenon in skeletal muscles of frog.

Above studies clearly indicate the involvement of G protein during EC coupling. When agonist (Ach) binds to the receptor, GTP binds to G protein in presence of Mg^{2+}. Reaction of hydrolysis of GTP to GDP is blocked by AlF_4^-. Then the signal is transmitted to enzyme phospholipase C for the production of DAG. PIP_2 pool is utilized for its production. DAG can mobilize Ca^{2+} from intracellular stores, stimulates the efflux of Ca^{2+} from PM and activates the enzyme protein kinase C to regulate the EC coupling phenomenon by acting through feedback pathways. Thus EC

coupling phenomenon is well programmed and regulated process even for stressed conditions like tetanization. Involvement of Gi is indicated by ADP-ribosylation of Gi by cholera toxin in presence of NAD^+. During incubation the action of Gi is inhibited and muscles remain in stimulated condition.

By all the studies made above following scheme can be drawn for EC coupling phenomenon in GNM of frog Rana hexadactyla

Stimulus

↓

Acetylcholine release from presynaptic junctions

↓

Binding of Ach to its receptors on the GNM membranes

↓

Binding of GTP to the G protein and its activation

↓

Transduction of signal of Ach binding to the receptor and then to phospholipase C by G protein

↓

Activation of phospholipase C.

↓

PIP_2 hydrolysis and production of IP_3 and DAG

↓

Release of Ca^{2+} from intracellular stores (SR) by combined action of DAG and IP_3 and its regulation.

↓

Binding of Ca^{2+} to tropnin

↓

Contraction of muscles

↓

Relaxation of muscles

EFFECT OF ATP AND ITS INVOLVEMENT

Experiments clearly show the involvement of ATP in DAG production and thus EC coupling phenomenon. The action of ATP can occur by various possible ways in EC coupling phenomenon:

1. It is possible that ATP maintains the activity of G protein by maintaining GTP levels through trans-phosphorylation.

2. ATP is required for resynthesis of phosphoinositides in the membrane, otherwise once phosphoinositide pool sensitive to agonist becomes utilized, its resynthesis would not be possible in absence of ATP.

3. Its requirement for opening and closing of channels by phosphorylation for the ions and Ca^{2+} homeostasis in particular.

4. For the activation of enzymes by phosphorylation which resynthesizes phosphoinositide pool.

5. Activation of phospholipase C. Requirement of -γp of ATP may be necessary by some protein to activate it. Evidence for the above functions of ATP comes by the studies of Litosch et al. (1985), Smith et al. (1985) Brass and Joseph (1985), Smith et al. (1985) and Uhing et al. (1986).

ATP was involved in phosphorylation and dephosphorylation through a protein which regulated the activity of phospholipase C.

Incubation of PMNs with labelled ATP in presence of $MgCl_2$ resulted in labelled phsophatidic acid, phosphoinositol monophosphate (PIP) and phosphatidyl inositol biphosphate (PIP_2).

PIP_2 metabolism was restricted to PM and incubation with pertussis toxin prevented PIP_2 breakdown. Dependence of IP_3 production on ATP has not been shown earlier.

In presence of ATP, GTP is almost as effective as its non-hydrolyzable analogues, GTP γ S and Gpp(NH)p in comparable concentrations.

Their studies also showed that GTP labelled was rapidly hydrolyzed by the high concentration of PM and ATP. When permeabilized neutrophils from man were incubated in a medium containing respective substrates and Mg^{2+}-ATP, they are able to maintain ambient (Ca^{2+}) steady state. This steady state was maintained by a vesicular and Mg^{2+}-ATP-dependent pools. So dependence on ATP directly suggested the mobilization from intracellular stores but not mitochondrial source (Prentki et al., 1985).

Above experimental evidence and studies clearly indicates the role of ATP in EC couplding phenomenon by participating in the resynthesis of inositol phospho-lipids in the membrane transphosphorylation of GTP and also as a source of energy for muscle contraction.

EFFECTS OF LITHIUM

Li^+ clearly shows the inhibition of DAG production during stimulation with Ach. It can be concluded that it has a role in inhibition of DAG through the following possible ways:

1. By increase in metabolic products of DAG and IP_3 and their accumulation.

2. Blocking of resynthesis of phosphoinositide pool in the PM by inhibition of enzyme activities of resynthesizing enzymes.

3. It can inhibit the binding of hormones to the receptors.

4. By partial inhibition of phospholipase C directly or indirectly.

Effect of Li^+ becomes important as it is of therapeutic value in case of <u>mania</u> and <u>depression</u>. Li^+ effects on phosphoinositide metabolism were investigated by many workers

(Berridge et al., 1982; Sherman et al., 1985; Sherman et al.. 1986). Their studies reveal the accumulation of Ins.1P by the Li^+. This accumulation was due to the inhibition of Ins.1 phosphatase. When Li^+ was withdrawn from the reacton mixture the activity of phosophatase was restored. No accumulation of Ins.5P was seen by them. No report is available regarding the IP_3 level, after Li^+ treatments. Lowering of myo-inositol pool in membranes was observed after prolonged stimulation with Li^+ by these workers due to inhibition of enzyme of resynthesis of phosphoinositides in the membrane. Half-maximal inhibition by Li^+ was observed by these workers at 1 mM Li^+ levels. In smooth muscles no effect on the hormone binding was observed in response to Li^+. Lowering of level of myo-inositol could lead to a decrease in concentration of phosphatidylinositol especailly in neurons which were stimulated excessively. Most of the Ins. IP accumulated came from phsophoinositides in the membrane by phospholipase C action and 10% from de novo synthesis of glucose.

The present experiment clearly shows that the Li^+ could affect the DAG production by the lowering of levels of pools sensitive to agonist and not by inhibition of phospholipase C and inhibition of Ach binding to its receptor. Half-maximal inhibition in this case was also observed at 1 mM Li^+ as reported earlier.

Studies made above regarding the EC coupling phenomenon in GNM of frog, Rana hexadactyla, clearly shows the involvement of G protein in EC coupling phenomenon. G Protein must be acting as transducer for the production of chemical messengers inside the muscle cells in response to Ach. Activation could occur by binding of Ach to its receptor and production of chemical messengers IP_3 and DAG occurred through phsopholipase C. IP_3 and DAG mobilizes Ca^{2+} from SR and after that it binds to troponin. EC coupling is completed by the sliding of thin and thick filaments with involvement of ATP. ATP played role in phosphoinositide metabolism besides as a source of energy for contraction.

Effects of Li^+ also showed that Li^+ had an inhibitory effect on phosphoinositide metabolism in PM. This phosphoinositide is utilized as substrate for the chemical messengers, IP_3 and DAG, production. Our studies showed the important position occupied by DAG in skeletal muscles during EC coupling. Cellular metabolism is also controlled by DAG through protein kinase C-dependent or protein kinase C-independent processes.

By understanding the chemical messengers and G protein involvement in EC coupling phenomenon it will become easy to fill up the gap which existed in understanding clearly the

phenomenon of EC coupling. In the frog skeletal muscle contraction the involvement of the second messenger shows that the EC coupling phenomenon is also mediated through the chemicals and is not electrical.

CONCLUSION

CONCLUSION

Experimental evidences given above indicated the involvement of DAG as chemical messenger in EC coupling phenomenon in skeletal muscle of frog Rana hexadactyla. When electrical stimulation is given through nerve, at sub-threshold levels of stimulus the DAG produced is very less of the order of 10%. The increease in DAG production at threshold level of stimulus is around ≈300% to the basal level, which on tetanization is ≈ 750%. The increase in DAG level was very less in complete exhaustive stimulus, ≈50%. At subthreshold stimulus the preparative phase for EC coupling and phosphoinositide metabolism occurred. At threshold stimulus its productivion was stimulated for its action was second messenger for mobilization of Ca^{2+} by protein kinase C-dependent and protein kinase C-independent processes. During tetanization a stressed condition more DAG was produced for regulation of cell metabolism and EC coupling. At irreversible exhaustion the muscles became almost inactive and whole of the phosphoinositides was used by or was not resynthesised.

DAG production in response to stimulus is caused by the release of acetylcholine and its binding to the receptor. Binding of Ach to the receptor activates the process through transmembrane signalling. Receptor, G protein and

phospholipase C are involved in the signalling. Snake venoms are Naja naja binds to the acetylcholine receptors and decreases theproduction of DAG.

When acetylcholine binds to the receptor, G protein is activated by the binding of GTP to it. Mg^{2+} is essential in the medium for binding of GTP to G protein. Hydrolysis of G protein bound GTP to GDP causes its deactivation. Continuous binding of GTP to G protein is maintained through the availability of AlF_4^-. AlF_4^- puts the GTP hydrolysis 'off' when it is bound to G protein. Gi is also involved in EC coupling phenomenon and is ADP-ribosylated with cholera toxin in presence of NAD^+.

The function of G protein is the transduction of signal from receptor to phospholipase C. DAG production occurs by hydrolysis of PIP_2 in the PM bythe enzyme, phospholipase C activated by G protein.

ATP is required for the EC coupling phenomenon. One purpose is for energy required during the muscle contraction andrelaxation. Second purpose is for the transphosphorylation of GTP which is rapidly metabolized during the activation and deactivation of G protein. The third purpose isthat it is required for theresynthesis of phosphoinositides. Phosphoinositides are utilized as

substrate during EC coupling for the production of IP_3 and DAG and regulation of ionic as well as Ca^{2+} channels, phospholipase C activity indirectly.

Li^+ inhibitd the production of DAG after Ach stimulation. The inhibition occurred due to accumulation of products of IP_3 and DAG, and inhibition of pathway of resynthesis of phosphoinositides in the membranes. Thus phosphoinositide pool in membranes, which is the only source for IP_3 and DAG productionduring EC coupling phenomenon, gets depleted through inhibition by Li^+.

So by this study the phenomenon of EC coupling becomes clear in skeletal muscle of the frog. Ach binds to the receptor and phospholipase C is activated through a G protein, IP_3 and DAG are produced as second messengers. IP_3 and DAG mobilizes and regulates the Ca^{2+} levels from intracellular stores i.e. SR. Ca^{2+} binds to the troponin inthe muscles and contraction occurs. DAG regulates the process of EC coupling through protein kinase C-dependent and protein kinase C-independent pathways. Probably the same chemical mechanism operates in the skeletal muscle contraction of the higher vertebrates also.

BIBLIOGRAPHY

REFERENCES

Aidley D J (1971): In: The Physiology of excitable cells. Cambridge University Press, London.

Allan D and Cockcroft S (1983) The fatty acid composition of 1,2-sn-diacylglycerol and polyphosphoinositides from human erythrocyte membranes. Biochem. J. 213 : 555-557.

Anderson T et al. (1986) Leukotriene B_4 stimulation of phagocytes results in the formation of IP_3. Biochem. J. 240 : 333-340.

Asano T and Ross E M (1984) Reconstitution of catecholaminé-stimulated binding of guanosine 5'-o-(3-thiotriphosphate to the stimulatory GTP-binding protein of adenylate cyclase. Biochemistry. 23 : 5467-5477.

Ashendel C L. (1985) Activation of protein kinase C by monoacyl-derivative of DAG (OAG) or Phorbol esters. Biochim. Biophys. Acta 822 : 219-242.

Augustine G J (1987) Ca^{2+} action in synaptic transmitter release. Ann. Rev. Neurosci. 10 : 633-693.

Barry D T (1987) Acoustic signals from frog skeletal muscles. Biophys. J. 51 : 769-773.

Bazzi M D and Nelsestuen G L (1989) Properties of protein kinase C - Phorbol ester interaction. Biochemistry 28 (8), 3577-3585.

Berg J M (1986) Potential metal-binding domains in nucleic acid binding proteins. Science 232, 485-

Berridge M J et al (1982) LI^{+} amplified Agonist-dependent phosphoinositol responses in brain and salivary glands. Biochem. J. 206 : 587-595.

Berridge M J and Irvine R F (1984) IP_3 and DAG as second messengers. Biochem. J. 320 : 345-360.

Bertorella A and Aperia A (1989) $Na^{+}K^{+}$ ATPase is an effector protein for protein kinase C in several proximal tubule cells. Amer J. Physiol. 256 (2) P2 : F370.

Betz W J, Caldwell J H and Kinnamon S C (1984) Increased K^+ conductance in the synaptic region of rat skeletal muscle fibers. J. Physiol. 352 : 180-202.

Biden T J and Wollheim C B (1986) Ca^{2+} regulates the inositol Tris/Tetrakisphosphate pathway in intact and broken preparation of insulin-secreting cells. J. Biol. Chem. 261 :11931-11934.

Blackmore P F, Boccking S B, Warynick L E and Extron J H (1985) Role of Guanine nucleotide-binding regulatory protein in the hydrolysis of hepatocytes PIP_2 by Ca^{2+} mobilizing hormones and control of cell Ca^{2+} studies utilizing AIF^-_4. J. Biol. Chem. 260 : 14477-14483.

Bokoch G M et al (1983) Identification of the predominent substrate for ADP-ribosylation by IAP. J. Biol. Chem. 258, 2072-2075.

Bokoch G M et al. (1984) Purification and properties of inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. J. Biol. Chem. 259 : 3560-3567.

Bokoch G M and Gilman A G (1984) Inhibition of receptor-mediated release of Arachidonic acid by pertussis toxin. Cell 39. 301-308.

Boon A M, Beresford B J, and Mellars A. (1985) A tumor promotor enhances the phosphorylation of phosphoinositides while decreasing phosphatidylinositol labelling in lymphocytes. Biochem. Biophys. Res. Commun. 129: 431-436.

Brandt D R and Ross E M (1986) Catecholamine-stimulated GTPase cycle. J. Biol. Chem. 261 : 1656-1664.

Brass L F and Joseph S K (1985) A role of IP_3 in intracellular Ca^{2+} mobilization and granule secretion in platelets. J. Biol. Chem. 260 : 15172-15179.

Brass L F and Laposata M (1987) DAG causes Ca^{2+} release from the platelet dense tubular system : comparison with Ca^{2+} released by IP_3. Biochem. Biophys. Res. Commun. 142 (1): 7-14.

Breitwieser G E and Szabo G (1985) Uncoupling of cardiac muscarinic and B-adrenergic receptors from ion channel by a guanine nucleotide analogue. Nature 317, 538-540.

Brocklehurst K W, Morita K and Polard B H (1985) Activation of protein kinase c through Ca^{2+} entering via voltage-dependent Ca^{2+} channels. Biochem. J. 228 : 35-42.

Brown R D, Berger K D and Taylor, P. (1984) -adrenergic receptor activation mobilizes cellular Ca^{2+} in a muscle cell line. J. Biol. Chem. 259 : 7554-7562.

Burgees G M, Godfey P P, McKinney J S, Berridge M J, Irvine R F and Putney Jr. J W (1984) The second messenger linking receptor activation to internal Ca^{2+} release in liver. Nature 309 : 63-66.

Burgees G M, Irvine R F, Berridge M J, McKinney J S, Putney J W. (1984) Activation of inositol phosphates on Ca^{2+} pools guinea pig hepatocytes. Biochem. J. 224 : 741-746.

Carson, R L. et al (1985) DAG-kinase of E. coli : Purification, reconstitution and partial amino acid analyses of amino- and carboxyl-terminals. J. Biol. Chem. 260 (7) : 4091-4097.

Cassel D and Pfeuffer T (1978) Mechanism of cholera toxin action, Covalent modification of the guanyl nucleotide-binding protein of adenylate cyclase system. P.N.A.S (USA) 75(6) : 2669-2673.

Cassel D and Selinger Z (1976) Catecholamine-stimulated GTPase activity in turkey erythrocyte membranes. Biochim. Biophys. Acta 452 : 538-551.

Cerione R A et al. (1985) A role for Ni in hormonal activation of adenylate cyclase. Nature 318 : 293-298.

Cheng Y S E et al. (1985) Affinity purification of an interferon induced human guanine-binding protein and its characterization. J. Biol. Chem. 260 : 11834-15839.

Cockcrofts S and Gomperts B D (1985) Role of guanine-nucleotide-binding protein the activation of polyphosphoinositide phosphodiesterase. Nature 314 : 534-536.

Codina J et al. (1983) Pertussis toxin substrate, the putative Ni component of adenylate cyclase in an heterodimer regulated by guanine nucleotide and Mg^{2+}. P.N.A.S. 80 :4276-4280.

Codina J et al (1984) Ns and Ni, the stimulatory and inhibitory regulatory components of adenylate cyclase. J. Biol. Chem. 259 : 5871-5886.

Cooke R (1986) The mechanism of muscle contraction. CRC. Critical Rev. inBiochem. 21 : 53-118.

Coussens L et al (1986) Multiple, distinct forms of bovine and human protein kinase C suggests diversity in signalling pathways. Science 233 : 859-866.

Das S and Rand R P (1984) DAG causes major structural transitions in phospholipid bilayer memranes. Biochem. Biophys. Res. Commun. 124 : 491-496.

Dell K R and Severson D L (1989) Effect of cis-unsaturated fatty acid on aortic protein kinase C activation. Biochem. J. 258 : 171-175.

Deretic D and Hamm H E (1987) Topographic analysis of antigens determinants recognized by monocolonal antibodies to the photoreceptor guanyl nucleotide-binding protein, transducin. J. Biol. Chem. 262 : 10839-10847.

Dobson P R M et al (1989) Interleukin induces PT-sensitive increase in DAG accumulation in mouse thymoma cells. J. Mol. Endocrinology 2 : R5-R8.

Dougherty R W, Godfrey P P, Hoyle P C, Putney J W, Freher R J. (1984) Secretagogue-induced phosphoinositides metabolism in human erythrocytes. Biochem J 222 : 307 - 314.

Dougherty R W and Niedel J E (1986) Cytosolic Ca^{2+} regulates phorbol ester binding affinity inintact phagocytes. J. Biol. Chem. 261 : 4097-4100.

Downing J E G and Role L W (1987) Activators of protein kinase C enhance acetylcholine receptor desensitization in sympathetic ganglion neurons. P.N.A.S. (USA) 84 : 7739-7743.

Dreher M L and Henley M R (1988) Multiple modes of regulation of protein kinase C and their significance in signalling. Trends in Pharmaocological Sci. 9 (4): 114-116.

Ebashi S (1960) Ca^{2+} binding and relaxation in the actomyosin system. J. Biochem. 48 : 150-151.

Ebashi S (1976) Excitation contraction-coupling. Ann. Rev. of Physiol. 38 : 293-313.

Edman K A P, Reggiani C and Kronnie T G (1985) Differences in maximal velocity of shortening along single muscle fibers of frog. J.Physiol. 365 : 147-163.

Edstrom A and Mattison H (1972) Fast sxonal transport in vitro in the sciatic system of frog. J. Neurochem. 19: 205-211.

Eisenberg E, Hill T L and Chen Y (1980) Cross-bridges model of muscle contraction. Biophys. Journal 29 : 195-227.

Elliott D C, Fournier A and Kokke Y S (1988) Phosphatidylserine activation of protein kinase C. Phytochemistry 27 (12): 3725-3730.

Fabbro D et al. (1988) Role of protein kinase C in short- and long-term cellular responses : Inhibition of agonist-induced Ca^2 transients and down regulation of protein kinase C J. Cardiovas. Pharmacol. 12 (Suppl. 5) S73.

Ferriola P C, Cody V, Middleton E Jr (1989) Protein kinase C inhibition by flavonoids : kinetic mechanisms and structural activity relationships. Biochem. Pharmacol. 38 (10) :1617-24.

Fry M J et al (1985) Fragment of protein kinase C genrated after protolysis has kinase activity and is not dependent on Ca^{2+} and PS.
EMBO J 4 : 3173.

Fung B K K and Hubbell W L (1978) Organization of Rhodopsin in Photoreceptor membranes. Biochemistry 17 (21) : 4396-4402.

Fung B K K (1983) Characterization of transduction from bovine ROS. J. Biol. Chem. 258(7) : 10495-10502.

Fung B K K, Hurley J B, Stryer L (1981) Flow of information in the light triggered cyclic nucleotide cascade of vision. P.N.A.S. (USA) 78(1) : 152-156.

Fung B K K and Stryer L (1980) Photolyzed rhodopsin catalyzes the exchange of GTP for bound GDP in Retinal ROS. P. N. A. S (USA) 77 : 2500-2504

Gierschik P, Mclewish K and Jakobs K H (1988) Regulation of G protein mediated signal transfer by ions. J. Cardiovas. Pharmacol 12 (suppl. 5) : S20.

Gilman A G (1984) G proteins and dual control of adenylate cyclase. Cell 36 : 577-579.

Gilman A G (1987) G Proteins : Transducers of receptor-generatd signals. Ann Rev. of Biochem. 56 :615-649.

Gollinick P D, Riedy M, Quintinski J J and Bertocci L A (1985) Differences in metabolic potential of skeletal muscle fibers and their significance for metabolic control. J. Exp. Biol. 115 : 191-199.

Gomperts B D (1983) Involvement of guanine nucleotide-binding protein in the gating of Ca^{2+} by receptors. Nature 306 : 64-66.

Gouri P K and Habibulla M (1988) Role of IP_3 during EC coupling phenomenon in the skeletal muscle of frog. Ph. D. Thesis submitted to JNU, New Delhi, Mrs. Gouri P K.

Harris B A et al (1985) Molecular cloning of cDNA for the a-subunit of G protein that stimulates adenylate cyclase. Science 229 : 1274-1277.

Held I R, Sayers, S T, Yeoh H C and McLane J A (1987) Role of cholinergic neuromuscular transmission in the neuroregulation of at the autophosphorylation regulatory subunit of cAMP-dependent protein kinase type II and the acetylcholine receptor content and skeletal muscle. Brain Res. 407 :341-350.

Heymans F et al (1987) Alkyl analogs of DAG as activators of protein kinase C. FEBS Lett 218(1) : 35-40.

Higashijima T et al (1987) The effect of activating ligands on the intrinsic fluorescence of guanine nucleotide-binding regulatory protein J. Biol. Chem. 262 :752-756.

Higashijima T et al. (1987) Effects of Mg^{2+} and ßt-subunit complex on the interaction of guanine-nucleotide with G proteins. J. Biol. Chem. 262 :762-766.

Higashijima T, Ferguson K M, Smigel MD, Gilman A G (1987) Effect of GTP and Mg^{2+} on the GTPase activity and the fluorescent properties of Go. J. Biol. Chem. 262 : 757-761.

Hildebrandt J D et al (1984) Identification of alpha subunit associated with the adenylate cyclase regulatory proteins N_s and Ni. J. Biol. Chem. 259 : 2039-2042.

Hilderbrandt J D et al (1985) Characterization by two dimensional peptide mapping of the gamma subunits of Ns and Ni, the regulatory proteins of adenylyl cyclase and of transducin,the guanine nucleotide binding protein of ROS of eye. J. Biol. Chem. 260 : 14867-14872.

Hill A V (1951) The influence of temperature on the tension developed in an isometric twitch. Proc. R. Soc. Biol. 138 : 349-354.

Hirata N et al (1985) Irreversible inhibition of Ca^{2+} release in saponin-treated microphages by the photoaffinity derivative of IP_3. Nature 317 : 723-725.

Homma S, Nakajima Y, Hayashi K, and Tomas (1986) Conduction velocity of action potential measured from unidimensional latency topography in human and frog skeletal muscle fibers. Jap. J. Physiol. 36 : 15-24.

Huxley A F. (1957) Muscle structure and theories of contraction. Prog. Biophys. Chem. 7 :255-318.

Huxley A. F. (1974) Reiew lecture - Muscle contraction. J. Physiol. 243 : 1-43.

Huxley A F and Niedergerke R. (1954) Structural changes in muscles during contraction. Interference microscopy of linking muscle fibers. Nature 173 : 971-973.

Huxley A F and Taylor R E (1955) Functions of krause's membrane. Nature 176 : 106.

Huxley A F and Taylor R E (1958) Local activation of straited muscle fibers. J.Physiol. 144 :426-441.

Huxley H E (1965) The mechanism of muscle contraction. Sci. Amer. 213(6) : 18-27.

Huxley H E and Hanson J (1954) Changes in cross-striations of muscle during contraction and stretch and their structural interpretation. Nature 173 : 973-976.

Irvine R F (1982) How is level of force arachidonic acid controlled in mammalian cells. Biochem. J. 204 :3-16.

Irvine, R F, Letcher A J, Heslop J P, and Berridge M J (1986) Inositol tris/tetrakis phosphate pathway demonstraction of IP_3-kinase activity in animal tissues. Nature 320 : 631-634.

Irvine M (1985) Muscle contraction : Weak and strong cross bridges. Nature 316 : 292-293.

Itoh H et al. (1986) Molecular cloning and sequence determination of cDNAs for alpha subunits of guanine nucleotide-binding proteins Gs, Gi and Go from rat brain. P. N. A. S. 83 : 3776-3780.

Iwasa K and Tasaki I (1982) Molecular events that underlie membrane excitation. J. Theor. Biol. 99 : 87-99.

Johnson F N (1979) Psychopharmacology of Li^+. Neurosci. Behaviour Rev. 3: 15-30.

Jurnak F. (1985) Structure of GDP domain of EF-Tu Location of Amino acids. Homologous to ras oncogene proteins. Science 320: 32-36.

Kanaho Y et al. (1981) Rhodopsin-enhanced GTPase activity of the inhibitory GTP-bindingprotein of adenylate cyclase. J. Biol. Chem. 259 : 7378-7381.

Katada T, Ui M (1981) Direct modification of membrane adenylate cyclase system by IAP due to ADP-ribosylation of a membrane protein. P.N.A.S. 79 :3129-3133.

Katada, T. Ui M (1982) ADP-ribosylation of specific membrane protein of C6 cells by IAP associated with modification of adenylate cyclase activity. J. Biol. Chem. 257 : 7210-7216.

Katada T et al. (1984) The inhibitory Guanine nucleotide-binding regulatory component of adenylate cyclase. J. Biol. Chem. 259 : 3568-3577.

Kawata, H. (1985) Some features of the electrically induced contraction of frog skeletal muscle : Reexamination of the post-anodal inhibition. Jap.J.Physiol. 35: 953-972.

Kidwai A M (1974) Isolation of PM from smooth, skeletal and heart muscles. Methods in Enzymology XXXI : 134-144.

Kikkawa U, Takai Y, Minakachi R, Inohara S and Nishizuka, Y. (1982) Ca^{2+} activated, phospholipid dependent protein kinase C from rat. J. Biol. Chem. 257 : 13341-13348.

Kim D. et al. (1989) G protein beta-gamma subunits activate cardiac muscarinic K^+ channels via phospholipase A_2. Nature 337 : 557-560.

Kitazawa T et al. (1989) Receptor coupled, permeabilized smooth muscle. Role of phosphatidylinositol cascade, G protein and modulation of contractile response to Ca^{2+}. J. Biol. Chem. 264 (10) : 5339-5342.

Kobayashi E et al. (1989) Calphostin C (UCN-1028C), a novel microbial compound is a highly potent and specific inhibitor of protein kinase C. B.B.R.C. 159(2) :548-553.

Kojima I, Kojima K, and Rasmussen H. (1985) Role of Ca^{2+} fluxes in the sustained phase of Angiotensin II mediated Aldosterone secretion from Adrenal Glomerulose cells. J. Biol. Chem. 260 :9177-9184.

Konishi M., Kurihara S, and Sakai T. (1985) Change in intracellular Ca^{2+} ion concentration induced by caffeine and rapid coupling in frog skeletal muscle fibers. J. Physiol. 365 : 131-146.

Kuhn H (1980) Light and GTP-regulatd interaction of GTPase and other proteins with bovine photoreceptor membranes. Nature 283 : 587-589.

Kurachi Y, Itoh H, Sugimota T, Kotada T, and Ui M. (1989) Speciofic activation of atrial muscarinic K^+ channels by low concentration of G protein beta-gamma subunits. Pflugers. Archiv. European J. of Physiol. 413(3) : 425.

Lea J L, Griffiths D J, Treager R T, and Ashley C C (1986) An examination of ability of IP_3 to induce Ca^{2+} release and tension development in skinned skeletal muscle fibers of frog and crustacea. FEBS Lett. 207 : 153-161.

Levitzki A (1985) Reconstitution of membrane receptor system. Biochim. Biophys. Acta 822 : 127-153.

Litosch I, Cristopher W and Fain J N (1985) 5-Hydroxytryptamine stimulates IP_3 production in a cell free system from blowfly salivary glands. J. Biol. Chem. 260 (9) : 5464-5471.

Llinas R and Precht W. (1976). Frog Neurobiology. Springer-Verlag Bertin Heidelberg, New York.

Low M G and Saltiel A R (1988) Structural and Functional roles of Glycosyl-Phosphatidyl inositol in membranes. Science 239 :268-275.

Lowry O H, Rosenbrough N J, Farr A L and Randall R J (1981) Protein measurement with folin phenol reagent. J. Biol. Chem. 193 : 265-275.

Manning D R and Gilman A G (1983) The regulatory components of adenylate cyclase and transducin. J. Biol. Chem. 258 : 7059-7063.

Masayoshi Go et al. (1987) Further studies on the specificity of DAG for protein kinase C activation. B. B. R. C. 144 (2) : 598-605.

Mathias R T et al.(1980) Electrical Models of EC coupling and charge movement in skeletal muscle. J. Gen. Physiol. 76 : 1-31.

May D C et al. (1985) Reconstitution of catecholamine-stimulated adenylate cyclase activity using three purified proteins. J. Biol. Chem. 260 (24) : 15829-33.

May W S, Sohyun N, Wolf M and Cuatracas S (1985) Role of intracellular Ca^{2+} mobiliztion in the regulation of protein kinase C mediated membrane processes. Nature 317 : 549-551.

Medynski D C et al.(1985) Amino acid sequene of transducin deduced from cDNA sequence. P.N.A.S.82: 4311-4315.

Milligan R A and Flickeer P.F. (1987) Structural relationship of actin, myosin and tropomyosin revealed by electron microscopy. J. Cell Bill. 105 : 29-39.

Murayama T and Ui M. (1985) Receptor-mediated inhibition of adenylate cyclase and stimulation of arachidonic acid release in 3T3 fibroblasts. J. Biol. Chem. 260 : 7226-7233.

Nakamura T, and Ui M (1985) Simultaneous inhibition of inositol-phospholipid breakdown, arachidonic acid release, histamine secretion in most cells by IAP, pertussis toxin. J. Biol. Chem. 260 : 3584-3594.

Nishizuka Y (1984) Role of protein kinase C in cell surface signal transduction and tumor promotion. Nature 308 : 693-698.

Nishizuka Y (1986) Studies and perspectives of protein kinase C. Science 233 : 305-312.

Northup J K et al.(1980) Purification of regulatory component of adenylate cyclase. P.N.A.S. 77 (1) : 6516-6520.

Northup J K, Smigel M D, Sternweis P C, and Gilman A G (1983) The subunits of the stimulatory regulatory component of adenylate cyclase. J. Biol. Chem. 258 : 11369-11376.

Nosek T M, Fender R Y and Godt R E (1987) Is it diprotonated inorganic phosphate the depressed force in skeletal muscle fibers. Science 236 : 191-193.

Nosek T M, Williams M F, Zeigler J T and Godt R E (1986) IP_3 enhances Ca^{2+} release in skeletal and cardiac muscle. Am. J. Physiol. 250 : C807-811.

Nukada T et al. (1986) Primary structure of alpha subunit of bovine adenylate cyclase-stimulating G protein deduced from the cDNA sequences. FEBS Lett 195 : 220-224.

Nukada T et al. (1986) Primary structure of alpha subunit of bovine adenylate cyclase-inhibiting G protein deduced from the cDNA sequences. FEBS Lett 197 : 305-310.

Obinata T, Maruyama K, Sugita H, Kohama K and Ebashi S (1981) Dynamic aspects of structural proteins in vertebrate skeletal muscle. Muscle and Nerve 4 : 456-488.

Ohta H, Okajima F and Ui M (1985) Inhibition by IAP of a chemotactic peptide induced early breakdown of inositol phospholipids and Ca^{2+} mobilization in Guinea Pig Neutrophils. J. Biol. Chem. 260 : 15771-15780.

Orly J, and Schramm M. (1976) Coupling of catecholamine receptor from one cell with adenylate cyclase from another cell by cell fusion. P.N.A.S. (USA) 73 : 4410-4414.

Paris S and Pouysoegur J (1987) Further evidence for a phospholipase C coupled G protein in hamster fibroblasts. J. Biol. Chem. 262(5) : 1970-1976.

Parker P J et al. (1986) The complete primary structure of protein kinase C - major phorbol ester receptor. Science 233 : 853-866.

Pederson S E and Ross E M (1982) Functional reconstitution of B-adrenergic receptors and the stimulating GTP-binding protein of Adenylate cyclase. P.N.A.S. 79 : 7228-7232.

Pette D (1985) Metabolic heterogeneity of muscle fibers. J. Exp. Biol. 115 : 179-189.

Pfaffinger P J, Martin J M, Hunter D D, Nathanson N M and Hille B (1985) GTP-binding couples cardiac muscarinic receptors to a K^+ Channel. Nature 317 : 536-538.

Pfeuffer T (1977) GTP-binding proteins in membranes and the control of adenylate cyclase activity. J. Biol. Chem. 252 : 7224-7234.

Pfeuffer T and Metzger H (1982) 7-o-Hemisuccinyl-deacetyl forkolin-sepharose-a novel affinity support for purification of adenylate cyclase. FEBS Lett. 146 (2) : 369-375.

Prentki M et al. (1985) IP_3 and endoplasmic reticulum cycle of Ca^{2+} in rat insulinoma cell line. J. Biol. Chem. 260 : 9185-9190.

Prentki M., Biden T J, Janjic D, Irvine K F, Berridge M J, and Willheim C B (1984) Rapid mobilization of Ca^{2+} homeostasis in permeabilized human neurophils. J. Bill. Chem. 259 (22) : 13777-13782.

Ramsdell J S and Tashiyain A H (1986) Thyrotropin-releasing hormone : Elevation of IP_3 and cytosolic Ca^{2+} is dependent on receptor number. J. Biol. Chem. 261 : 5301-5306.

Rana R S et al. (1986) Confinement of hormone sensitive pool to plasma membrane. Arch. Biochem. Biophys. 245 :411-416.

Reddy T S and Rajan N G (1987) Arachidonic acid, stearic acid DAG accumulation correlates wit loss of phsophatidyl 4,5 biphosphate in cerebrum 2 sec after electroionvulsive shock. Complete reversion of changes in 5 min after stimulation. J. Neurosci Res. 18 : 449-455.

Reibman J. et al. (1988) Changes in DAG labelling, cell shape and protein phosphorylation distinguish "triggering" from "Activation" of human neutrophils. J. Biol. Chem. 263 : 6322-6328.

Reynolds E E and Dubyak G R (1985) Activitation of Ca^{2+} mobilization and Ca^{2+} influx by Alpha adrenergic receptors in smooth muscle cell line. B.B.R.C. 130 : 627-632.

Rink T J, Sanchez A and Hallam T J (1983) DAG and phorbol ester stimulate secretion wihout raising cytoplasmic free Ca^{2+} in human platelets. Nature 305 :317-319.

Robinshaw J D et al. (1986) Deduced primary structure of the a-subunit of the GTP-binding stimulatory protein of adenylate cyclase. P.N.A.S. (USA) 83 : 1251-1255.

Rodbell M et al.(1971) The glucagon-sensitive adenylate cyclase system in plasma membrane of rat liver I-V. J. Biol. Chem., 246: 1849-1852.

Ross E M (1977) Relationship between the B-adrenergic receptor and adenylate cyclase. J. Biol. Chem. 252(16) : 5761-5775.

Sasaguri T, Hirata M, Kuriyama H (1985) Dependence on Ca^{2+} of the activities of phosphoinositol 4,5-biphosphate-phosphodiesterase and IP_3-phosphatase in smooth muscle of porcine coronary artery. Biochem. J. 231 : 497-503.

Schneider M F and Chandler W K (1973) Voltage dependent sharp movement in skeletal muscle - a possible step in EC coupling. Nature 242 : 244-246.

Sherman W R et al. (1985) Effects of systematically administered Li^+ on phosophoinositide metabolism in rat brain, kidney and testis. J. Neurochem. 44 : 798-807.

Sherman W R et al. (1986) Effect of Li^+ on phosphoinositide metabolism in vivo. Fed Proceedings 45(11): 2639-2646.

Smal J, Kathuria S and DeMeyts P (1989) Acridine orange, an inhibitor of protein kinase C abolished insulin and GH stimulation of lipogenesis in rat adipocytes. FEBS lett. 244(2) : 465-468.

Small J V (1988) Cell motility : Myosin filaments on move. Nature 331 : 568-569.

Smith C D, Lane B S, Nusaka I, Verghese K W, Snyderman R. (1985) Chemoattractant receptor-induced hydrolysis of PIP_2 in human PMN membranes. J. Biol. Chem. 260(10) :5875-5878.

Smith J B, Smith L, Higgins B L. (1985) Temperature and nucleotide dependence of Ca^{2+} release by myo-Ins-1,4,5 P_3 in cultured vascular smooth cells. J. Bioll. Chem. 260 (27) : 14413-14416.

Somlyo A P (1985) EC coupling : the messenger across the gap. Nature 316 : 298-299.

Spicher K et al. (1988) Immunochemical detection of alpha subunit of G protein in membrane and cytosol of mammalian cells. B. B. R. C. 157(3) :883-890.

Squire J (1981) 1. Introduction of muscle physiology (16-38). 2. Vertebrate skeletal muscle (4-15). In: the structural basis of muscle contraction. Plenum Press, New York.

Steinback J H and Stevens C F (1976) Neuromuscular transmission In: Frog Nuerobiology Eds. LLinais R and Precht W., Light Springer-Verlag, New York, pp. 33-42.

Sternweis P C (1986) The purified alpha subunit of G_o and G_i from bovine brain require ßt for association with phospholipid vesicles J. Biol. Chem. 261 : 631-637

Sternweis P C et al. (1981) Regulatory component of adenylate cyclase. J. Biol. Chem. 256: 11517-11526.

Sternweis P C and Gilman A G (1982) Aluminium : A requirement for activation of the regulatory component of adenylate cyclase by fluoride. P.N.A.S. (USA) 79:4888-4891.

Sternweis P C and Robinshaw J D (1984) Isolation of 2 proteins with high affinity for guanine nucleotides from membranes of bovine brain. J. Biol. Chem. 259 : 13806-13813.

Storey D J et al. (1984) Stepwise enzymatic dephosphorylation of IP_3 to inositol in liver. Nature 312 :374-376

Streb H, Bayerdreffer E, Haase W, Irvine R F and Schulz I (1984) Effect of IP_3 in isolated subcellular fraction on rat pancreas. J. Memb. Biol. 81 :241-253.

Streb H, Irvine R F, Berridge M J, Schulz I (1983) Release of Ca^{2+} from the nonmitochondrial intracellular store in pancreatic acinar cells by IP_3. Nature 306 : 67-68.

Stryer L (1988) Excitable membranes and sensory neurons. Biochemistry. 3rd Edition, pp. 1005, W H Freeman and Co., New York.

Szent-Gyorgi A (1951) In: Chemistry of muscular contraction. 2nd revised edition, pp. 10, Academic Press, New York.

Takagi T and Itabashi Y (1987) Rapid separations of Diacyl- and Dialkylglycerol enantiomers by HPLC on a chiral stationary phase. Lipids. 22(8) : 596-600.

Taylor M V et al. (1984) Mitogens increase phosphorylation of phosphoinositides in thymocytes. Nature 312 : 462-465.

Taylor S S (1987) Protein kinases : A diverse family of related proteins. Bioassays 7(1) : 24-29.

Thomas A P, Beaven M A, Moore J P, Smith G A, Hesketh T R, Metcalfe J C (1984) The Ca^{2+} signal and phosphoinositol breakdown in 2HZ cells. J. Biol. Chem. 259 : 7137-7142.

Turk J et al. (1986) Glucose-induced accumulatin of IP_3 in isolated pancreatic islets. Biochem. J. 237 : 259-263.

Uhiag R J, Popic V, Jiang H and Exton J H (1986) Hormone-stimulated phosphoinositide breakdown in rat liver PM. J. Biol. Chem. 261 : 2140-2146.

Vergara J et al. (1983) The physiology of Excitable cells. Grinnel and Moody ed. pp. 343-355. Alan R Liss, New York.

Vergara J et al., (1985) IP_3 : A possible chemical link in EC coupling in muscle. P.N.A.S. (USA) 82 :6352-6356.

Volpe P et al.(1986) Role of IP_3 in EC coupling in skeletal muscle. FEBS Lett. 197 : 1-4.

Volpe P, Salviati G, DiVirgilio F and Pozzen T (1985) IP_3 induces Ca^{2+} release from SR of skeletal muscle in rabbit. Nature 316 :347-349.

Warrick H M and Spundick J A (1987) Myosin : structure and function in cell motility. Ann. Rev. Cell Biol. 3 : 379-422.

Williamson J R, Copper R H, Joseph S K, and Thomas A P (1985) IP_3 and DAG as intracellular second messengers in liver. Amer. J. Physiol. 248 : C203-C216.

Wolf M et al. (1985) A model for intracellular translocation of protein kinase C including synergism between Ca^{2+} and phorbol esters. Nature 317 : 546-551.

Wolfman A and Macara I G (1987) Eelevated levels of DAG and decreased phorbol ester sensitivity in ras-transformed fibroblasts. Nature 325 : 359-361.

Yee R, and Liebman P A (1978) Light activated phosphodiesterase of rod outer segments. J. Biol. Chem 253 : 8902-8909.