THE ROLE OF CYCLIC NUCLEOTIDES IN MODULATION OF CRAYFISH NEUROMUSCULAR JUNCTIONS BY A NEUROPEPTIDE

BY

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ABSTRACT

DF_2, a heptapeptide, is a member of the family of FMRFamide-like peptides and has been shown to increase the amount of transmitter released at neuromuscular junctions of the crayfish, *Procambarus clarkii.* Recent evidence has shown that protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II (CaMKII) and the cAMP-dependent protein kinase (PKA) play a role in the neuromodulatory pathway of DF_2. The involvement of these kinases led to the prediction that a G-protein-coupled receptor (GPCR) is activated by DF_2 due to the role that each kinase plays in traditional GPCR pathways seen in other organisms and in other cells. G-proteins can also act on an enzyme that generates cyclic guanosine monophosphate (cGMP) which mediates its effects through a cGMP-dependent protein kinase (PKG). This thesis addresses the question of whether or not DF_2's effects on synaptic transmission in crayfish are mediated by the cyclic nucleotides cAMP and cGMP.

The effects of DF_2 on synaptic transmission were examined using deep abdominal extensor muscles of the crayfish *Procambarus clarkii*. An identified motor neuron was stimulated, and excitatory post-synaptic potentials (EPSPs) were recorded in abdominal extensor muscle L1. A number of activators and inhibitors were used to determine whether or not cAMP, PKA, cGMP and PKG mediate the effect of this peptide.

Chemicals that are known to activate PKA (Sp-cAMPS) and/or PKG (8-pCPT-cGMP) mimic and potentiate DF_2's effect by increasing EPSP amplitude. Inhibitors of either PKA (Rp-cAMPS) or PKG (Rp-8-pCPT-cGMPS) block a portion of the increase in EPSP amplitude induced by the peptide. When both kinase inhibitors are applied simultaneously, the entire effect of DF_2 on EPSPs is blocked. The PKG inhibitor blocks

the effects of a PKG activator but does not alter the effect of a PKA activator on EPSP amplitude. Thus, the PKG inhibitor appears to be relatively specific for PKG. A trend in the data suggests that the PKA inhibitor blocks a portion of the response elicited by the PKG activator. Thus, the PKA inhibitor may be less specific for PKA.

Phosphodiesterase inhibitors, which are known to inhibit the breakdown of cAMP (IBMX) and/or cGMP (mdBAMQ), potentiate the effect of the peptide. These results support the hypothesis that cAMP and cGMP, acting through their respective protein kinase enzymes, mediate the ability of DF_2 to increase transmitter output.

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ABBREVIATIONS

5-HT	Serotonin (5-hydroxytryptamine)
8-pCPT-cGMP	8-(*para*-chlorophenylthio)guanosine-3',5'-cyclic monophosphate
AC	Adenylyl Cyclase
CaMKII	Calcium/Calmodulin-Dependent Protein Kinase, Type II
cAMP	cyclic adenosine-3',5'-monophosphate
CAP	Catabolite Gene Activator Protein
CF	Cystic Fibrosis
cGMP	cyclic guanosine-3',5'-monophosphate
CHH	Crustacean Hyperglycemic Hormone
cNMP	cyclic nucleotide monophosphate
CNS	Central Nervous System
CRP	cAMP Receptor Protein
DA	Dopamine (3-hydroxytyramine)
DAE	Deep Abdominal Extensor
DAG	diacylglycerol
EPP	End-Plate Potential
EPSP	Excitatory Postsynaptic Potential
ER	Endoplasmic Reticulum
FaRP	FMRFamide-Related Peptide
FMRFamide	Phe-Met-Arg-Phe-amide
GC	Guanylyl Cyclase
GPCR	G-Protein Coupled Receptor
G-protein	Heterotrimeric Guanine Nucleotide-Binding Protein
IBMX	3-isobutyl-1-methylxanthine
IP_3	inositol-1,4,5-trisphosphate
L	Lateral Deep Abdominal Extensor Muscle
LTD	Long-Term Depression
LTF	Long-Term Facilitation
LTP	Long-Term Potentiation
m	quantal content (as in m=np)
M	Medial Deep Abdominal Extensor Muscle
mdBAMQ	4-{[3',4'-(methylenedioxy)benzyl]amino}-6-methoxyquinazoline
mEPP	Miniature End-Plate Potential
n	number of quanta released (as in m=np)
NO	nitric oxide
NMJ	Neuromuscular Junction
OA	Octopamine
p	probability of quantal release (as in m=np)
PDE	phospodiesterase
PIP_2	phosphatidylinositol-4',5'-bisphosphate
PKA	cAMP-Dependent Protein Kinase
PKC	Ca^{2+}/phospholipid-Dependent Protein Kinase
PKG	cGMP-Dependent Protein Kinase
PLC	Phospholipase C
PO	Pericardial Organ
PNS	Peripheral Nervous System
RpA	Rp-cAMPS
RpG	Rp-8-pCPT-cGMPS
Rp-cAMPS	adenosine-3'5'-cyclic monophosphorothioate, Rp-isomer
Sp-cAMPS	adenosine-3'5'-cyclic monophosphorothioate, Sp-isomer
TSH	Thyroid Stimulating Hormone
VASP	Vasodilator-Stimulated Phosphoprotein

1. INTRODUCTION AND LITERATURE REVIEW

1.1 The Neuron

The nervous system is made up of an organized web of cells called neurons. One function of these neurons is to transmit information from one area of the organism to another. This information is coded in the neurons through electrical signals. The neurons, however, have a limited capacity to code specific information on their own; rather they simply generate electrical signals or do not. For example, nerve cells in the visual system generate impulses in fundamentally the same manner as cells responsible for responding to tactile stimulation of the skin. The code lies in how many, and what type of, neurons are activated and the way they are activated, i.e. the frequency and duration of impulse bursts. In order to integrate these electrical signals, they must be decoded by a higher system and, with the exception of reflexes, this higher system is the brain.

The human brain consists of many neurons, estimated to be in the range of 10^{10} to 10^{12} cells (Nicholls, et.al., 1992), organized into sections responsible for specific tasks. For example, cells generating signals from the visual system are connected to a region of the brain responsible for the decoding of these specific signals, and those cells that respond to tactile stimulation of the skin are connected to another region of the brain. This physical separation, or functional segregation of the brain into specific regions, is only the beginning of the task in decoding signals generated by neurons. The brain must also be able to distinguish between different types of stimulation. For example, it is necessary to tell the difference between a pleasant feeling on the skin from one that is

undesirable or painful. How the brain is able to perform these tasks remains one of the major enigmas in understanding the human brain.

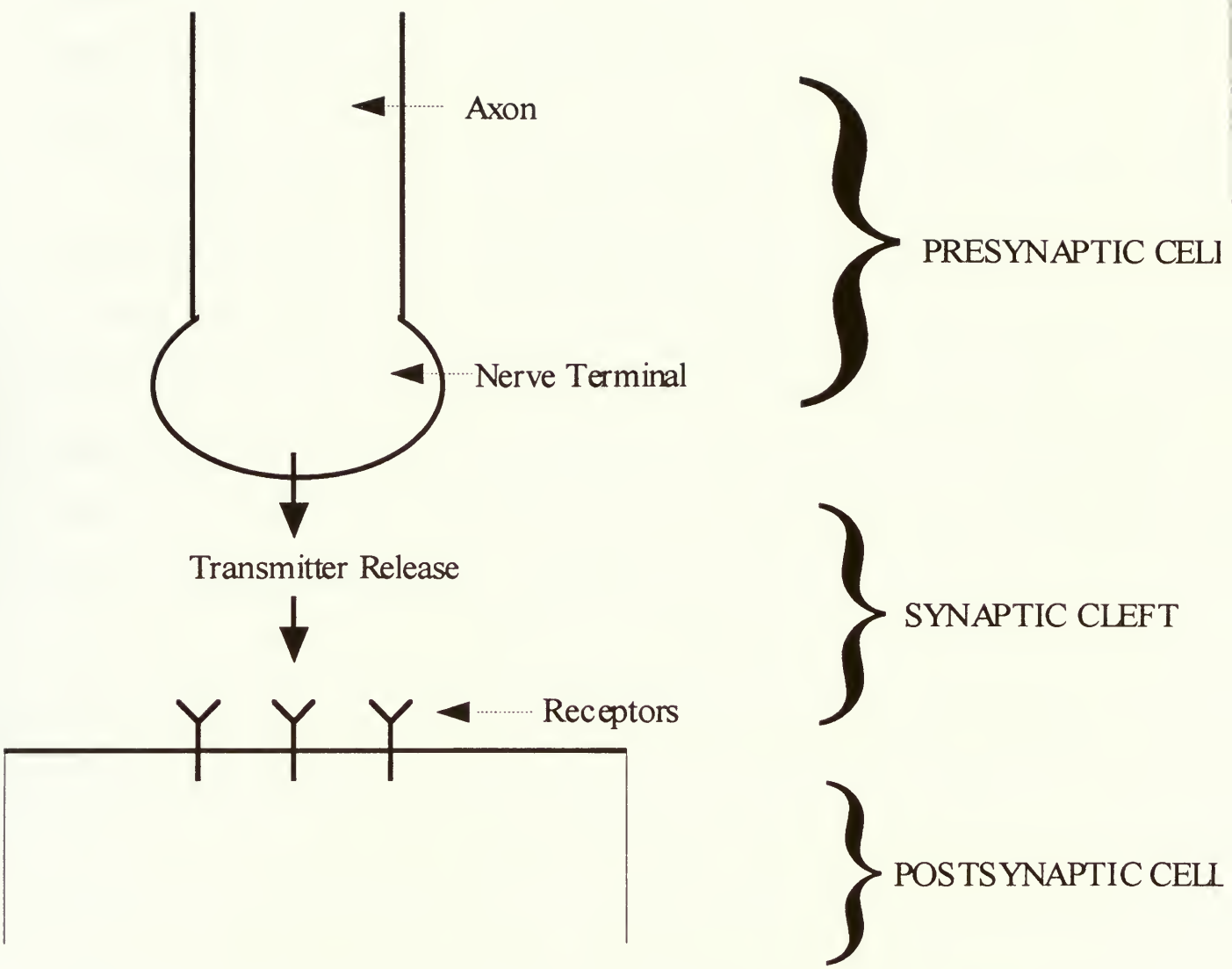
Regardless of how the brain processes the information it receives from the body, it must also be able to respond to this information. The body would be quite useless if we were only able to sense information and not do anything about it. The real beauty of the brain lies in its ability to not only separate and decode information that it receives, but also to coordinate this information and initiate a behavioral response to these stimuli. In order to move an arm to pick up a glass that is seen on the table, the brain must generate signals in the neurons that ultimately tell the muscles of the arm to move and to grasp the glass. In order for these and other types of signals to be communicated with their target, the signal must be passed on from one neuron to another along the pathway. How one neuron initiates a signal in the next neuron along the pathway is what will be discussed in the next section.

1.2 The Synapse and Neuromodulation

In order for one neuron to communicate with either the next successive neuron in a specific neural pathway or directly with an effector cell, such as a muscle fibre, it must transmit a signal, in the form of excitation or inhibition, onto it. In most cases, there is no direct contact between the neuron and its target. Rather, there exists a small space between the nerve terminal membrane and the membrane of either the next neuron or the target cell. This small space is called the synaptic cleft, and the whole anatomical specialization is called the synapse. The cell that is to pass on the excitation is termed the presynaptic cell, and the cell that is to receive this information is called the postsynaptic

FIGURE 1-1: Schematic Drawing of a Chemical Synapse

Upon stimulation, the pre-synaptic cell, the neuron, releases its chemical neurotransmitter into the synaptic cleft. The transmitter traverses the cleft and binds to receptors on the post-synaptic membrane, resulting in the electrical transduction of the signal from the pre-synaptic cell to the post-synaptic cell.


Axon
Nerve Terminal
Transmitter Release
Receptors
PRESYNAPTIC CELL
SYNAPTIC CLEFT
POSTSYNAPTIC CELL

cell (Fig. 1-1). In the CNS, the postsynaptic cell is another neuron and this specific junction is called a neuron-neuron synapse, and in the PNS the second cell can be either another neuron or some effector cell within a muscle or a gland. When the synapse exists between a neuron and a muscle cell, the junction is called a neuromuscular junction (NMJ).

In the early 1900's, many scientists believed that the excitation was passed across a synapse electrically, whereby the action potential was transmitted directly from the presynaptic cell to the postsynaptic cell (Fox, 1990). With the advent of newer and more detailed histological techniques, coupled with experimental evidence that the effect of some autonomic nerves could be mimicked with the addition of exogenous chemicals, physiologists came to the conclusion that the transfer of information across some synapses occurs chemically (Loewi, 1921 as cited in Fox, 1990). These chemicals, or neurotransmitters, are released from the presynaptic terminal when stimulated to do so by an action potential in the presynaptic cell. Upon release, these neurotransmitter molecules traverse the synaptic cleft and bind to receptors on the postsynaptic membrane. Once bound to these receptors, the postsynaptic cell becomes excited and in some cases generates action potentials of its own. This system of transmission allows the propagation of an electrical signal from one neuron to another in the case of a neural pathway, or would generate a response in the effector cells in the case of muscles or glands.

1.2.1 The Quantal Nature of Release

After it had been established that transmission of the neural impulse is accomplished using a chemical neurotransmitter, studies by del Castillo, Katz and Dudel (del Castillo and Katz, 1954b, Dudel and Orkland, 1960) demonstrated that transmission occurs by releasing packets of transmitter from the presynaptic nerve terminal. Each of these packets, or quanta, contains many, and a fixed amount of, neurotransmitter molecules. When triggered to do so, the nerve terminal releases the contents of many of these quanta into the synaptic cleft. This is accomplished through the fusion of the vesicle membrane with the cell membrane, which triggers the exocytotic release of the neurotransmitter contained within them. The neurotransmitters bind to receptors on the postsynaptic membrane, and a change in the membrane potential results. When dealing with the NMJ, this change in the membrane potential is called an end-plate potential (EPP).

When the NMJ is at rest, there is still some spontaneous activity. This activity is seen as miniature EPPs (mEPPs). Each mEPP is the result of the spontaneous release of an individual quantum of transmitter (del Castillo and Katz, 1954a&b, Dudel and Kuffler, 1961a&c). Even in the absence of nerve activity, some transmitter is able to be released from the presynaptic terminal and traverse the synaptic cleft causing a specific postsynaptic response, the mEPP. When the presynaptic cell becomes activated, many quanta are released simultaneously, and the EPP that results is an integral multiple of the unitary mEPP (Fatt and Katz, 1952).

There are at least two major factors that determine how many quanta are released per nerve impulse. These are the number (n) of synaptic vesicles that are available for

release and the probability (p) of release of one synaptic vesicle. The average number of quanta released per nerve impulse is called the quantal content (m) and is equal to the product of 'n' and 'p' [m=np] (del Castillo and Katz, 1954c; Nicholls, et.al., 1992). Any modification in either of these two major factors will alter the quantal content, 'm'. For example, if some presynaptic event increases the number of available vesicles (n), then m will increase. Likewise, if something increases the probability of release (p), the quantal content will also go up. One thing that can alter p is the level of intracellular Ca^{2+} within the nerve terminal. This will be explained in the next section.

1.2.2 The Role of Calcium in Neurotransmitter Exocytosis

It is well established that calcium is necessary to release the transmitter vesicles into the synaptic cleft (del Castillo and Katz, 1954a; Dudel, et.al., 1984; Zucker, 1974). Calcium is used as the trigger for exocytosis of the vesicle at the nerve terminal membrane. As the electrical impulse approaches the nerve terminal, voltage-gated, P-type, Ca^{2+} channels open, causing the intracellular concentration of Ca^{2+} to increase (Araque, et.al., 1994; Peterson, et.al., 1994). The specific role for calcium remains elusive. However if its intracellular concentration is reduced through the use of the calcium chelating agent EGTA (Katz and Miledi, 1968), or if Mg^{2+}, a competitive inhibitor of Ca^{2+} is present in sufficient amounts, transmission is inhibited (del Castillo and Katz, 1954a; Johnson and Wernig, 1971). Conversely, if the intracellular concentration of Ca^{2+} in increased, so too is the level of neurotransmitter release (Dudel, et.al., 1982). To put this into the context of the binomial nature of release introduced in the previous section (m=np), higher calcium concentrations increase the overall synaptic

output (m) by increasing the probability of release (p). Reducing the intracellular calcium concentrations, or introducing magnesium into the extracellular fluid, has the opposite effect on 'p' and, therefore, on 'm' (del Castillo and Katz, 1954b&c).

The subject of much recent research is the determination of the specific role of Ca^{2+} in activating the release machinery. There also exists much debate as to how many Ca^{2+} ions are associated with the exocytosis of each transmitter vesicle. According to Dodge and Rahamimoff (1967) and their work on the frog neuromuscular junction, quantal release increases proportional to the fourth power of $[Ca^{2+}]_o$, suggesting that four calcium ions act cooperatively and are necessary for transmitter release. This does not appear to be the case in crayfish. In at least one study, it was found that at the crayfish neuromuscular junction, a near linear dependence of quantal release on $[Ca^{2+}]_o$ exists (Zucker, 1974). Yet another study performed on crayfish, although a different species of crayfish, suggested that release is mediated by the association of two calcium ions with the release machinery (Nickell and Boyarski, 1980). This apparent species-specific calcium dependence of release is the root of many discussions and the cause of much debate into determining the specific role of Ca^{2+}. Regardless of how many calcium ions associate with the release machinery, the effect of $[Ca^{2+}]_o$ on 'm' is directly on the release probability 'p' (Dudel, 1981; Parnas, H., et.al., 1982).

1.3 EPSPs

When excitatory neurotransmitters are released upon an excitable cell, for example a neuron or a muscle cell, the excitation is passed on. In most cells, this excitation exists as an action potential, the all-or-none response (Hille, 1992). In order to

generate an action potential, the membrane potential of the excitable cell must depolarize to reach a certain threshold. These depolarizations are called excitatory postsynaptic potentials (EPSPs). In most systems, these EPSPs summate to reach this threshold and a full excitatory response, the action potential, results (Hille, 1992). If one can control the amount of transmitter released from the presynaptic cell, it is possible to elicit EPSPs, but not allow the postsynaptic membrane to depolarize sufficiently to reach this threshold. Therefore, only EPSPs result, and in the case of most types of muscle, no contraction is seen. In order to decrease the amount of transmitter released by the presynaptic cell, 'm', Mg^{2+} can be added to the bathing solution to inhibit Ca^{2+} entry, and therefore reducing 'p' (Johnson and Wernig, 1971). Lowering the Ca^{2+} concentration in the bathing solution further reduces 'p'. With 'p' and therefore 'm' reduced, the amount of transmitter released can be decreased to a level at which EPSPs can be recorded from the postsynaptic cell, but no action potentials will be elicited. This is desirable when recording intracellularly from muscle cells because contractions in the muscle could break the intracellular electrode and/or damage the muscle cell.

1.4 Crustacean Muscles

In general, there are two major classes of muscles, those whose contractile elements, the actin and myosin filaments, are loosely arranged (smooth muscle) and those with a highly ordered array of these elements (striated muscle). Unlike most other animals, crustaceans and other arthropods contain only striated muscle (Atwood, 1976; Atwood, et.al., 1965). Due to the fact that the muscle fibres are of two types depending on the speed of contraction, either fast or slow (Govind and Atwood, 1982), and that each

muscle can be stimulated by one of two types of motor neurons, tonic or phasic (Govind and Atwood, 1982; Atwood, et.al., 1991; Hill and Govind, 1981; King and Atwood, 1996), a surprisingly large amount of functional diversity is exhibited by these muscles. Tonic motor neurons are those which do not fatigue as quickly. They generate EPSPs that are generally smaller in amplitude, yet the EPSPs maintain their amplitude and show minimal synaptic depression with prolonged stimulation. Phasic neurons, on the other hand, can generate an initially larger EPSP that quickly becomes smaller, or depressed (Atwood, et.al., 1991; Atwood, 1977; Atwood and Marin, 1983).

Not surprisingly, there is a certain degree of linkage between the muscle fibre type and the type of motor neuron that innervates it (Atwood, 1976; Bradacs, et.al., 1997). Many slow muscles are innervated by tonic axons and are usually associated with postural type movements where there is little need for quick muscle activity and a high degree of fatigue is a disadvantage. This system is referred to as the tonic muscle system. Conversely, muscles that are responsible for quick movements (as in escape) are of the fast type and are innervated by the large EPSP-generating phasic motor neurons, comprising the phasic muscle system (Govind and Atwood, 1982; Chapple, 1982).

1.5 Neuropeptides

There exists a large number of extracellular signaling molecules that have been shown to exhibit a wide range of effects upon neural tissue systems. A subset of this group is the neuropeptides. These small proteins have been shown to modify neuronal function in a variety of tissues and can be found in all animal species studied. Some of them can be released locally and have only directed effects, and some can be released

into the general circulation and have an array of effects on a number of different target tissues. One subfamily of neuropeptides, called FMRFamide-Related peptides has been widely studied and has been shown to have a number of neuromodulatory functions in all animals.

1.6 DF_2 and other FMRFamide-Related Peptides

There are over 100 members of the FMRFamide-Related Peptide (FaRP) family, so named because they all share the carboxy-terminal sequence –RFamide (Arg-Phe-NH_2) and bear a sequential resemblance to the first member of the family, FMRFamide (Phe-Met-Arg-Phe-NH_2), isolated from the nervous system of a mollusk (Price and Greenberg, 1977). Representatives of this family can be found in every class of animals from the nematode to man (Cottrell, 1997). These peptides can act as neurotransmitters, but in many cases they act as neuromodulators, either increasing or decreasing the efficacy of synaptic transmission.

As mentioned above, the first such peptide was isolated from the clam by Price and Greenberg (1977) where the tetrapeptide FMRFamide is found to be cardioexcitatory. Since that time, many members of the family have been found in a wide range of animals and exhibit a diverse range of effects. Sometimes, the same peptide can have opposite effects in two different animals. For example, FMRFamide has been shown to be excitatory in the clam (Price and Greenberg, 1977) and inhibitory in the snail (Haydon, et.al., 1991; Linacre, et.al., 1990). In the snail, FMRFamide is found to inhibit synaptic release (Man-Son-Hing, et.al., 1989). This modulation of synaptic release is not unique to the snail. Evidence for the involvement of FaRPs in

modulation of the transmitter release machinery exists in the lobster (Goy, 1990; Pavloff and Goy, 1990; Worden, et.al., 1995), insects (specifically the locust; Cuthbert and Evans, 1989; Lange and Orchard, 1998), squid (Cottrell, et.al., 1992), cuttlefish (Loi and Tublitz, 1997), shrimp (Meyrand and Marder, 1991), crayfish (Mercier, et.al., 1990; Skerrett, et.al.) and mammals such as the calf (Khananshvili, et.al., 1993).

One member of this family of peptides, and the one most important to this thesis, has been recently isolated from the pericardial organs (POs) of the crayfish *Procambarus clarkii* (Mercier, et.al., 1993). Earlier, two FaRPs were isolated from the lobster referred to as F_1 (amino acid sequence TNRNFLRFamide) and F_2 (SDRNFLRFamide). Both peptides were found to increase the rate and amplitude of heart contractions and increase the amount of transmitter released from NMJs in the lobster (Worden, et.al., 1995; Trimmer, et.al., 1987). These two peptides have similar effects on crayfish hearts (Mercier and Russenes, 1992) and on NMJs of the crayfish deep-abdominal extensor (DAE) muscles (Mercier, et.al., 1990). It was therefore thought that F_1 and F_2, or very similar peptides, were also native to the crayfish. Immunocytochemical techniques indicated that crayfish POs contain FMRFamide-like immunoreactive material that elutes similarly to these lobster peptides on reverse-phase high performance liquid chromatography (Mercier, et.al., 1991b). It was later determined that these two peptides are very similar to their counterparts in the lobster, and they were named for their sequence homology with F_1 and F_2. The two peptides were called NF_1 (NRNFLRFamide) and DF_2 (DRNFLRFamide; Mercier, et.al., 1993). When applied exogenously, NF_1 and DF_2 were found to be cardioexcitatory, and also to increase the amount of transmitter released by nerve terminals onto DAE muscles (Mercier, et.al.,

1993; Skerrett, et.al., 1995). The ability of the peptide to enhance transmitter release is more pronounced when the temperature is decreased to about 7-9°C, which decreases EPSP amplitude (Friedrich, et.al., 1994).

How do these neurohormones modulate transmitter output at NMJs? Evidence for the involvement of a number of second messengers exists. Recently, a great deal of research has been directed to elucidate the intracellular signaling systems involved in neuromodulation of crayfish neuromuscular junctions by DF_2. For example, inhibiting the calcium/calmodulin-dependent Protein Kinase type II (CaMKII) results in the inhibition of an early component of synaptic modulation by DF_2, resulting in an increase in the time it takes for EPSPs to reach their peak amplitude (Noronha and Mercier, 1995). Likewise, inhibition of Protein Kinase C (PKC), results in the inhibition of a late component of neuromodulation, resulting in a more rapid decline in EPSP amplitudes when the peptide is removed from the bathing solution (Friedrich, et.al., 1998). When the cAMP-dependent Protein Kinase is inhibited, a large portion of the modulation is blocked, resulting in a decrease in the peak amplitude of modulated EPSPs (Weston, et.al., 1997). These results implicate the involvement of more than one second messenger system (Fig. 1-2).

1.7 Intracellular Signaling and Signal Transduction

There are two ways that a signaling molecule can cause a reaction in its effector cell. The first involves the messenger actually crossing the cell membrane to initiate the appropriate response. The second involves the extracellular (first) messenger increasing the intracellular concentrations of other, second messengers. The most basic type of

second messenger system involves the first messenger opening an ion channel, for example a Ca^{2+} channel, which allows the passive entry of the ion, the second messenger, into the cell (Cottrell, 1997; Hille, 1992). Therefore, the response is directly mediated by the second messenger and indirectly mediated by the first messenger. Another major second messenger pathway involves the activation of a heterotrimeric guanine nucleotide-binding protein (G-protein). In this pathway, the first messenger binds to a receptor, which is coupled to a G-protein (Fox, 1990; Nicholls, et.al., 1992). Therefore, upon activation of this receptor by its ligand, the G-protein becomes activated. Activation of this G-protein-coupled receptor (GPCR) initiates the generation of a number of second messengers and, therefore, a number of different responses, or a single coordinated response. One such second messenger system involves the G-protein-mediated activation of membrane bound cyclases (Gilman, 1984). These cyclases catalyze the conversion of nucleotide tri-phospates into cyclic nucleotide mono-phosphates (cNMPs). These cNMPs act as second messengers. Two such second messengers are cAMP and cGMP, whose generation is catalyzed by adenylyl cyclase and guanylyl cyclase, respectively. These cNMPs are discussed in further detail in sections 1.6.1 and 1.6.2. Another second messenger system initiated by GPCRs involves the conversion of a membrane-bound inositol lipid, phosphatidylinositol 4,5-bisphosphate (PIP_2) into two second messengers. This reaction is catalyzed by the membrane-bound Phospholipase C (PLC). PIP_2 is converted into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$, or simply IP_3). The roles of DAG and IP_3 are discussed further in section 1.6.3.

1.7.1 cAMP

As briefly introduced, cAMP is very important in intracellular signaling. Not only is it a second messenger in the GPCR pathway, but it is also used in cell-cell communication. For example, in the slime mold *Dictyostelium discoideum*, cAMP is used as a signal for the individual organisms to aggregate into a community to begin the spore-forming process (Flaadt, et.al., 1993). During this process, when cAMP is detected by cell-surface receptors, Ca^{2+} channels become activated, and Ca^{2+} influx follows. This influx acts as a trigger to aggregate towards the source of the elevated cAMP concentrations and also to release cAMP into the environment in an effort to transmit this signal (Van Haastert, et.al., 1984). This is an example of how cAMP can act as a first messenger to bring about a behavioural response. A more important, and more widely reported role for cAMP is in the intracellular signaling pathway where it acts as a second messenger in response to activation of GPCRs by specific ligands. Upon activation, the GPCR causes an increase in activity of a specific class of enzymes called the cyclase enzymes. One such cyclase is adenylyl cyclase (AC). AC is responsible for converting ATP into cAMP, the second messenger. cAMP is broken down into AMP by a phosphodiesterase (PDE). This increase in cAMP concentration results in an increase in activity of certain targets, one of which is the cAMP-dependent protein kinase (PKA). PKA, and therefore cAMP, have been shown to have a wide range of effects, some of which will be discussed here.

It has been shown that in response to undetermined first messengers, cAMP is responsible for inhibiting tumor-cell growth in humans (Yokozaki, et.al., 1992; Pepe, et.al., 1991). The specific mechanism of activation and the specific role of cAMP remain

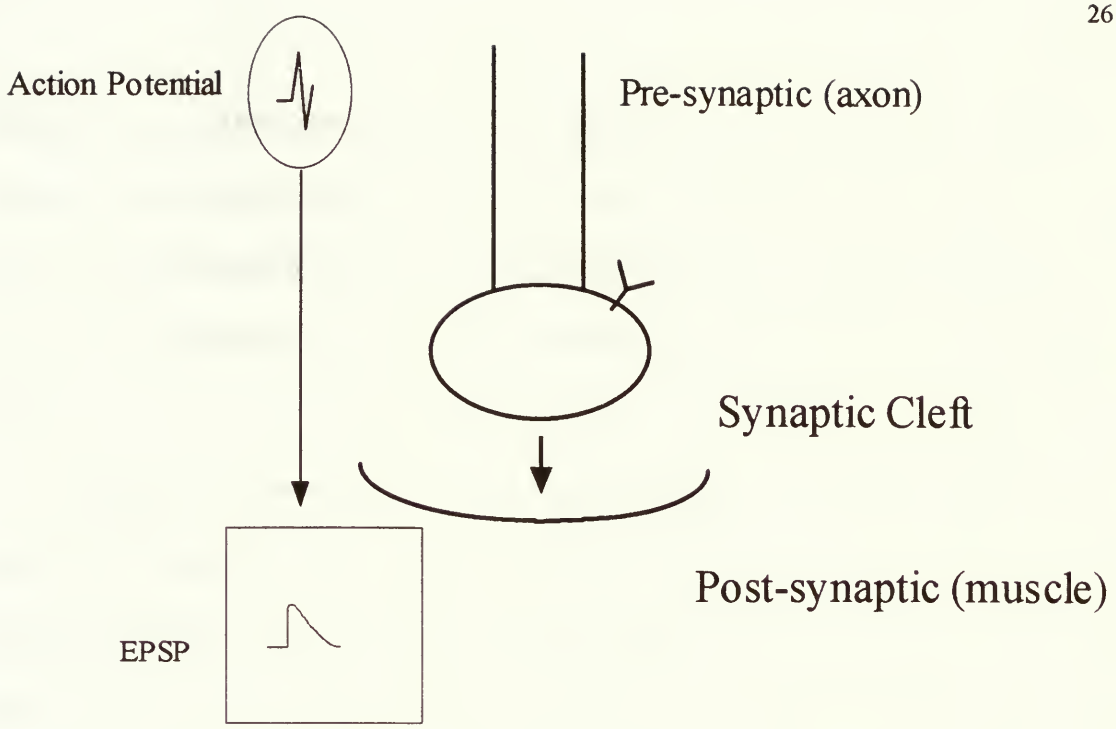
elusive, but perhaps work done on transcription of certain bacterial genes and their expression can help to provide the answers. cAMP plays an important role in controlling the transcription and translation of a variety of genes in *Escherichia coli* through its interaction with one of two proteins called catabolite gene activator protein (CAP) or cAMP receptor protein (CRP) both of which are activated by cAMP (Scholübbers, et.al., 1984). Regulation by CAP is exerted at the transcriptional level when activated by cAMP. The cAMP-CAP complex is responsible for the initiation of transcription of a variety of genes by recognition of DNA sequences near the promotor of several operons such as *lac* and *gal* (Scholübbers, et.al., 1984).

cAMP has also been shown to be involved as a signaling intermediate in other biosythetic pathways (Rothermel, et.al., 1984; Pereira, et.al., 1987; Schwarzschild and Zigmond, 1991). During the production of some steroids and other hormones, cAMP has been shown to be involved as an intracellular messenger. Upon activation of a receptor by some initiation signal, cAMP triggers the production of certain hormones. For example, thyroid hormone synthesis and secretion is triggered by the presence of thyrotropin (or thyroid stimulating hormone, TSH) which rapidly increases the intracellular concentration of cAMP (Erneux, et.al., 1986). Synthesis of this hormone is inhibited by inactive cAMP analogues, and is initiated by the exogenous addition of active cAMP analogues (Erneux, et.al., 1986).

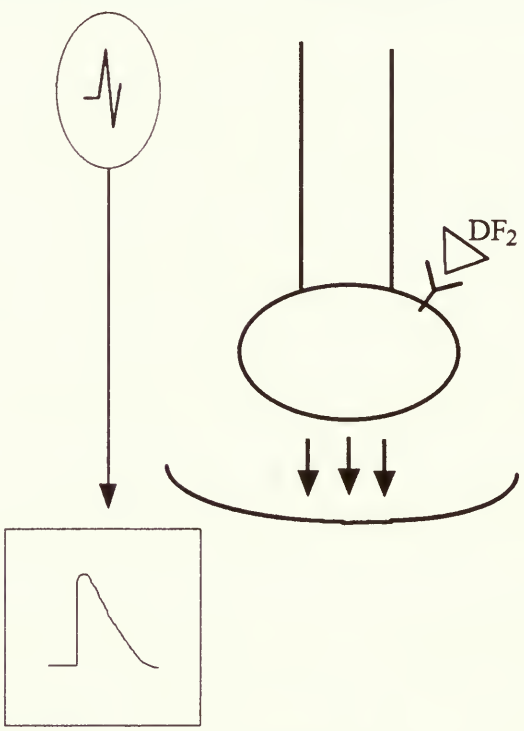
At least one more area in which second messengers play a major role, is in modulation of neuromuscular synapses. During Long-Term Potentiation (LTP) or Long-Term Facilitation (LTF), the synaptic output of a neuron remains higher than normal for a

FIGURE 1-2: Increase in Transmitter Output by DF_2

In the absence of the neuromodulator DF_2, an action potential travelling down the axon initiates the release of a certain amount of transmitter into the synaptic cleft. When DF_2 is bound to the receptor, the amount of transmitter released is increased, and therefore the post-synaptic response (the EPSP) is also increased.



Pre-peptide (no DF_2)



With DF_2 (note the increase in EPSI Amplitude)

prolonged period of time, and this is generally brought about by a brief period of high-frequency stimulation in crayfish (Dixon and Atwood, 1989a). This phenomenon is believed to be mediated by AC and therefore the cAMP/PKA pathway both at the crayfish NMJ (Dixon and Atwood, 1989a) and in rat hippocampal neurons (Frey, et.al., 1993).

Olfaction in vertebrates is also partially mediated by cAMP. When odorants interact with receptors on cilia, a G-protein is activated, initiating the neural signal (Moon, et.al., 1998). This odorant-induced neural signal *is* the sense of smell. cAMP is also involved in the sympathetic regulation of cardiac rhythms in rats (Hartzell, et.al., 1991) and in the control of the rhythmic outputs of bag cells in *Aplysia* (Kaczmarek, et.al., 1980).

cAMP has also been shown to be involved in the neuromodulatory pathway of a number of neuropeptides and biogenic amines. For example, serotonin (5-HT) has been shown to cause an increase in neurotransmitter output at lobster neuromuscular junctions (Goy, et.al., 1984) and crayfish neuromuscular junctions (Dudel, 1965a). This increase has been at least partially attributed to the intermediate signaling properties of cAMP/PKA (Goy and Kravitz, 1989; Kravitz, et.al., 1980). Octopamine (OA) has also been shown to have an effect upon contraction at insect visceral muscles, specifically locust oviduct (Lange and Nykamp, 1996). This modulation of synaptic transmission appears to be mediated through the activation of a GPCR and, therefore, through cAMP and PKA (Lange and Nykamp, 1996; Nykamp and Lange, 1998). It has been shown that in the case of still another amine, dopamine (DA), cAMP and therefore PKA are involved

in the modulation of hindgut contractions in crayfish (Mercier, et.al, 1991; Knotz and Mercier, 1995).

In the case of neuropeptides, it has been shown that the cAMP/PKA pathway plays a role in the modulation of synaptic output and/or in the control of muscular contractions by proctolin (Lange, et.al., 1987), schistoFLRFamide (Lange and Orchard, 1998) and DF_2 (Weston, et.al., 1997).

1.7.2 cGMP

As stated above, in the G-protein intracellular signaling system, cAMP is not necessarily the only cNMP that can act as a second messenger. Upon activation of the G-protein, AC can become active. Another cyclase that is sometimes involved is guanylyl cyclase (GC). As the name implies, it doesn't convert ATP into cAMP; rather it converts GTP into cGMP. cGMP can then go on to activate the cGMP-dependent Protein Kinase (PKG) analogous to the cAMP/PKA pathway. Though cAMP is found to be a second messenger in nearly all vertebrate and invertebrate cells, cGMP appears to have a more restricted distribution within the animal kingdom, playing a larger role in invertebrates than in vertebrates (Pavloff and Goy, 1990). That notwithstanding, these parallel pathways can have similar behavioural effects, or the targets of these two kinases can be quite different within individual cells.

Unlike cAMP, there exists no direct evidence that the cGMP/PKG system plays any role in modulation of synaptic output by neuropeptides or other neurohormones. However, like the cAMP/PKA system, the cGMP/PKG system does influence LTP and LTF in rat hippocampus (Arancio, et.al., 1995). LTP and LTF appear to be dependent

upon increases in the concentration of intracellular cGMP levels and in PKG activity, in a manner similar to the results found with cAMP (Frey, et.al., 1993). Interestingly, in cells of the rat Dentate Gyrus, cGMP and PKG have no effect on LTP, however Long-Term Depression (LTD) of synaptic transmission is cGMP/PKG dependent (Wu, et.al., 1998). These opposing effects on synaptic transmission on two different regions of the brain appear to be involved in short-term and long-term memory (Arancio, et.al., 1995; Frey, et.al., 1993; Wu, et.al., 1998).

Another behavioural response that has been shown to be mediated, at least in part, by cGMP and therefore PKG, is the relaxation of human vascular endothelial cells which leads to vasodilation (Draijer, et.al., 1995; Geiger, et.al., 1992; Sane, et.al., 1989). When stimulated to do so, cGMP levels rise, presumably through the activation of GC via stimulation through a GPCR, which in turn activate PKG. PKG then phosphorylates the vasodilator-stimulated phosphoprotein (VASP) which initiates vasodilation (Draijer, 1995). Further evidence of this comes from studies indicating that well established nitrovasodilators such as nitroprusside and endothelium-derived relaxing factor are potent activators of cGMP elevation (Geiger, et.al., 1992). cGMP has also been shown to initiate platelet aggregation, which accompanies vasodilation in humans (Sane, et.al., 1989), in rats (Rapoport, et.al., 1982) and in cows (Ruth, et.al., 1991). These data explain why humans suffering from heart and circulatory problems take nitric oxide (NO), an agent known to increase cGMP levels in many systems, including the stomatogastric system of the crab (Scholz, et.al., 1996).

Another role for cGMP in mammals, including humans, is in Cl^- conductance modulation. In human colon cells, increased levels of cGMP are linked to an increase in

Cl^- conductance (Tien, et.al., 1994). This Cl^- conductance control is important in maintaining a proper salt level in the body. It is not known whether cGMP directly modulates Cl^- conductance through direct gating of the channels, or if the effect is mediated via PKG. In patients with cystic fibrosis (CF), a specific type of Cl^- channel is not produced, the cAMP/cGMP-inducible Cl^- channel (Tien, et.al., 1994). It is the lack of this channel that leads to the symptoms of this disease.

Recent studies have found a hormone in lobster and other crustaceans that is known to selectively increase cGMP levels in muscles. This peptide, called crustacean hyperglycemic hormone (CHH), is secreted from the sinus gland of the lobster into the circulation, from which receptors on the muscle receive it. These GPCRs then activate GC and thereby increase intracellular cGMP levels (Goy, 1990). This hormone, also called peptide G_1 for its effects on cGMP levels, can increase the intracellular cGMP concentrations by nearly 200-fold in all lobster tissues that were tested (Pavloff and Goy, 1990). The physiological significance of this peptide and its associated increase in cGMP levels however, were not determined.

1.7.3 IP_3 and DAG

Upon activation of a G-protein through the binding of a ligand to a GPCR, one pathway for signal transduction is to activate the cyclase enzymes generating cNMPs as second messengers. Another pathway involves the activation of a membrane-bound enzyme called phospholipase C (PLC; Takahashi, et.al., 1998). PLC catalyses the breakdown of PIP_2 into membrane-bound DAG and cytosolic IP_3. These two entities act as second messengers, transducing the activity of the G-protein into activation of other

kinases. DAG activates PKC, which initiates a variety of intracellular responses. PKC requires DAG and Ca^{2+} to become activated. However, DAG increases the affinity of PKC to Ca^{2+} to such an extent that the enzyme can become fully active without an increase in intracellular Ca^{2+} levels. Thus the basal Ca^{2+} concentration is significant. When coupled with the property of IP_3 to increase intracellular Ca^{2+} concentrations (see below), the activity of PKC is complete (Nishizuka, 1986; Salter and Hicks, 1995). PKC has been shown to increase the membrane conductance to certain ions, such as Ca^{2+}, helping to describe the role it plays in increasing transmitter output (Nishizuka, 1986; Friedrich, et.al., 1998). It is believed that PKC increases ionic conductance by phosphorylating membrane proteins such as channels, pumps and ion exchange proteins (Nishizuka, 1986), and that its effects reverse relatively slowly. This could explain the long-term, sustaining role that PKC appears to play in neuromodulation by the neuropeptide DF_2 (in crayfish: Friedrich, et.al., 1998; in *Aplysia*: Sugita, et.al., 1992).

As mentioned above, the other product of PIP_2 hydrolysis by PLC is IP_3. IP_3 has been shown to elevate intracellular Ca^{2+} concentration by facilitating its release from internal stores, primarily the endoplasmic reticulum (Nishizuka, 1986; Berridge and Irvine, 1989; Burgess, et.al., 1984). Coupled with the DAG-mediated activation of PKC and the subsequent increase in the influx of calcium, the IP_3-mediated release of Ca^{2+} from internal stores results in a substantial increase in cytosolic Ca^{2+} which can have a wide range of effects, one of which may be an increase in transmitter output at a nerve terminal (Berridge and Irvine, 1989; Friedrich, et.al., 1998). Another role for Ca^{2+} in the nerve terminal is the activation of the calcium/calmodulin-dependent protein kinase type II (CaMKII). This kinase has been shown to be involved in the modulation of

neuromuscular transmission at the squid giant synapse (Llinas, et.al., 1991) and a crayfish neuromuscular junction (Noronha and Mercier, 1995).

Therefore, all of the results taken together indicate that the modulation of neuromuscular transmission at crayfish abdominal NMJs by DF2 involves at least three kinases, PKC (Friedrich, et.al., 1998), CaMKII (Noronha and Mercier, 1995) and PKA (Weston, et.al., 1997).

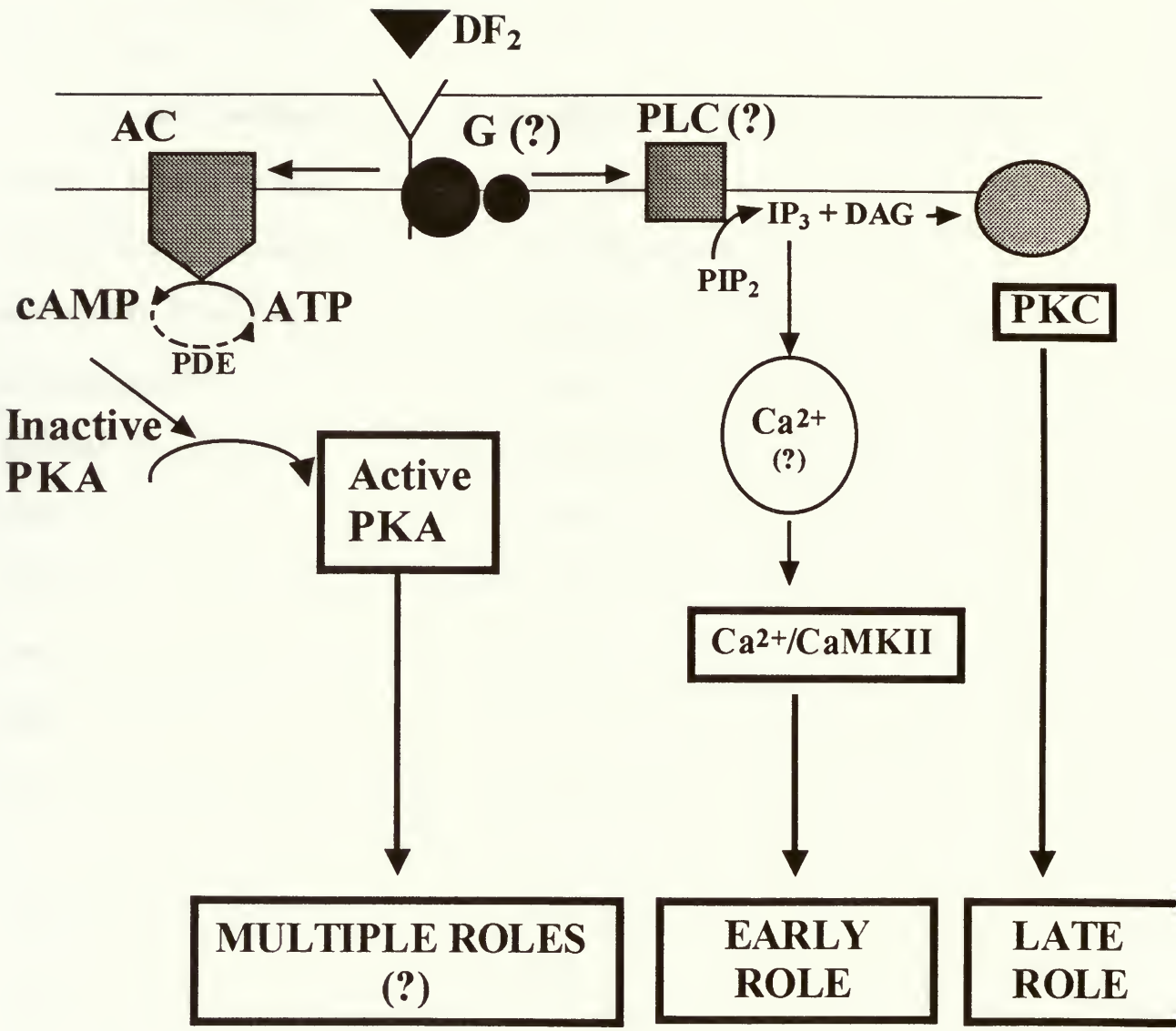
1.8 Objectives

The main purpose of this thesis is to provide further insight into the intracellular mechanisms involved in the increase in transmitter output at a crayfish neuromuscular junction in response to the neuropeptide DF_2. As described above, it has been established that DF_2 increases the quantal content of the neuron rather than increasing the sensitivity of the muscle to the transmitter. It is proposed that this peptide acts by binding to a presynaptic G-protein-coupled receptor, activating second messengers and increasing the activity of a number of kinases including PKC, PKA and CamKII (all described above). It is also possible that PKG plays a role in this neuromodulation. In order to demonstrate this, a variety of activators and inhibitors of some of the enzymes typically associated with the G-protein pathway will be employed to see if they, and therefore the enzyme with which they are interacting, play a role in DF_2's effect on the presynaptic terminal. The prediction is that in the presence of an inhibitor of a kinase that is believed to be involved in transducing the peptide's effect, the response to DF_2 will be partially blocked. Conversely, increasing the activity of one (or more) of these kinases should mimic the effect that the peptide has on postsynaptically recorded EPSPs.

A typical G-protein-coupled receptor pathway along with the previous evidence discussed in the previous sections, were used as a guide in determining what kinases may be involved in DF_2's modulatory pathway (Fig. 1-3). It has been inferred that if the kinases associated with this pathway are involved, than so too is a G-protein, although no direct evidence exists of a receptor of this type for DF_2.

FIGURE 1-3: Proposed Mechanism for Modulation by DF_2

This scheme is based upon the traditional GPCR pathway. Once bound to a receptor, DF_2 initiates the generation of at least three second messengers. Activation of adenylyl cyclase catalyses the production of cAMP which goes on to activate PKA. Activation of PLC converts PIP_2 into IP_3 and DAG. DAG turns on PKC and IP_3 causes Ca^{2+} to be released from internal stores resulting in activation of CaMKII.


DF2
AC
G (?)
PLC (?)
IP3 + DAG
PIP2
cAMP
ATP
PDE
PKC
Inactive PKA
Active PKA
Ca2+ (?)
Ca2+/CaMKII
MULTIPLE ROLES (?)
EARLY ROLE
LATE ROLE

2. MATERIALS AND METHODS

2.1 Chemicals and Solutions

2.1.1 Salines

In order to maintain activity in the excitable tissues of the dissected preparation, bathing it in a solution closely matching the haemolymph of crayfish is ideal. The chemical composition of this saline is based on van Harrevald (1937) as cited in Mercier et.al. (1993). If, however, the preparation is bathed in this 'normal' saline, the neurons release enough transmitter to make the muscles in the preparation twitch. This is not desirable due to the fact that intracellular electrodes placed inside the twitching muscle cells could either break, or the cell membrane could tear. Either way, the muscle cell would become damaged, and the preparation would be ruined. Also, the electrical recording would be affected by the movement of the muscle. For all these reasons a new saline had to be employed to prevent the twitching without detrimentally changing the ionic environment. Reducing the calcium ion concentration by a factor of two and increasing the magnesium ion concentration by a factor of five accomplishes this feat (Mercier and Atwood, 1989; Mercier et.al., 1990). To compensate for the change in osmolality, the NaCl and the KCl concentrations were modified. The lower calcium ion concentration/higher magnesium ion concentration allows for normal nerve activity, but with a reduced amount of transmitter being released at the neuromuscular synapse. This prevents twitching of the muscle cell, yet excitation can still be recorded as an EPSP. Therefore, for all recordings, this low-Ca^{2+}/high-Mg^{2+} saline was used. This saline contained the following dissolved in distilled water : NaCl (200.65mM, BDH Inc., Toronto, Ontario), KCl (5.37mM, Caledon Laboratories, Georgetown, Ontario),

$CaCl_2 \cdot 2H_2O$ (6.5mM, Sigma, St. Louis, Missouri), $MgCl_2 \cdot 6H_2O$ (12.3mM, BDH Inc., Toronto, Ontario) and HEPES (5.0mM, Sigma, St. Louis, Missouri). The pH of the saline was adjusted to 7.4 using NaOH (1N, Caledon Laboratories, Georgetown, Ontario).

2.1.2 Neuromodulator

As described earlier, the neuromodulator used was the heptapeptide DF_2. Solid DF_2 (Bachem Inc., Torrance, California) was dissolved in low-Ca^{2+}/high-Mg^{2+} saline to yield a 10^{-4}M stock, which was then divided into 100μL aliquots. Both the solid and the stock solution were stored at -4°C, the solid under desiccation. The stock solution was diluted to the final, experimental concentration using low-Ca^{2+}/high-Mg^{2+} saline.

2.1.3 Phosphodiesterase Inhibitors

Two different phosphodiesterase (PDE) inhibitors were used in this study. The first, 3-Isobutyl-1-methylxanthine, or IBMX (Calbiochem, La Jolla, California) is a non-specific PDE inhibitor (Scamps et.al., 1993). The solid was dissolved in dimethylsulphoxide (DMSO) and then was diluted with low-Ca^{2+}/high-Mg^{2+} saline to yield a stock concentration of 10^{-3}M containing 0.05% v/v DMSO and stored at -4°C. The stock was then diluted two-fold in saline to obtain the final experimental concentration of 10μM.

The other phosphodiesterase inhibitor was more specific for the cyclic GMP-specific PDE, cGMP-PDE type V (Takase et.al., 1994). This inhibitor was

4-{[3',4'-(Methylenedioxy)benzyl]amino}-6-methoxyquinazoline, or mdBAMQ (Calbiochem, La Jolla, California). Again the solid was diluted to a stock concentration of 10^{-4}M in low-Ca^{2+}/high-Mg^{2+} saline and was stored at -4°C in 100μL aliquots. The stock was diluted using the same saline to a final experimental concentration of 1μM.

Both chemicals, when received from the supply company in solid form, were stored in the freezer at -4°C under desiccation.

2.1.4 Protein Kinase Activators and Inhibitors

In order to determine what effects DF_2 may have on the protein kinases that are typically associated with the G-protein pathway, specifically protein kinase A (PKA) and protein kinase G (PKG), specific activators (agonists) and inhibitors (antagonists) to these kinases were used.

The PKA agonist that was employed was the membrane-permeant analogue of cAMP called adenosine-3',5'-cyclic monophosphorothioate, Sp-isomer (Sp-cAMPS; Calbiochem, La Jolla, California). The solid was stored under desiccation at -4°C. Preparation of the stock solution required diluting the solid in low-Ca^{2+}/high-Mg^{2+} saline to a concentration of 10^{-3}M. The stock was then divided into 100μL aliquots and was stored at -4°C.

An inactive analogue of cAMP was used as an antagonist to PKA. Adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer, Rp-cAMPS (Calbiochem, La Jolla, California) is a membrane-permeant inhibitor of PKA (DeWit, et.al., 1984). Preparation and storage of the solid and stock solution were the same as for Sp-cAMPS.

Storage and preparation of the stock solution is similar to the methods described above for Rp- and Sp-cAMPS except that the stock concentration was 10^{-4}M.

In order to activate the PKG, an analogue of cGMP was used. This analogue, 8-(*para*-chlorophenylthio)guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP; Calbiochem, La Jolla, California), has been shown to activate PKG in human endothelial cells (Draijer et.al., 1995) and human platelets (Geiger et.al., 1992). Storage and preparation of the solid and the stock solution was similar to all other kinase activators and inhibitors, with the stock solution made at a concentration of $4x10^{-4}$M. In order to determine what concentration of 8-pCPT-cGMP would be considered just subthreshold, a dose-response curve was generated. Once the appropriate concentration was chosen, an experiment, using the same protocols described for the PDE inhibitors, was performed with 10^{-8}M DF_2, which would provide evidence to support or refute the role of PKG in neuromodulation by DF_2.

2.2 Animals

2.2.1 Storage and Care of Animals

The animals used were crayfish, *Procambarus clarkii,* obtained from the Atchafalaya Biological Supply (Raceland, Louisiana). They were stored in tanks of dechlorinated freshwater at a temperature of 15°C and were fed a tender variety of solid cat food every second day. The water was continuously aerated and filtered. Before the animals were euthanized for study, they were anesthetized on ice for 15 to 20 minutes to dull nervous transmission in any sensory structures that might be associated with pain,

although there is no evidence that such structures exist. This protocol met with the approval of the Animal Care and Use Committee at Brock University.

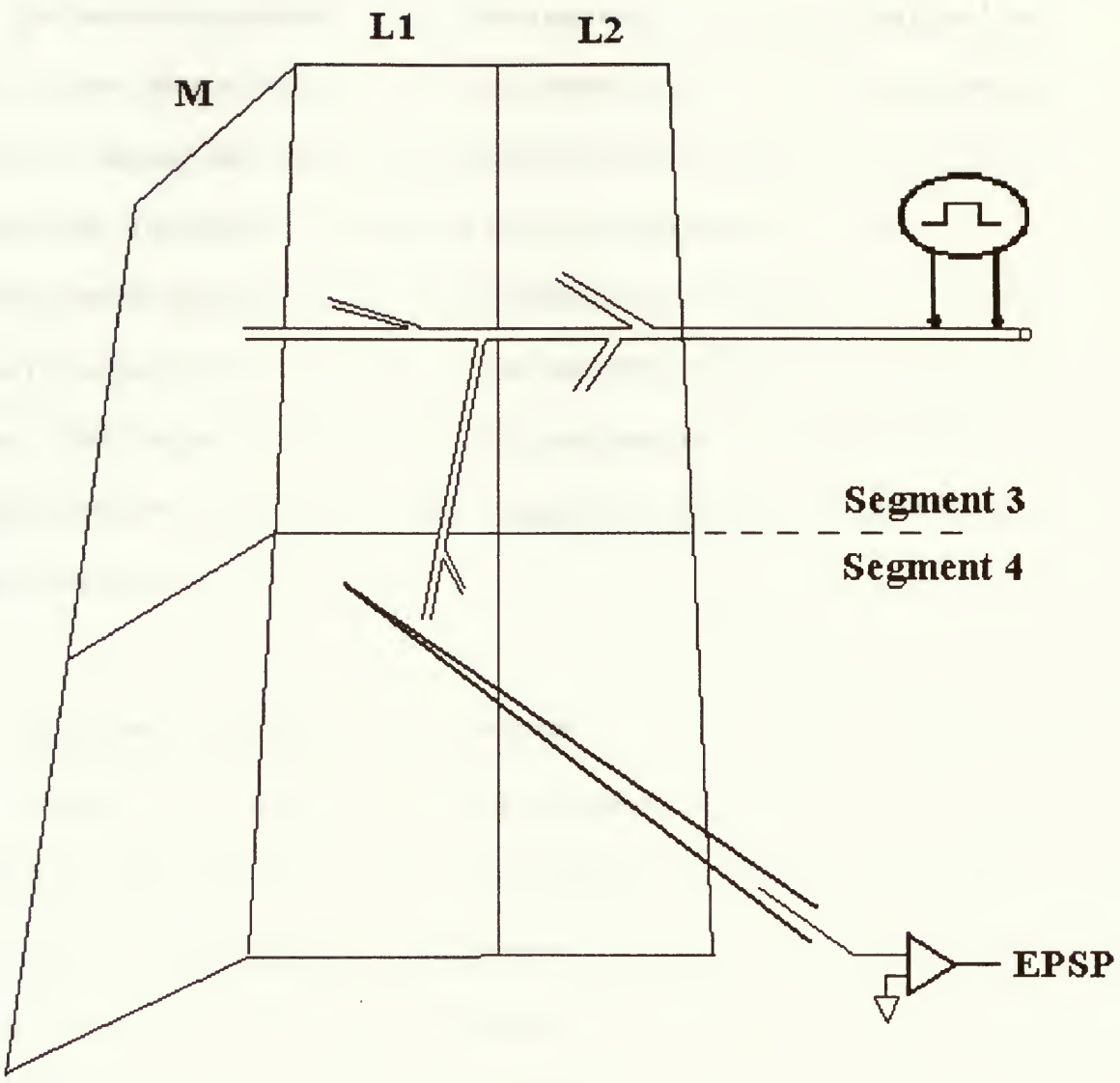
2.2.2 Preparation of Animals

Following euthanasia, the legs of the crayfish were removed, and the abdomen was cut away from the thorax. The dorsal shell of the abdomen was removed by making shallow cuts along both lateral sides beginning at the anterior end and continuing to the end of the sixth and most posterior abdominal segment about midway along the dorsal-ventral axis. A very shallow lateral cut was made just posterior to the sixth abdominal segment on the dorsal shell to connect the two previous cuts. This set of cuts allowed the removal of the dorsal shell by gently grasping the sixth segment and carefully peeling all six segments away from the rest of the abdomen, working from Segment 6 anteriorly through Segment 1. The dorsal shell, containing the deep abdominal extensor muscles, was then placed ventral side up in a sylgard-lined dissecting dish containing the low-Ca^{2+}/high-Mg^{2+} crayfish physiological saline.

Within each segment are a pair of medial muscles (designated M) and two pairs of lateral muscles (designated L1 and L2) as described by Parnas and Atwood (1966). The M muscles were removed from all six segments in order to isolate the L muscles. The sixth, first and second segments were then completely removed, leaving only the third, fourth and fifth segments. The L muscles of segment four and the nervous innervation from segment three were left intact (Fig 2-1). The preparation was then cut in half along the anterior-posterior axis, yielding a pair of preparations, one of which was saved in low Ca^{2+}/high Mg^{2+} saline in the refrigerator for later use. The other preparation was

FIGURE 2-1: Schematic Representation of the Preparation

This representation shows one side of the third (S3) and fourth (S4) segments of the crayfish deep abdominal extensor muscles. In the final preparation, the M muscles and the L muscles of S3 are removed leaving only the L muscles of segment 4 and its innervation from the nerve root from S3 (note: preparation in this way allows for the recording of EPSPs generated by only one excitatory axon (see text)).


L1
L2
M
Segment 3
Segment 4
EPSP

transferred and pinned ventral side up into a sylgard-lined recording dish with a 2 mL chamber volume.

Each muscle is innervated by axons from a nerve in its own segment and by at least one axon from a branch of the nerve in the next anterior segment. For these experiments the nerve in segment three was stimulated, and EPSPs were recorded from muscle L1 in segment four. This muscle is innervated by two axons from segment three. One is excitatory and the other is inhibitory. Thus, EPSPs elicited in this muscle by stimulating the nerve in segment three are generated by one identifiable excitatory axon (Mercier and Atwood, 1989; Parnas and Atwood, 1966). The muscle studied is also innervated by a common excitatory axon, a specific excitatory axon and an inhibitory axon from its own segment, but these were not stimulated.

2.3 Recording Equipment and Apparatus

Once the preparation had been dissected, and placed ventral side up in a 2 mL chamber in a sylgard-lined recording dish, it was maintained at 9-10°C by a circulating bath of antifreeze with its temperature controlled by a refrigerated circulator and thermostat (Model 2095, Forma Scientific, Marietta, Ohio). The temperature in the recording dish was continuously monitored with a digital thermocouple-thermometer (Model 8500-40, Cole-Parmer Instruments, Chicago, Illinois), which showed that the temperature during any one experiment fluctuated by less than ±0.5°C.

In most experiments, the preparations were constantly superfused with fresh saline that had been cooled to the appropriate temperature. The saline was pumped into the chamber at a constant rate of 2.5 mL/min by means of a Masterflex peristaltic pump

(Cole-Parmer Instruments, Chicago, Illinois). The overflow was taken away by a suction pump to maintain a constant volume of about 2 mL in the recording chamber.

In some experiments, however, the static bath method was used. In this method, instead of constantly superfusing the preparation, the pumps were turned off and the bath was held static at a known volume, 2 mL. To this volume, the appropriate amount of test chemical was added to achieve the proper final, experimental concentration. This methodology was employed for the experiments using the protein kinase activators/inhibitors.

The preparation and the cooling coils of the refrigerated circulation system, were placed under a 6.4-40x dissecting microscope (Wild Instruments, Switzerland) equipped with a 10x ocular. The preparation was illuminated with a grounded fiber-optic light source. A chlorided silver wire connected to ground was placed in the bath. The preparation and the microscope were situated in a grounded copper (Faraday) cage to filter out ambient electromagnetic room noise.

2.4 Recording of Intracellular Signals

Once the preparation was placed inside the Faraday cage and under the microscope, intracellular recording could begin.

2.4.1 Stimulating Electrode

In order to record EPSPs from the muscles, it is necessary to stimulate the neurons that innervate them. As described earlier, preparation of the animals in this way allows for the recording of EPSPs elicited by only one excitatory axon. A suction

electrode was used to stimulate the nerve of segment 3 (Fig. 2-1) following the same protocols used in earlier work (Mercier and Atwood, 1989). The suction electrode was mounted in a micromanipulator (Narishige) to prevent any undue movements of the tip relative to the preparation. Once the tip was aligned just above the nerve trunk, a slight negative pressure was applied to draw in the nerve.

In order to stimulate the nerve to fire action potentials along its axons, the suction electrode was attached to a Model S-88 stimulator (Grass Instruments, Quincy, Massachusetts) via an SIU-5 stimulus isolation unit (Grass Instruments, Quincy, Massachusetts). Stimulation was delivered at a frequency of 1 pulse every 10 seconds (0.1Hz) for a duration of 5ms. The intensity of the stimulus varied from 2 to 15 V depending upon the preparation.

2.4.2 Intracellular Recording

Stimulating the abdominal nerve in the above way will generate post-synaptic potentials in the abdominal muscles. As previously described, axon 3 innervates the L1 muscle of segment 4 and is the only excitatory axon to do so. In order to record the EPSPs generated by this axon in this muscle, it is necessary to place an electrode inside one of the muscle cells.

An intracellular electrode holder was mounted onto a Narishige micromanipulator in order to control the fine movements of the electrode tip. The electrode tip was made by pulling a glass capillary tube with a fine glass filament inside (cat.no. 6030, A-M Systems, Inc., Everett, Washington) to a very fine tip (eventual electrode resistance of 15-50 MΩ) using a Model 700-D vertical pipette puller (David Kopf Instruments,

Tujunga, California). The electrode holder was connected to the probe of a Cyto 721 Electrometer (World Precision Instruments, New Haven, Connecticut) to detect transmembrane voltages. Signals were viewed on a Hameg Model 205-3 digital storage oscilloscope (Hameg, Frankfurt, Germany).

2.4.3 Data Acquisition

To digitize the signal, the output voltage from the electrometer was connected to a DSP Pre-Amplifier (Brock University, St. Catharines, Ontario). The output of the Pre-Amp, in turn, was connected to an IBM Compatible 386 computer for acquisition and storage. In order to ensure synchronous recordings, the triggering pulse output from the stimulator was also connected through the Pre-Amp to the computer and the oscilloscope. To store and analyze the data, a computer program entitled Evoke (Brock University, St. Catharines, Ontario) was used to acquire the data and, Graph Pad Prism (Graph Pad Software, San Diego, California) was used for graphical representation of the data and for statistical analyses. Each data point collected was an average of 6 stimuli. Therefore, at a frequency of 1 pulse every 10 seconds, each data point represents the average recording in 1 min.

A schematic representation of the entire set-up can be seen in Fig. 2-3.

2.5 Procedures

Once the dissection was complete, the preparation was placed in the recording dish and situated under the microscope in the cooling apparatus, where the temperature

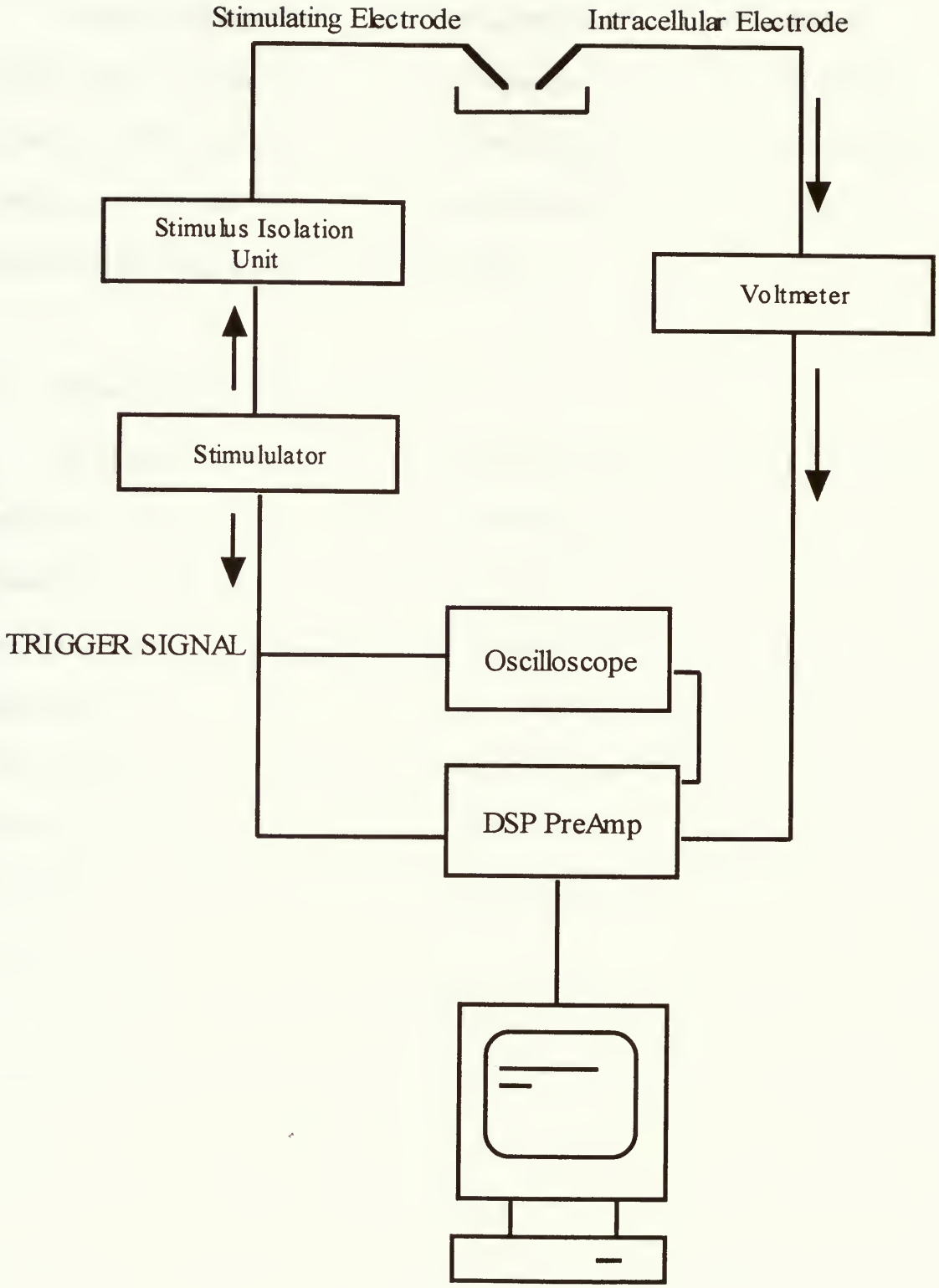
was maintained at approximately 10°C. The bathing solution was maintained and changed as described above for the constant perfusion and the static bath methods.

2.6 Statistical Analysis

When comparing the initial and final values within one experimental condition (eg. treatment with IBMX alone), the Wilcoxan Signed Rank Test for correlated samples was used in order to determine if there is a significant level of difference between initial and final values (Ferguson, 1971). Initial conditions were calculated as the average amplitude of EPSPs during the first 15 min (time 0-15 min.). Comparisons between different experimental conditions (eg. comparing DF_2 + IBMX with DF_2 alone) were made using the Mann-Whitney U-test.

FIGURE 2-2: Flow Diagram of Apparatus

Stimulation of the nerve root of segment 3 initiated an EPSP in the L1 muscle of segment 4. This EPSP was detected with the intracellular electrode which was connected to a computer for data acquisition and an oscilloscope via the Pre-Amp.


Stimulating Electrode
Intracellular Electrode
Stimulus Isolation Unit
Voltmeter
Stimululator
TRIGGER SIGNAL
Oscilloscope
DSP PreAmp

3. RESULTS

As previously described, it has been proposed that DF_2 confers its effect on synaptic output, or quantal content, through a G-protein-coupled receptor. In order to determine how DF_2 is able to increase the number of synaptic vesicles released per nerve impulse, certain activators and inhibitors of enzymes and other intermediates in the G-protein-coupled receptor pathway were employed.

3.1 Phosphodiesterase Inhibitors

If the peptide does activate a G-protein, then it would follow that there would be an increase in the activity of a cyclase enzyme, among other things including phospholipase C which would eventually result in an increase in PKC activity. For example, when DF_2 binds to its receptor, the G-protein could activate adenylyl cyclase which catalyses the conversion of ATP to cAMP. cAMP would then, in turn, act as a second messenger by activating other enzymes further down this intracellular signalling pathway eventually resulting in the increased transmitter output and increased EPSP amplitude. Therefore, there would be an increase in intracellular cAMP associated with the presence of DF_2.

If a cyclic nucleotide mediates DF_2's effects, artificially increasing the concentration of the cyclic nucleotide should mimic at least a portion of the peptide's response. Likewise, if the concentration of the cyclic nucleotide is increased to a level that just fails to elicit an increase in EPSP amplitudes (ie- a cyclic nucleotide concentration that is just sub-threshold) in the presence of a concentration of DF_2 that is considered just sub-threshold, than the two moderate increases in cyclic nucleotide

concentration would combine to generate an increase EPSP amplitudes. There are at least two methods to increase the concentration of the cyclic nucleotide. One involves increasing the activity of the enzyme responsible for its genesis, and the other would be to decrease the activity of the enzyme responsible for its breakdown. It is the latter method that was employed. There exists a class of enzymes whose purpose is to break the cyclic nucleotide down into a non-cyclic form, thereby removing its ability to act as a second messenger in this system. These enzymes are called phosphodiesterase (PDE) enzymes. Inhibiting these enzymes would prevent the breakdown of the cyclic nucleotide, increasing its intracellular concentration.

3.1.1 IBMX

One inhibitor of these PDE's is 3-isobutyl-1-methylxanthine, abbreviated as "IBMX". IBMX is a broad spectrum PDE inhibitor, which inhibits the breakdown of at least two types of cyclic nucleotides, cAMP and cGMP (Scamps, et.al., 1993). Previous research has shown that IBMX has a dose-dependent effect on EPSP amplitudes in this preparation with a threshold concentration lying just above 10μM (Friedrich, 1994). As shown in Fig. 3-1, a subthreshold dose (10μM) of IBMX did not alter EPSP amplitude even after more than 80 minutes (%change of EPSP = -1.7±0.7%*; $p>0.08$, Wilcoxan Test). When a subthreshold concentration of DF_2 was added in the presence of IBMX, an immediate increase in EPSP amplitudes occurred. Therefore, neither IBMX nor DF_2 (-0.181±1.675%; $p>0.08$) at the concentrations used had any effect on EPSP amplitudes

* All statistics reported will be the % change in EPSP amplitude recorded at time = 84 min, the final recording made, except where indicated otherwise. Comparisons between different experimental conditions were made using the Mann-Whitney U-test. Comparisons of initial and final values within one experimental condition were made using the Wilcoxan Signed Rank Test for correlated samples.

when added alone, but when added together, an increase in EPSP amplitude resulted (23.3±2.3%) which was significantly different from the effect of DF_2 alone ($p<0.005$, Mann-Whitney Test). Thus, the peptide and the PDE inhibitor acted synergistically.

3.1.2 mdBAMQ

Another PDE inhibitor, one more specific for the cGMP-specific PDE was also used. This inhibitor, 4-{[3',4'-(Methylenedioxy)benzyl]amino}-6-methoxyquinazoline, abbreviated mdBAMQ, would, through inhibiting only the cGMP-specific PDE, selectively increase intracellular cGMP levels and should have no effect on cAMP levels at low concentrations. The results of the experiments performed with mdBAMQ are plotted in Fig. 3-2. Like IBMX, at a low enough concentration, one order of magnitude below the reported EC_{50} of 30μM (Takase, et.al., 1994), 3μM mdBAMQ had no effect on EPSP amplitudes (-1.9±4.2%; $p>0.08$, Wilcoxan Test). When DF_2 was added, however, EPSP amplitudes began to increase (23.7±5.7%). Again, a synergy can be seen between the peptide and the PDE inhibitor ($p<0.005$, Mann-Whitney Test). One quite interesting result is that the magnitudes of the effect on EPSP amplitudes in the presence of DF_2 are similar between IBMX (Fig. 3-1) and mdBAMQ (Fig. 3-2).

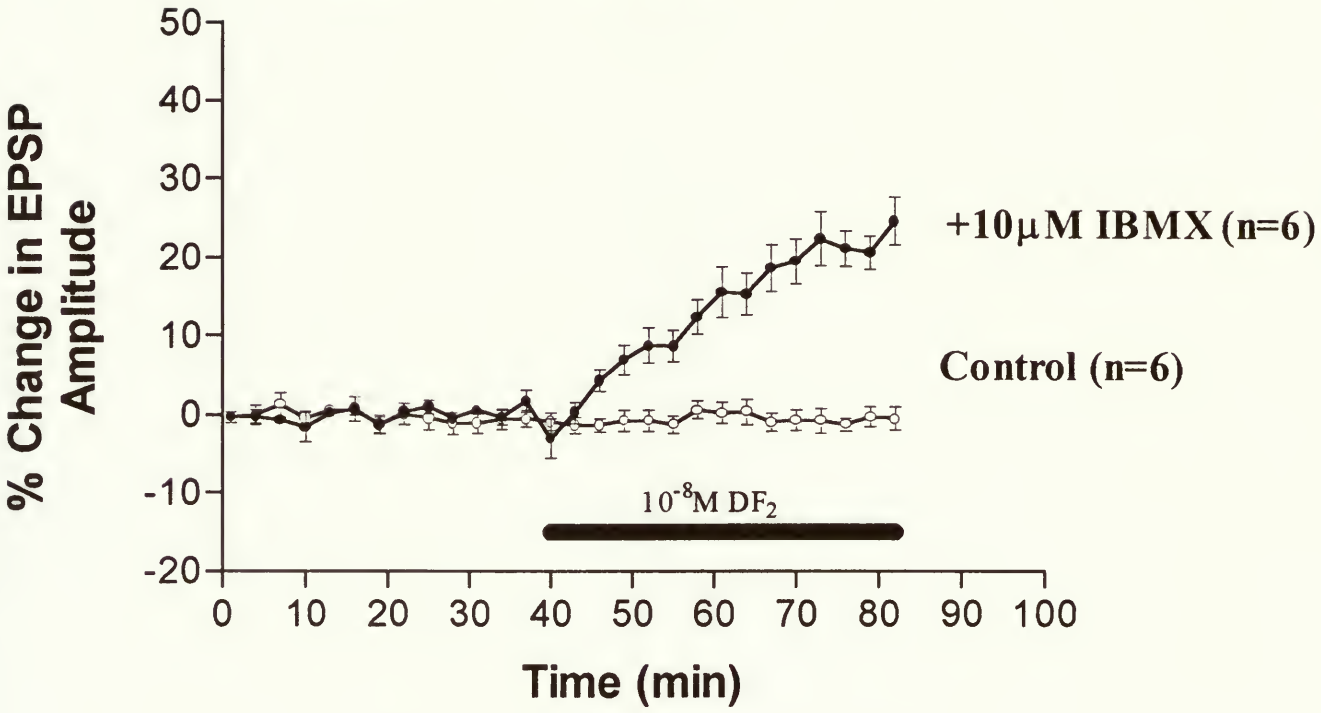
3.2 Protein Kinase G

The above work with the PDE inhibitors along with earlier work (Friedrich, 1994; Weston, et.al., 1997), provided evidence for the involvement of cyclic nucleotide monophophates, specifically cAMP and cGMP, in mediating the peptide's response. When acting as intracellular second messengers, these compounds invariably convey

FIGURE 3-1: Effect of IBMX on EPSP Amplitude

In the lower graph, 10μM IBMX was added at t=0min. Note that on its own, this concentration of IBMX has no effect even after 84min (-1.7±0.7%; $p>0.08$, Wilcoxan Test). The upper graph shows the effect of IBMX in the presence and in the absence of a low dosage of DF_2. The lower trace of the upper graph shows the effect of 10^{-8}M DF_2 added for 44min beginning at t=40min. Note that this sub-threshold concentration of DF_2 had no effect on its own (-0.2±1.7%; $p>0.08$, Wilcoxan Test). However, in the presence of the IBMX (added at t=0min), an increase in EPSP amplitude occurred (23.3±2.3%; $p<0.005$, Mann-Whitney Test).

Effect of Pre-Incubation With IBMX on EPSP Amplitude



Effect of IBMX on EPSP Amplitude in the Absence of DF2

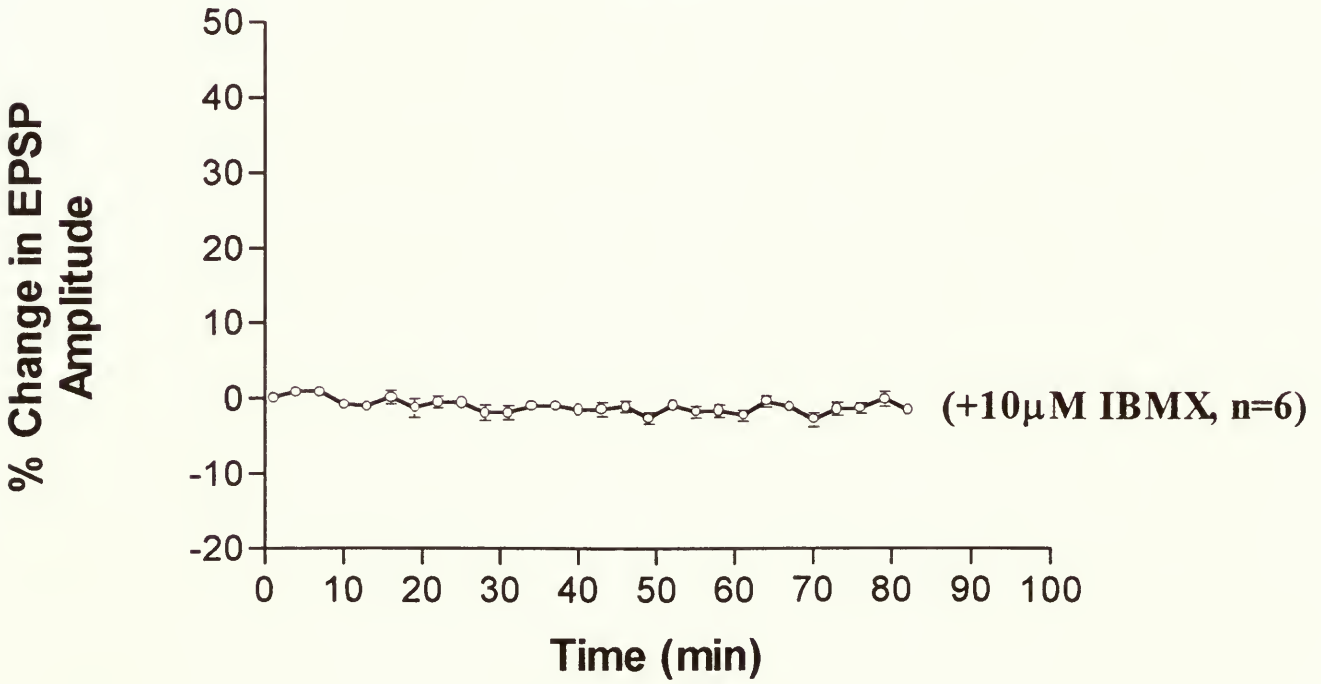
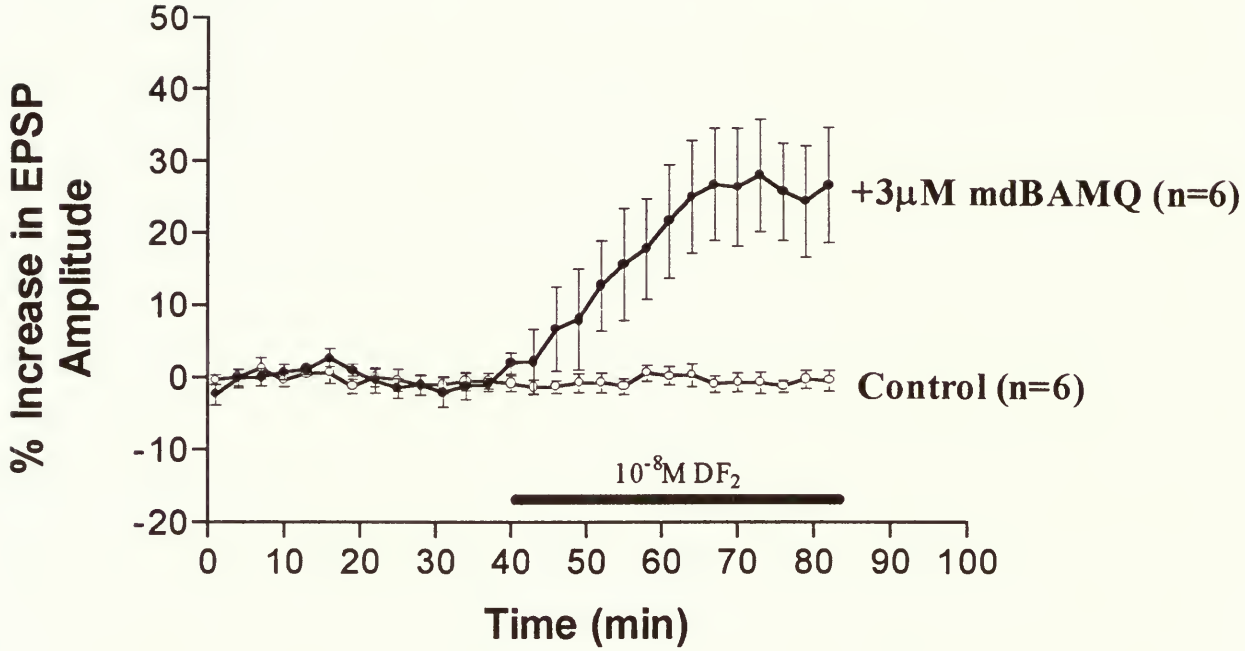


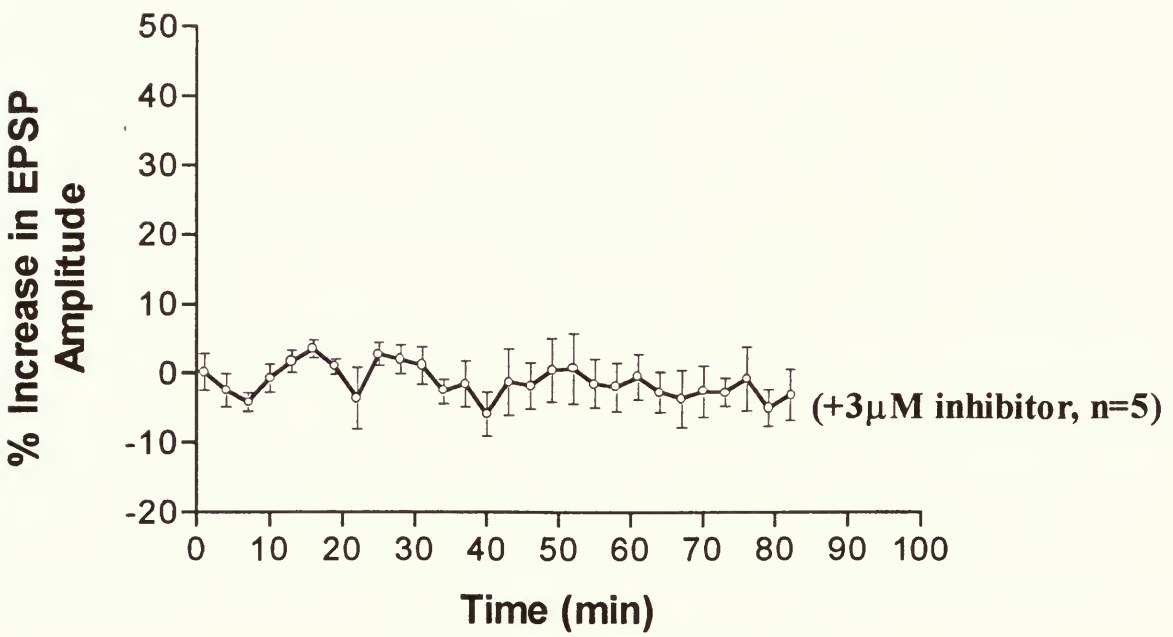
FIGURE 3-2: Effect of mdBAMQ on EPSP Amplitude

The lower graph shows the effect of 3μM mdBAMQ alone (added at t=0min) on EPSP amplitude. Note that even after 84min, this concentration of mdBAMQ had no effect (-1.9±4.2%; $p>0.08$, Wilcoxan Test). The upper graph shows the effect of mdBAMQ in the presence and absence of 10^{-8}M DF_2. The lower trace shows that this sub-threshold concentration of DF_2 had no effect on EPSP amplitude (peptide added at t=40min for a duration of 44min; -0.2±1.7%; $p>0.08$, Wilcoxan Test). However, when the preparation was pre-incubated in mdBAMQ, this low concentration of DF_2 caused an increase in EPSP amplitude (23.7±5.7%; $p<0.005$, Mann-Whitney Test).

Effect of Pre-Incubation With mdBAMQ on EPSP Amplitude



Effect of mdBAMQ on EPSP Amplitude in the Absence of DF_2



their effects by activating their appropriate target kinase. Specifically activating or inhibiting these kinases should also mimic or block the response to DF_2, respectively. Cyclic AMP is known to activate the cAMP-dependent Protein Kinase (PKA), and there exists evidence that PKA is involved in this neuromodulatory pathway (Friedrich, 1994; Weston, et.al., 1997). Cyclic GMP would activate the cGMP-dependent Protein Kinase (PKG). Until now, there was no evidence that cGMP or PKG played a role in the response to DF_2.

3.2.1 8-pCPT-cGMP

Activation of PKG by an analogue of cGMP should mimic at least part of the effect of DF_2. One such analogue, 8-pCPT-cGMP was used and, as can be seen in Fig. 3-3, increased EPSP amplitude in a dose dependent manner. At a concentration of 0.4μM, 8-pCPT-cGMP failed to have any effect on EPSP amplitude (0.3±1.9%; $p>0.08$, Wilcoxan Test). At concentrations of 4μM and 400μM, EPSP amplitudes increased by 9.8±3.6% ($p<0.04$, Wilcoxan Test) and 22.5±3.3± ($p<0.02$, Wilcoxan Test) respectively. These data indicate that a threshold of activation lies somewhere between 0.4μM and 4μM (Fig. 3-4). This dose-response curve was generated by plotting the maximal increase in EPSP amplitudes for each dose seen in Fig. 3-3.

Because activating PKG artificially using 8-pCPT-cGMP was able to increase EPSP amplitudes, as does DF_2, it was desirable to see if the increase in EPSP amplitudes resulting from peptide application can be potentiated by activation of PKG by 8-pCPT-cGMP. In order to determine this, a similar experimental protocol to that used with the PDE inhibitors was employed. A subthreshold concentration of 8-pCPT-cGMP (0.4μM)

FIGURE 3-3: Effect of Different Concentrations of 8-pCPT-cGMPS on EPSP Amplitude

This PKG agonist increased EPSP amplitudes in a dose-dependent manner. In all trials, 8-pCPT-cGMPS was added at t=15min for a duration of 30min. The lower trace shows that 0.4μM 8-pCPT-cGMPS appeared to be sub-threshold (0.3±1.9%; $p>0.08$, Wilcoxan Test), whereas a 10-fold increase in concentration resulted in an increase in EPSP amplitude (9.8±3.6%; $p<0.04$, Wilcoxan Test). At 400μM, the EPSP amplitude was increased by 22.5±3.3% ($p<0.02$, Wilcoxan Test).

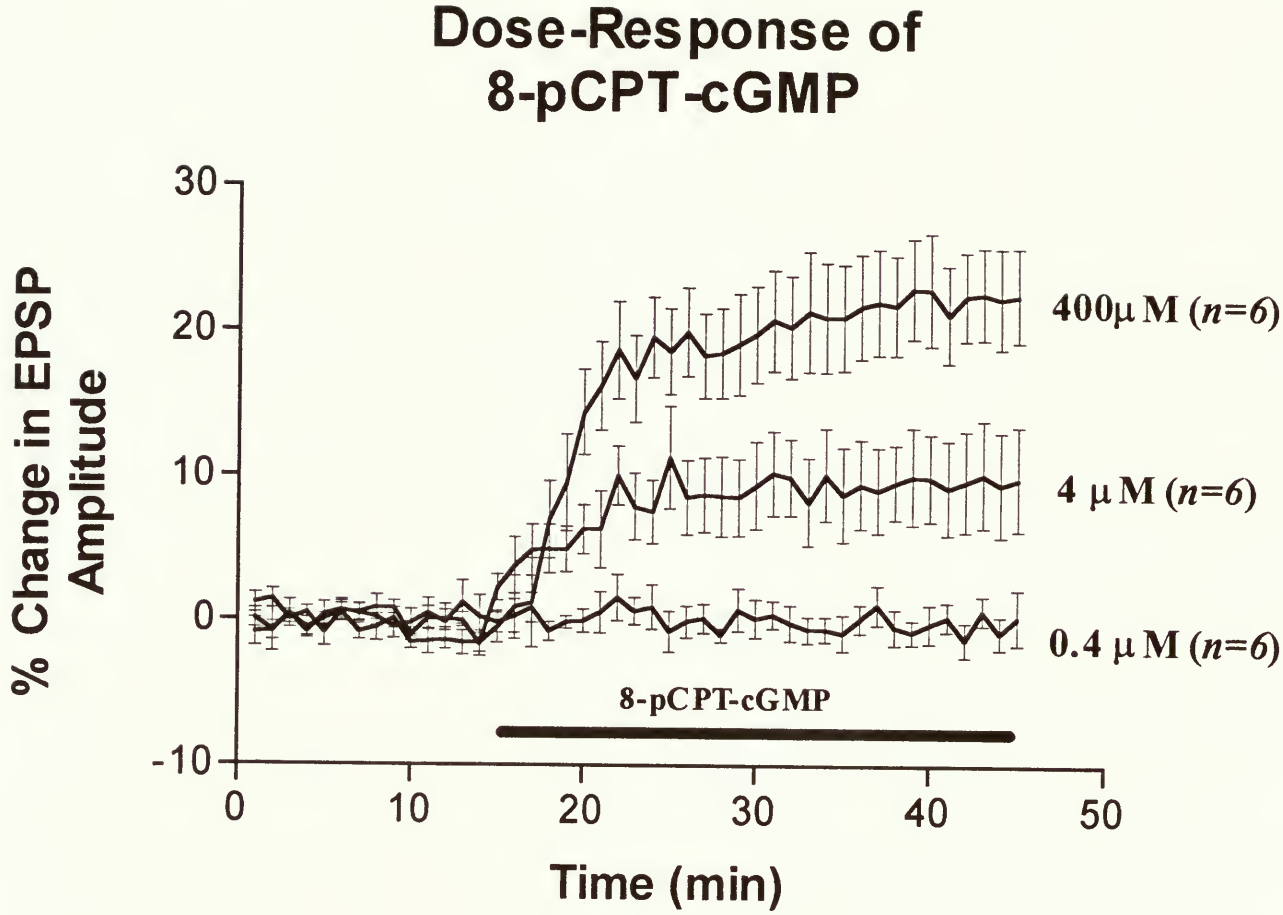
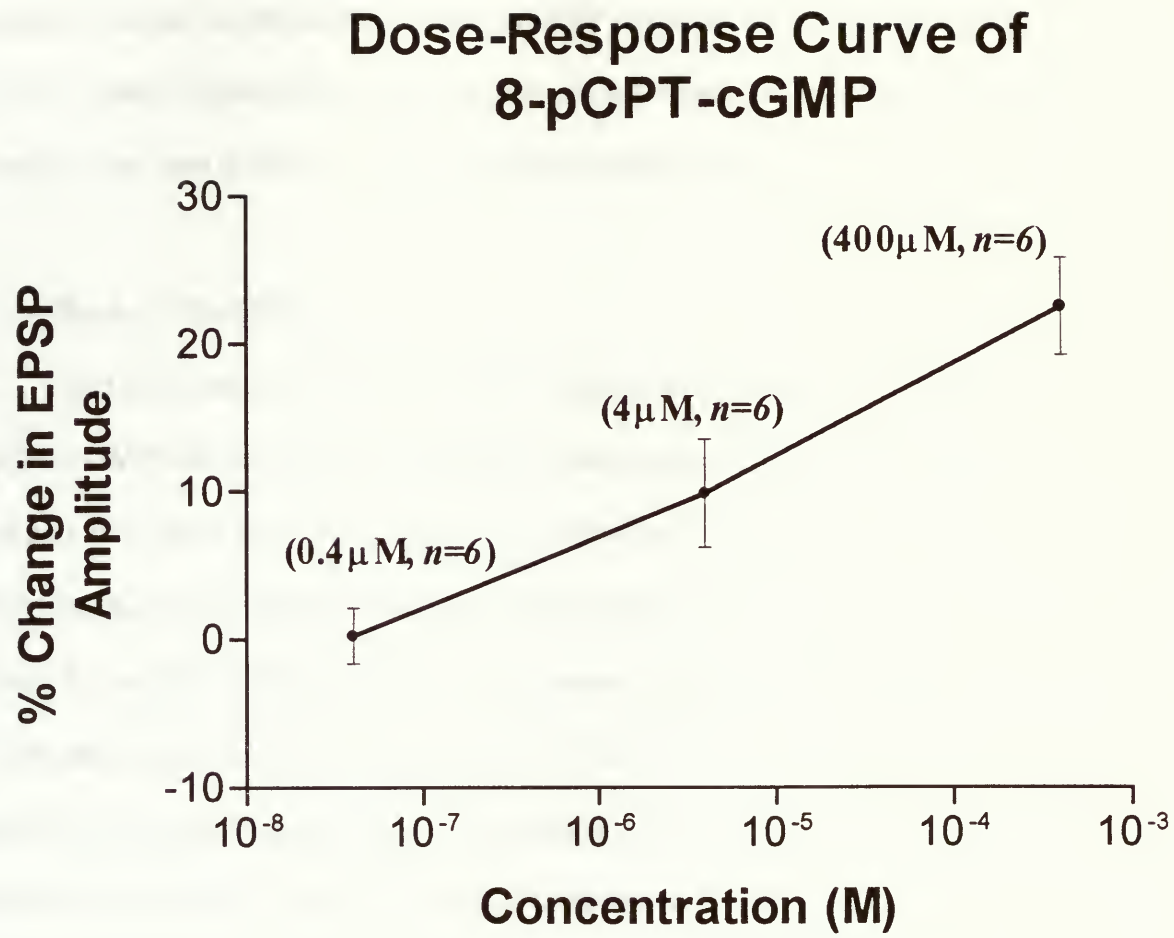

Dose-Response of
8-pCPT-cGMP
% Change in EPSP Amplitude
30
20
10
0
-10
0
10
20
30
40
50
Time (min)
400μM (n=6)
4 μM (n=6)
0.4 μM (n=6)
8-pCPT-cGMP

FIGURE 3-4: Dose-Response Curve for 8-pCPT-cGMPS

This figure shows the dose-dependence for the effect of 8-pCPT-cGMPS on EPSP amplitude. At a concentration of 0.4μM, there was no effect on EPSP amplitude, but with higher concentrations, EPSP amplitudes increased in a dose-dependent manner.


Dose-Response Curve of
8-pCPT-cGMP
% Change in EPSP
Amplitude
30
20
10
0
-10
(400μM, n=6)
(4μM, n=6)
(0.4μM, n=6)
10-8
10-7
10-6
10-5
10-4
10-3
Concentration (M)

was used in the presence of a subthreshold concentration of the peptide (10^{-8}M). Neither compound, on its own, altered EPSP amplitudes (Fig. 3-5; 8-pCPT-cGMP: -2.8±1.8%; $p>0.08$, Wilcoxan Test; DF_2: -4.6±2.8%; $p>0.08$, Wilcoxan Test). When present together, however, an immediate increase in EPSP amplitude was seen (40.4±4.9%; $p<0.005$, Mann-Whitney Test). This increase can more than likely be attributed to the synergistic increase in PKG activity bringing the overall activity above threshold.

3.2.2 Rp-8-pCPT-cGMPS

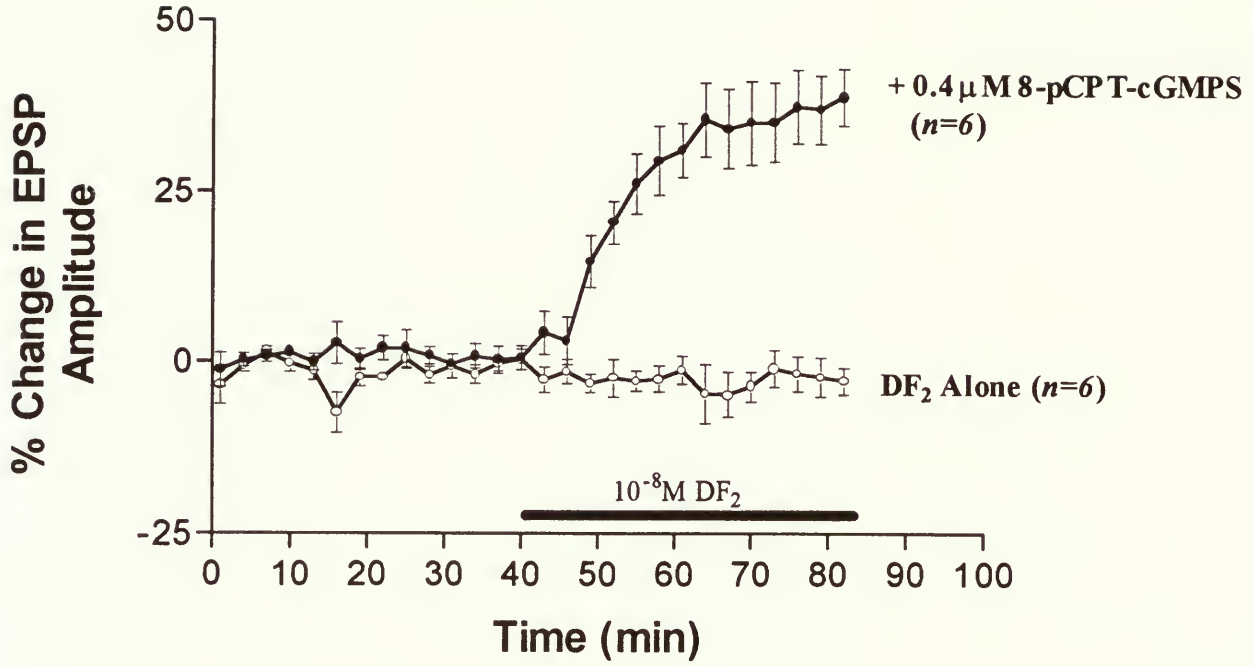
Artificially activating PKG with the membrane permeant cGMP analogue 8-pCPT-cGMP was able to mimic the effect of the peptide. If, in fact, DF_2 confers at least part of its effect through PKG, than one should be able to block some of its effect on EPSP amplitudes with a PKG antagonist. This was tested using the inactive analogue of cGMP, Rp-8-pCPT-cGMPS (RpG). In the presence of $2x10^{-7}$M DF_2, EPSPs were shown to increase in amplitude by 103±14.2% ($p<0.02$, Wilcoxan Test; Fig. 3-6). When the preparation was preincubated in RpG, this increase was reduced to 58.2±13.5% ($p<0.05$, Mann-Whitney Test). Therefore, blocking PKG effectively reduces a portion (approximately 40%) of the response, which further implicates PKG as an intermediate in neuromodulation by DF_2.

If both the PKG agonist and the PKG antagonist were specific (or equally non-specific) for PKG, preincubating the preparation in RpG should inhibit the increase in EPSP amplitudes elicited by the PKG agonist. These data are displayed in Fig. 3-7. As can be seen from the graph, the effect of the PKG agonist on EPSP amplitude was

FIGURE 3-5: Effect of 8-pCPT-cGMPS on EPSP Amplitude

The lower graph shows that 0.4μM 8-pCPT-cGMPS did not alter EPSP amplitudes when present alone (added at t=0min for the full 84min; -2.9±1.8%; $p>0.08$, Wilcoxan Test). The upper graph shows that this concentration of 8-pCPT-cGMPS potentiated the effect of a sub-threshold concentration of DF_2 (40.4±4.9%; $p<0.005$, Mann-Whitney Test). 0.4μM 8-pCPT-cGMPS was added at t=0min and was present for the entire 84min; 10^{-8}M DF_2 was added for the final 44min beginning at t=40min.

Effect of 8-pCPT-cGMPS on EPSP Amplitude



8-pCPT-cGMPS Alone

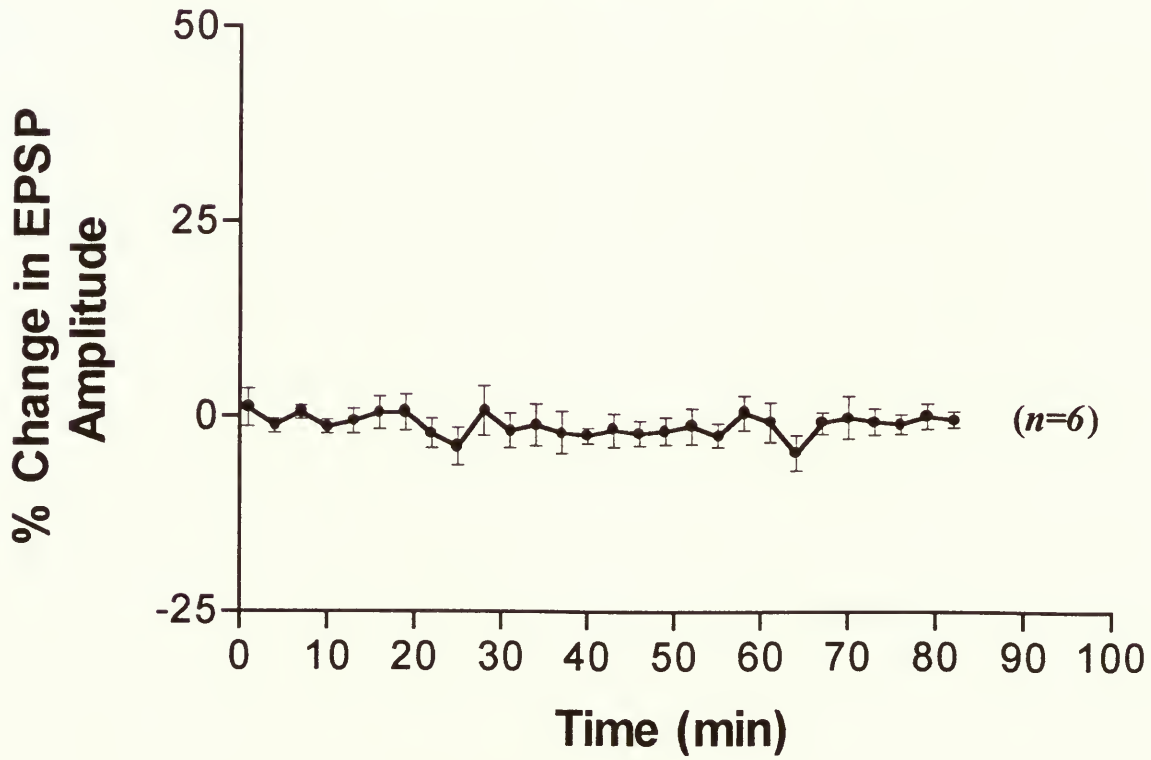
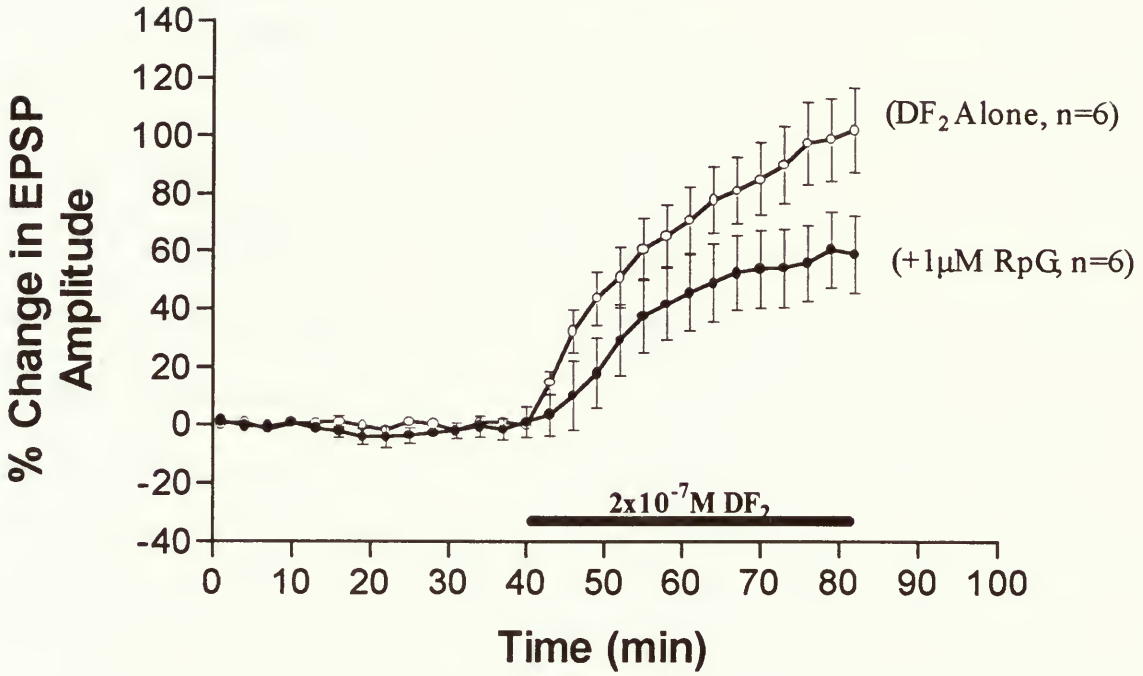


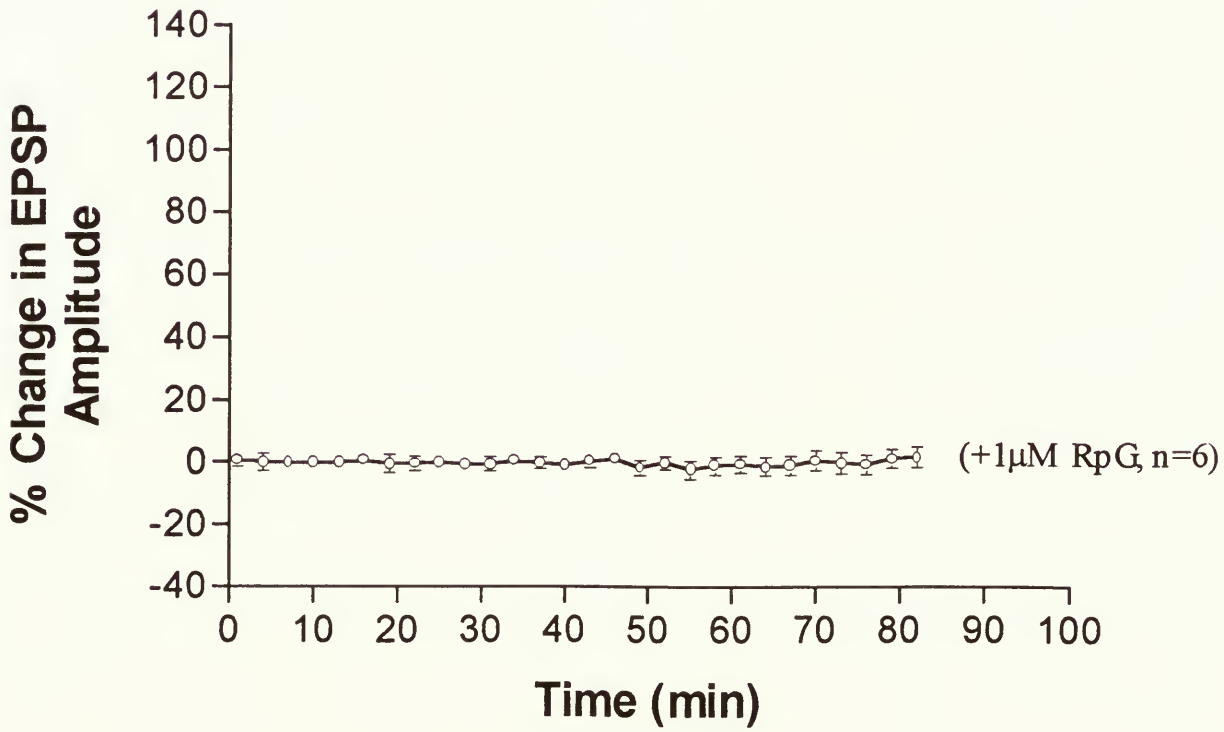
FIGURE 3-6: ***Effect of Rp-8-pCPT-cGMPS (RpG) on EPSP Amplitude***

By itself, 200μM DF_2 increased EPSP amplitude by 103.6±14.2% ($p<0.02$, Wilcoxan Test). When preparations were preincubated with 1μM RpG (added at t=0min), 200μM DF_2 increased EPSP amplitude by only 58.2±13.5% ($p<0.02$, Wilcoxan Test). Thus, RpG inhibited the effect of the peptide by approximately 40% ($p<0.05$, Mann-Whitney Test).

Effect of Rp-8-pCPT-cGMPS on EPSP Amplitude



Effect of Rp-8-pCPT-cGMPS Alone



completely blocked when the preparation was preincubated with the PKG antagonist ($p<0.05$; Mann-Whitney Test).

3.2.3 Specificity of Activators/Inhibitors

It was desirable to determine whether or not the 8-pCPT-cGMP and the Rp-8-pCPT-cGMPS were specific for PKG or if they had some degree of effect on PKA. The results in the above experiment (Fig. 3-7), could have been obtained if both the agonist and the antagonist were completely specific for PKG at the concentrations used. However, the same results could occur if both were equally non-specific for PKA, such that any moderate activation of PKA by 8-pCPT-cGMP along with any activation of PKG would be blocked by the RpG. Either of these two situations would produce 100% block of 8-pCPT-cGMP by RpG as in Fig. 3-7.

To help determine which of the above scenarios is actually taking place, a set of specificity tests was performed. These experiments tested whether or not a PKA agonist could be blocked at all by a PKG antagonist and, conversely, if a PKG agonist could be blocked by a PKA antagonist.

First, the question of whether any effect of the PKG activator, 8-pCPT-cGMP, could be blocked by a PKA antagonist, Rp-cAMPS (RpA) was addressed. As shown in Fig. 3-8, 100μM RpA appeared to block a portion of the increase in EPSP amplitudes resulting from the presence of the PKG agonist (at a concentration of 4μM). When the endpoints (t=45min) of each condition were compared, the level of inhibition approached significance ($p<0.10$, Mann-Whitney Test) but was not significant at the 0.05 level. A Mann-Whitney U Test was performed on all data points from t=20min to t=45min, and a

FIGURE 3-7: ***Inhibition of 8-pCPT-cGMP by Rp-8-pCPT-cGMPS (RpG)***

The top trace shows that an increase in EPSP amplitude of 9.8±3.6% ($p<0.04$, Wilcoxan Test) results from the addition of 4μM 8-pCPT-cGMP at t=15min (see also Figs. 3-3 and 3-4). This increase is completely blocked when the preparation is pre-incubated in 1μM RpG (0.9±0.7%; $p<0.05$, Mann-Whitney Test).

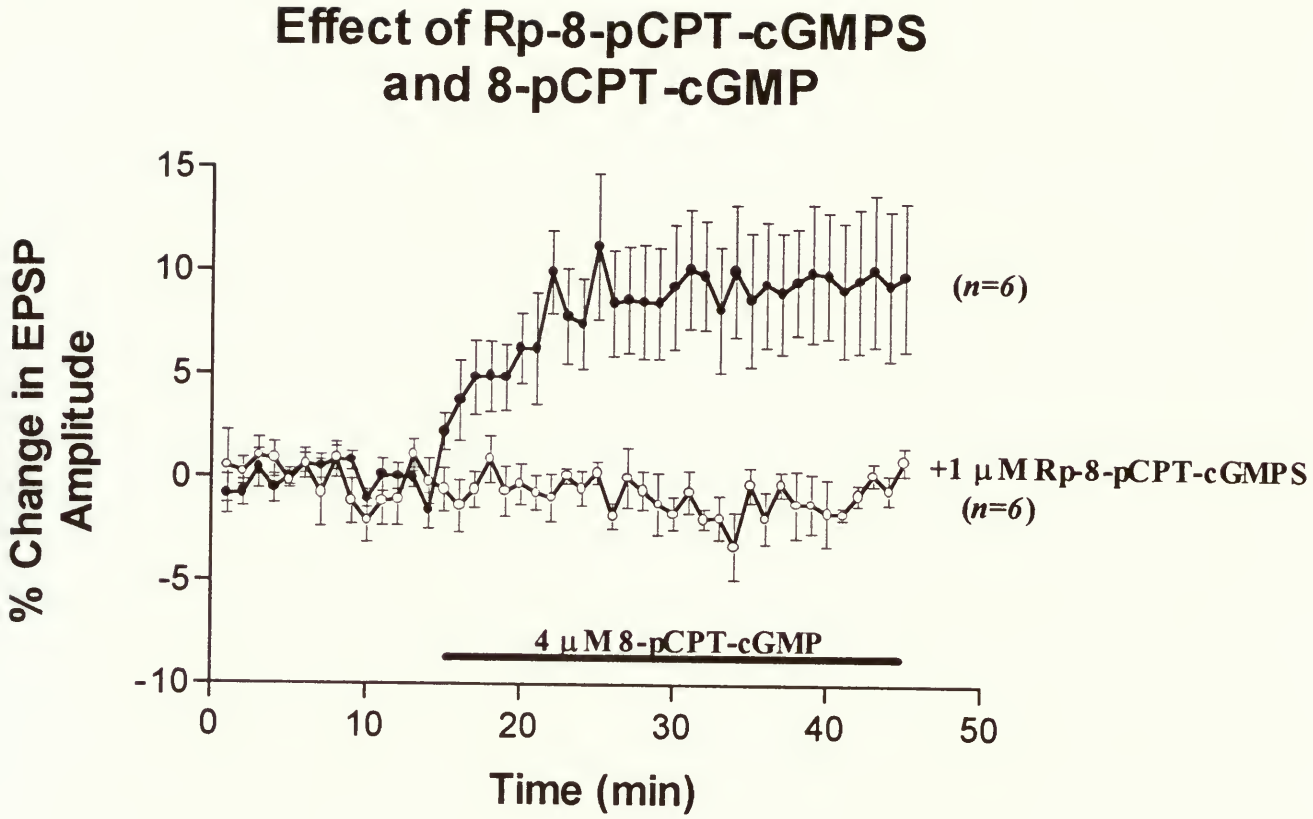
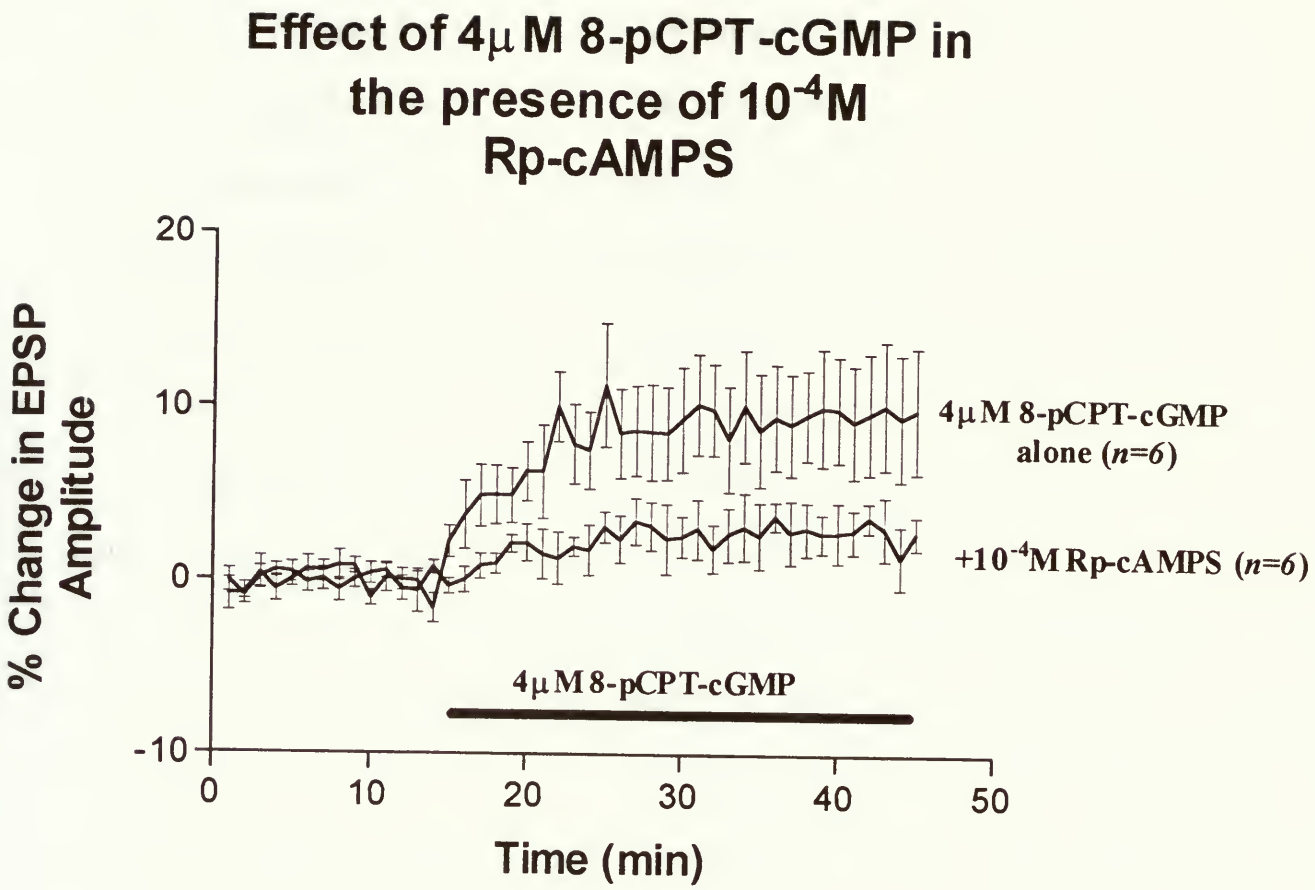

Effect of Rp-8-pCPT-cGMPS
and 8-pCPT-cGMP
% Change in EPSP
Amplitude
15
10
5
0
-5
-10
0
10
20
30
40
50
Time (min)
(n=6)
+1 μM Rp-8-pCPT-cGMPS
(n=6)
4 μM 8-pCPT-cGMP

FIGURE 3-8: Effect of Rp-cAMPS (RpA) on 8-pCPT-cGMP

The upper trace shows the increase in EPSP amplitude seen in the presence of 4μM 8-pCPT-cGMP added for 30min.beginning at t=15min. The lower trace shows that pre-incubation in 100μM Rp-cAMPS (added at t=0) blocks an insignificant portion of this increase ($p<0.10$, Mann-Whitney Test).


Effect of 4μM 8-pCPT-cGMP in the presence of 10^{-4}M Rp-cAMPS
% Change in EPSP Amplitude
20
10
0
-10
0
10
20
30
40
50
Time (min)
4μM 8-pCPT-cGMP alone (n=6)
+10^{-4}M Rp-cAMPS (n=6)
4μM 8-pCPT-cGMP

significant difference was found only at t=22min; no other values showed a significant difference at the 0.05 level. Within each preparation, the percent change in EPSP amplitude was averaged between t=25min and t=45min, and data were compared. By itself, the PKG agonist increased EPSPs by 9.4±3.0% over this time period, but in the presence of Rp-cAMPS, the PKG agonist only increased EPSPs by 2.8±1.4% over the same period. This suggests that Rp-cAMPS decreased the effectiveness of the PKG agonist by 70%. However, data for the two conditions were not significantly different at the 0.05 level.

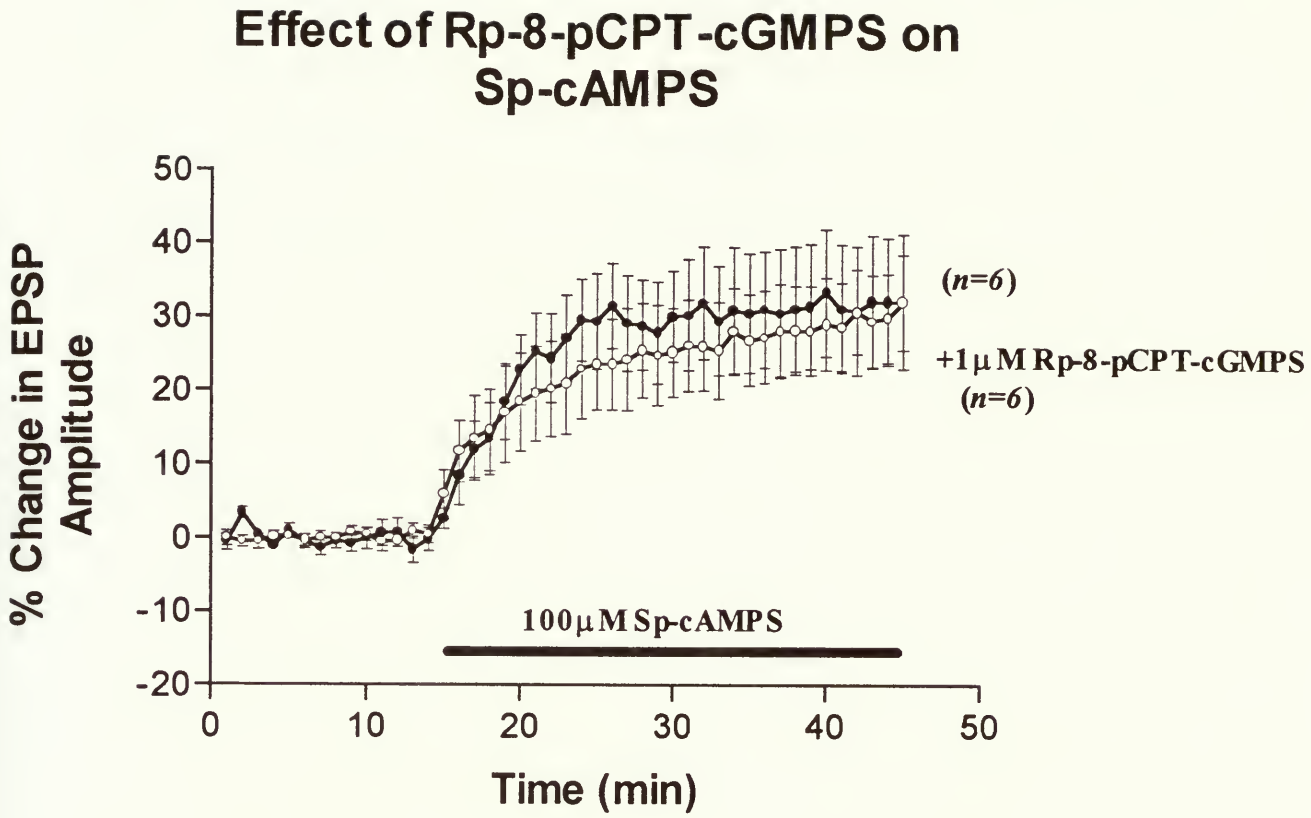
To gain further insight into the specificity of the agonists and antagonists, the reciprocal test was performed. A PKA agonist known to be highly specific for PKA at the concentrations used (Scholübbers, et.al., 1984), Sp-cAMPS, was used to stimulate PKA and increase EPSP amplitudes (Fig. 3-9; 32.1±6.5%; $p<0.02$, Wilcoxan Test). This effect was previously described in Weston, et.al. (1997). RpG was added in an attempt to block at least some portion of the effect of Sp-cAMPS. If RpG was able to block any of this effect, than, it could be inferred that RpG was not completely specific for PKG but it had at least a moderate effect on PKA. However, RpG did not inhibit the effect of Sp-cAMPS on EPSPs at all (Fig. 3-9; 32.2±9.1%; $p<0.005$, Mann-Whitney Test), indicating that RpG is also highly specific.

3.3 Inhibition of the Entire DF_2 Response

It was predicted that if both PKA and PKG were inhibited simultaneously, most of the effect of the peptide on EPSP amplitude would be inhibited, but a small portion of the modulation would remain due to the activity of the other kinases known to play a role

FIGURE 3-9: Effect of RpG on Sp-cAMPS

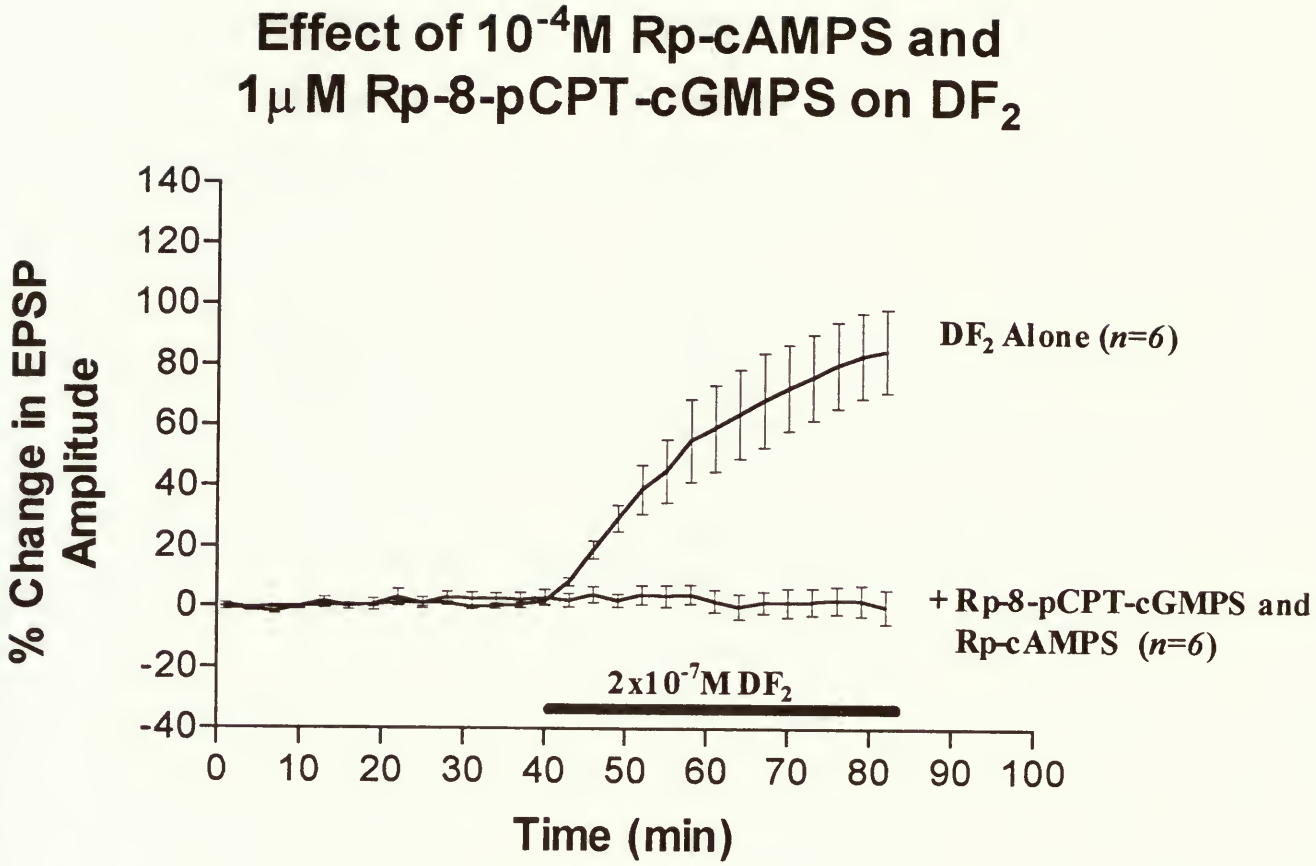
The solid circles indicate that 100μM Sp-cAMPS increases EPSP amplitudes by 32.1±6.5% ($p<0.02$, Wilcoxan Test; added at t=15min). This increase is unaffected if the preparation is pre-incubated in 1μM RpG (32.2±9.1%; $p<0.005$, Mann-Whitney Test; open circles; beginning at t=0 min).


Effect of Rp-8-pCPT-cGMPS on Sp-cAMPS
% Change in EPSP Amplitude
50
40
30
20
10
0
-10
-20
0
10
20
30
40
50
Time (min)
100μM Sp-cAMPS
(n=6)
+1μM Rp-8-pCPT-cGMPS
(n=6)

in DF_2's effect. Fig. 3-10 shows the results from modulation of the preparation with $2x10^{-7}M$ DF_2 and the inhibition of this response with both RpG and RpA together. The result was that the peptide's effect (85.2±13.7%; $p<0.02$, Wilcoxan Test) was completely blocked by only these two kinase inhibitors (2.6±5.2%; $p<0.005$, Mann-Whitney Test). This result was surprising because the other two kinases, namely PKC and CaMKII, should presumably still be active and a small amount of modulation should exist. However, this was not the case.

FIGURE 3-10: Inhibition of DF_2 by RpA and RpG

The upper trace shows the nearly 90% increase in EPSP amplitudes seen in the presence of $2x10^{-7}$M DF_2 (85.2±13.7%; $p<0.02$, Wilcoxan Test; added at t=40min for 44min). If the preparation is pre-incubated in both RpA and RpG (bottom trace), then the entire effect of DF_2 on EPSP amplitudes is blocked (2.6±5.2%; $p<0.005$, Mann-Whitney Test).


Effect of 10-4M Rp-cAMPS and
1μM Rp-8-pCPT-cGMPS on DF2
% Change in EPSP Amplitude
140
120
100
80
60
40
20
0
-20
-40
0
10
20
30
40
50
60
70
80
90
100
Time (min)
DF2 Alone (n=6)
+ Rp-8-pCPT-cGMPS and Rp-cAMPS (n=6)
2x10-7M DF2

4. DISCUSSION

Several criteria have been established to demonstrate unequivocally that cyclic nucleotides, in this case cAMP and cGMP, mediate a biological response (Sutherland, 1972; Levitan and Drummond, 1980; Robison, et.al., 1971;Greengard, 1976). These criteria can be summarized as follows:

a) The response must be mimicked with cAMP/cGMP analogues and/or treatments with agents that increase PKA/PKG activity.
b) The response must be blocked with agents that block the rise in cAMP/cGMP and/or the activities of PKA/PKG.
c) The response must be potentiated with agents that slow the breakdown of cAMP/cGMP.
d) Levels of cAMP/cGMP and activities of the associated cyclase enzymes must rise in the presence of the agonist, in this case the peptide DF_2.
e) Pharmacological agents which activate or block the receptor to the agonist must increase or decrease, respectively, cyclic nucleotide levels.

The purpose of this research project was to determine whether or not cyclic nucleotides mediate the neuromodulatory effects of DF_2 on crayfish neuromuscular synapses. The results satisfy three of the five criteria listed above (a, b and c), providing strong evidence for the involvement of cAMP, cGMP and their associated kinase enzymes. These results, coupled with previous data (Weston, et.al., 1997; Friedrich, et.al., 1998; Noronha and Mercier, 1995), implicate a number of second messengers and kinases that are typically associated with the G-protein intracellular signaling pathway.

4.1 Activation of PKA and PKG

Weston et.al. (1997) reported that the PKA activator, Sp-cAMPS, mimics the effect of DF_2 on EPSP amplitude, and this is confirmed in Fig. 3-9. Figs. 3-2, 3-3, 3-4 and 3-5 show that a cGMP analogue, 8-pCPT-cGMP, is also able to increase EPSP

amplitude in a dose-dependent manner with a threshold between 0.4 and 4.0μM. The EC_{50} for PKG = 0.04μM and for PKA = 7μM in intact human platelet cells (Butt, et.al., 1994). The latter results comprise the first direct evidence that the cGMP/PKG intracellular signaling system plays a role in neuromodulation by a FMRFamide-like peptide. Cyclic GMP and PKG have been shown to play intracellular signaling roles in other systems.

At the dactyl opener neuromuscular junction of lobsters, cGMP and cAMP have been shown to increase the level of serotonin-induced phosphorylation of a protein that increases the amount of transmitter released (Goy, et.al., 1984; Goy and Kravitz, 1989). Another role for cGMP in the lobster was outlined by Goy (1990), where a specific peptide, called crustacean hyperglycemic hormone (CHH), activates a membrane-associated guanylyl cyclase, resulting in an increase in cGMP levels in skeletal muscle. This causes, among other things, an increase in the responsiveness of the muscle to neurotransmitter. PKG and cGMP have also been shown to lower calcium levels in human platelets, preventing aggregation and clotting (Geiger, et.al., 1992). Cyclic GMP has been shown to be directly involved in the modulation of transmitter release in long-term potentiation in human hippocampal neurons (Arancio, et.al., 1995).

Together, these studies provide evidence that cGMP and PKG play a role in intracellular signaling in several systems, and underlie modulation of chemical synapses in a least some cases. The data presented here implicate PKG as an intermediate in DF_2's effect. This satisfies the first of the five criteria presented earlier.

4.2 Inhibition of PKA and PKG

If the peptide's effect involves PKA and/or PKG, inhibiting one or both of the kinase enzymes should block at least a portion of the increase in EPSP amplitude seen in the presence of DF_2. Inhibition of PKA by Rp-cAMPS, an inactive analogue of cAMP, blocks 60-80% of the increase in EPSP amplitude caused by $2x10^{-7}$M DF_2 (Weston, et.al., 1997). This provides further evidence of the involvement of PKA and, therefore, cAMP in synaptic modulation by the peptide.

Figure 3-6 shows that a PKG antagonist, Rp-8-pCPT-cGMPS (1.0μM) blocks nearly 40% of the effect of a superthreshold concentration ($2x10^{-7}$M; Skerrett, et.al., 1995) of the peptide. The EC_{50} in intact cells for PKG = 0.5μM and for PKA = 8.3μM (Butt, et.al., 1994). The concentration of the agonist that was used (1.0μM) is below the EC_{50} for effects on PKA and only slightly above the EC_{50} for effects on PKG. We now have evidence that a PKG agonist mimics the effect of DF_2 on EPSP amplitude, and a PKG antagonist blocks a portion of the peptide's effect. This further strengthens the conclusion that cGMP and PKG are involved as second messengers.

The only way that one can be confident in these results is if the inhibitors are specific for their targets. For example, is the PKG antagonist inhibiting any effects of PKA? At the concentrations used, the inhibitors should be specific according to the reported EC_{50}'s (the concentration at which the chemical tested has an effect 50% of its maximum effect). The ideal situation would be to select a concentration of the inhibitor significantly higher than the EC_{50} for its target, and significantly lower than the EC_{50} for its counterpart. This would help to ensure that the target enzyme is being affected and

effects on non-target enzymes are negligible. The EC_{50}'s that have been reported are the results of tests done by Butt, et.al. (1990; 1994) on intact human platelets.

Because the antagonists and agonists were tested in human platelets and not in the tissues of the preparation used in these experiments, it was important to perform specificity tests with crayfish. Because the PKG antagonist, Rp-8-pCPT-cGMPS, had no effect on the increased EPSP amplitude resulting from the application of the PKA agonist, Sp-cAMPS (Fig. 3-9), this inhibitor appears to be specific for PKG. The reciprocal experiment, the use of the PKA antagonist, Rp-cAMPS in the presence of the PKG agonist, 8-pCPT-cGMPS, yielded results that were not as straightforward (Fig. 3-8). The data suggest that there is partial inhibition of the increase in EPSP amplitude seen in the presence of the PKG agonist by the PKA antagonist. However, this level of inhibition is not statistically significant at the 0.05 level ($p<0.10$, Mann-Whitney Test) except for one time point. Further confirmation of the specificity of the PKG agonist and antagonist can be seen in Fig. 3-7. The inhibition of the PKG agonist-induced increase in EPSP amplitude by the PKG antagonist is complete ($p<0.005$, Mann-Whitney Test; $p>0.08$, Wilcoxan Test).

The most compelling evidence that *both* PKA and PKG are necessary intermediates in the DF_2 neuromodulatory pathway is that the application of the PKA antagonist, Rp-cAMPS, inhibits only a portion of the increase in EPSP amplitudes resulting from peptide application (60-80%; Weston, et.al., 1997). Likewise, the application of the PKG antagonist, Rp-8-pCPT-cGMPS, inhibits only approximately 40% of the effect (Fig. 3-6). If, however, the preparation is exposed to both kinase antagonists simultaneously, the peptide's effect is completely blocked (Fig. 3-10). This result is

surprising as previous data have shown that PKC and CaMKII are involved in the intracellular signalling pathway (Friedrich, et.al., 1998; Noronha and Mercier, 1995). Figure 3-10 indicates that not only are PKA and PKG involved in neuromodulation, but appear to be *necessary* as well.

4.3 The Use of Phosphodiesterase (PDE) Inhibitors

In this investigation, PDE inhibitors were used with the aim of increasing the levels of cyclic nucleotide-monophosphates (cNMPs) in the presynaptic terminal. Phosphodiesterases are a class of enzymes which catalyse the breakdown of cAMP, cGMP and any other cNMP which may be present. There are five known types of PDEs, each with either a different target and/or a different sensitivity to certain compounds (Takase, et.al., 1994; Scamps, et.al., 1993). These five types are as follows:

Type I - calcium/calmodulin dependent PDE
Type II - cGMP stimulated PDE
Type III - cGMP inhibited PDE
Type IV - cAMP specific PDE
Type V - cGMP specific PDE

Depending upon the type of inhibitor used, it is possible to inhibit one specific PDE type or several of the PDEs. 3-isobutyl-1-methylxanthine, IBMX, can inhibit more than one of the PDE types. It has been shown that IBMX inhibits at least types IV and V in frog cardiac cells, resulting in an increase in intracellular cAMP and cGMP levels (Scamps, et.al., 1993). Traditionally, IBMX has been used in systems thought to involve cAMP. For example, IBMX has been shown to increase cAMP levels in locust oviduct tissues (Nykamp and Lange, 1998). In lobsters, IBMX potentiates the serotonin (5-HT)-induced increase in transmitter release at neuromuscular junctions of the walking leg

dactyl opener (Goy and Kravitz, 1989; Goy, 1990; Goy, et.al., 1984). There is also evidence that IBMX can affect crayfish tissues. In crayfish hindguts, dopamine (DA) enhances contractions and this response is thought to be mediated by cAMP as IBMX potentiates the dopamine's effect (Knotz and Mercier, 1995). Friedrich (1994) demonstrated that at the deep abdominal extensor neuromuscular junctions, IBMX results in a dose-dependent increase in EPSPs recorded from the L1 muscle, with a threshold of activation lying between 10μM and 30μM.

Although IBMX does increase cAMP levels, it does not do so selectively. There is evidence that IBMX can also increase cGMP levels. In the lobster dactyl opener muscle, the increase in cGMP levels initiated by Crustacean Hyperglycemic Hormone (CHH) is potentiated by IBMX (Pavloff and Goy, 1990).

These studies indicate that IBMX is a broad-spectrum PDE inhibitor, inhibiting at least types IV and V, resulting in an increase in cAMP and cGMP levels. The results seen in Fig. 3-1 should be viewed with this in mind. Although it is likely that the potentiation of the peptide's effect by IBMX is probably at least partially due to an increase in cAMP levels, a portion of this potentiation could be due to an increase in cGMP levels.

The results shown in Fig. 3-2 indicate that the use of a PDE inhibitor that specifically inhibits type V (the cGMP-specific PDE) also potentiates the peptide's effect. The PDE inhibitor 4-{[3',4'-(methylenedioxy)benzyl]amino}-6-methoxyquinazoline, mdBAMQ, has been shown to specifically increase intracellular cGMP concentrations in porcine aorta cells by inhibiting PDE type V with a high degree of specificity (EC_{50} for PDE type V = 0.23±0.03μM, EC_{50} for all other PDE types >100μM; Takase, et.al., 1994).

Therefore, the potentiation seen in Fig. 3-2 is probably due to an increase in cGMP levels. In this preparation, 3μM mdBAMQ failed to increase EPSP amplitude on its own. The subthreshold concentration of DF_2 (10^{-8}M) also failed to have any effect. When present together, mdBAMQ and DF_2 act synergistically, presumably through cGMP, to increase EPSP amplitude.

The IBMX data, the mdBAMQ data and the work done with the PKA and PKG agonists and antagonists, all taken together, indicate that both the cAMP/PKA pathway and the cGMP/PKG pathway are involved and are necessary for the increase in EPSP amplitude seen in the presence of DF_2.

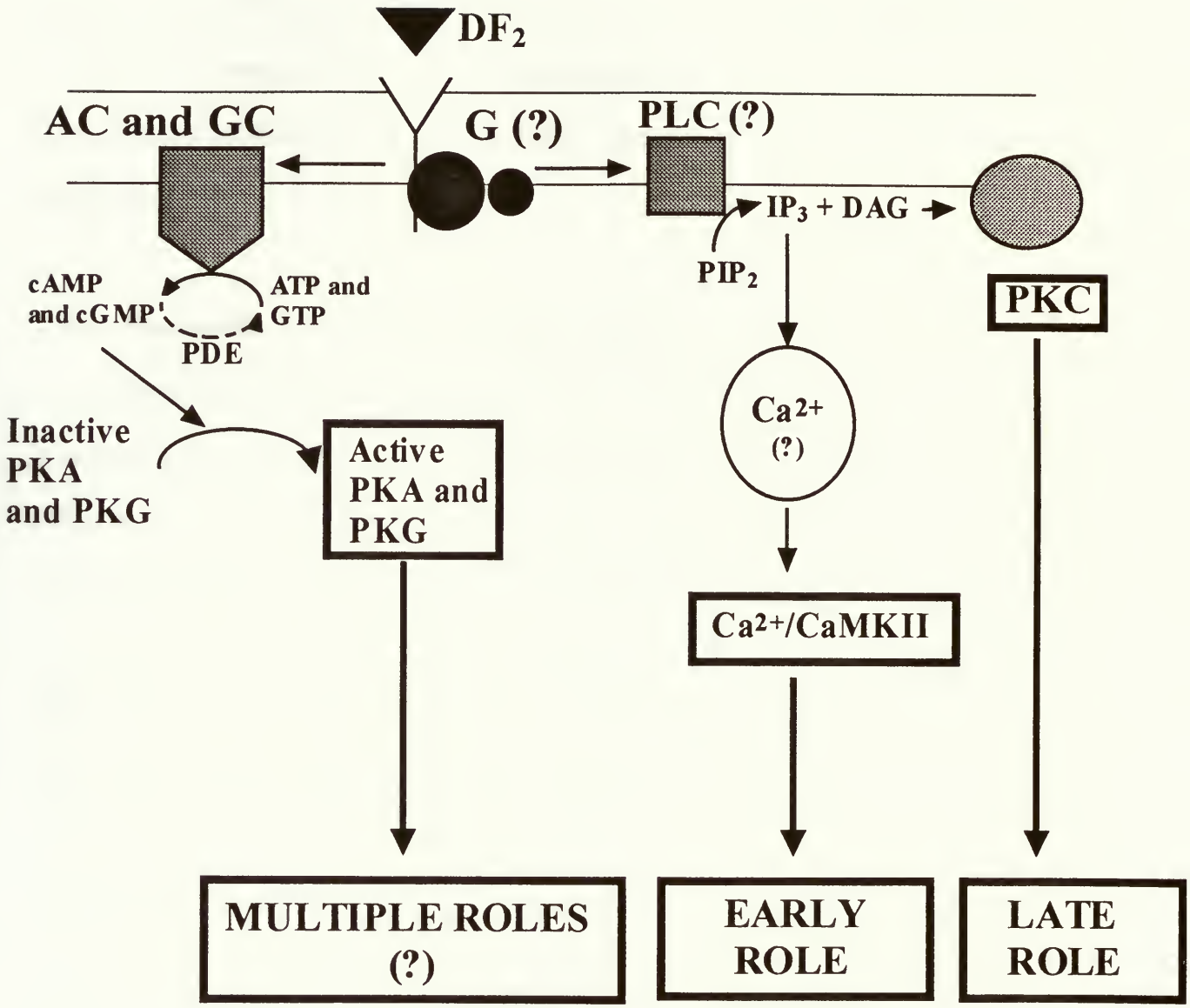
Since the present evidence indicates that both the cAMP/PKA and cGMP/PKG pathways are involved, and roles for PKC and CaMKII are already established (Friedrich, et.al., 1998; Noronha and Mercier, 1995), a new, revised mechanism of neuromodulation by DF_2 can be drawn. This proposed mechanism still involves the activation of a G-protein Coupled Receptor (GPCR) by DF_2, but includes the activation of guanylyl cyclase as well as adenylyl cyclase (Fig. 4-1 as compared to Fig. 1-3).

4.4 Possible Mechanisms/Targets for Kinases

The primary goal of this thesis was to provide further insight into the intracellular signalling systems involved in the neuromodulatory pathway of DF_2. The data discussed in the preceding sections satisfy three of the criteria introduced at the beginning of the discussion. This provides strong evidence for the involvement of cyclic nucleotides in modulating synaptic output by DF_2. This, coupled with earlier work (Friedrich, et.al.,

FIGURE 4-1: ***Revised Mechanism of Neuromodulation by DF_2***

This mechanism is the same as that presented in Fig. 1-3, except that here, the guanylyl cyclase-cGMP-PKG pathway is included.


DF2
AC and GC
G (?)
PLC (?)
IP3 + DAG
PIP2
cAMP
and cGMP
ATP and
GTP
PDE
PKC
Inactive
PKA
and PKG
Active
PKA and
PKG
Ca2+
(?)
Ca2+/CaMKII
MULTIPLE ROLES
(?)
EARLY
ROLE
LATE
ROLE

1998; Noronha and Mercier, 1995; Weston, et.al., 1997) implicate the involvement of at least four kinases: PKA, PKG, CaMKII and PKC. However, the targets for each of these kinases and the specific roles they play in increasing transmitter release at the neuromuscular junction have not been identified. Here, possible targets for these kinases will be discussed.

4.4.1 Postsynaptic vs. Presynaptic Activation of Kinases

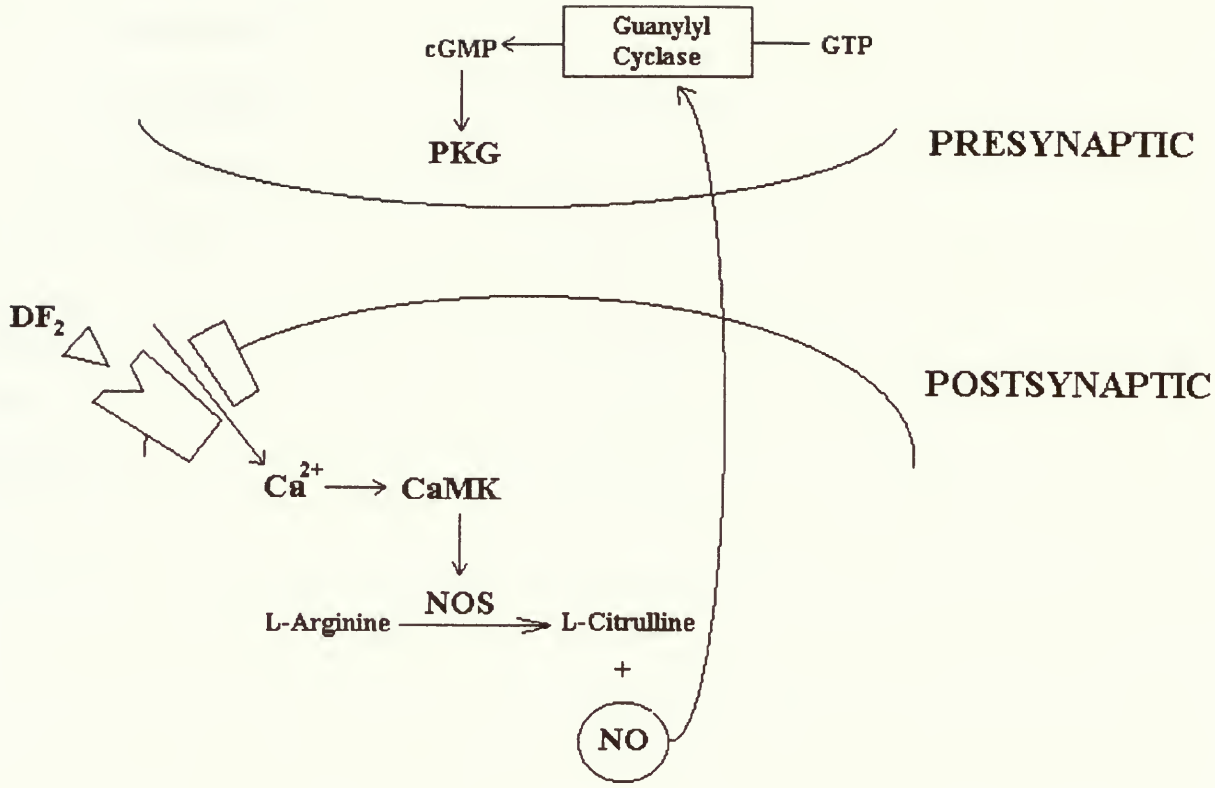
Quantal synaptic recordings of postsynaptic currents have been performed in the presence and absence of DF_2 at the neuromuscular junctions used in this experiment (Skerrett, et.al., 1995). It has been demonstrated that the peptide causes an increase in the amount of neurotransmitter released from the presynaptic terminal, without changing the size of the current elicited by the release the contents of a single vesicle of transmitter (the quantal size). The peptide also failed to alter the muscle fibre input resistance. Such results indicate that the peptide increases EPSP amplitude by presynaptic mechanisms. The involvement of the kinases, PKA, PKG, CaMKII and PKC, along with this presynaptic action of the peptide, allows us to propose the G-protein-coupled receptor (GPCR) pathway seen in Fig. 4-1. This mechanism can be used to explain the data generated with DF_2 thus far. The peptide binds to a GPCR (or possibly several GPCRs) on the plasma membrane of the neuron, initiating the various pathways which ultimately lead to, through these kinases, an increase in neurotransmitter output. This pathway is not the only possible mechanism.

A great deal of recent study has been into the roles of the widely employed retrograde signal, nitric oxide (NO). The increase in intracellular levels of cGMP has

been shown to be controlled by two types of enzymes. One is a membrane-bound guanylyl cyclase which can be activated by certain neuromodulators in humans (Arancio, et.al., 1995) and in invertebrates (Goy, 1990; Pavloff and Goy, 1990; Goy and Kravitz, 1989). The second is a class of soluble enzymes which can be stimulated by NO (Yuen and Garbers, 1992; Scholz, et.al., 1996). NO is referred to as a retrograde signal because it can be released from target cells in synapses to modulate the amount of transmitter released from the presynaptic cell (Hille, 1992; Bredt, et.al., 1991). Its production is catalysed by Nitric Oxide Synthase (NOS) which converts L-arginine into L-citrulline and NO. The activity of NOS can be increased by an increase in intracellular calcium and a subsequent increase in calcium/calmodulin dependent kinases (CaMKs; Zhang, et.al., 1997). In the crayfish neuromuscular system, the release of the transmitter glutamate from the presynaptic terminal onto the muscle increases the calcium currents through postsynaptic glutamate receptors (del Castillo and Katz, 1954a&b; Hille, 1992). This would result in an increase in intracellular calcium and therefore an increase in the activity of the CaMKs. Such increased activity might stimulate NOS-catalysed production of NO. NO can then leave the postsynaptic cell where, acting as an intercellular signal, it can activate a soluble guanylyl cyclase like that present in other invertebrates such as the crab (Scholtz, et.al., 1996). This would increase presynaptic intracellular cGMP levels and, therefore, increase presynaptic PKG activity (Fig. 4-2). If such a mechanism involving NO was occurring, than bath application of PDE inhibitors and PKG agonists should still potentiate the response and PKG antagonists should inhibit a portion of it.

FIGURE 4-2: Possible Role for NO in DF_2 Neuromodulation

Possible mechanism for the involvement of DF_2-induced NO production. The peptide increases the intracellular Ca^{2+} by directly gating a Ca^{2+} channel. This increased level of Ca^{2+} results in an increase in the activity of CaMKs. One target CaMKs is NOS. Once NOS is activated by CaMKs, NO production begins. NO can act as a retrograde signal, increasing the concentration of cGMP in the presynpatic cell, and therefore increase the activity of PKG.


Guanylyl Cyclase
cGMP
GTP
PKG
PRESYNAPTIC
DF_2
POSTSYNAPTIC
Ca^{2+}
CaMK
NOS
L-Arginine
L-Citrulline
+
NO

The NO pathway could account for the roles of PKG and CaMKII, but what of PKA and PKC?

4.4.2 Modulation of Presynaptic K^+ Currents

In some invertebrates, there is evidence that cAMP plays a role in modulating the current through some presynaptic K^+ channels. In *Aplysia*, the binding of serotonin (5-HT) to a GPCR results in presynaptic spike broadening through a decreased current through K^+ channels. This effect is mediated by cAMP and PKA (Sugita, et.al., 1992; Sugita, et.al., 1997) and, to a lesser extent, PKC (Sugita, et.al., 1992). The broadened spike results in an increase in transmitter release (Hille, 1992). FMRFamide has the opposite effect. When the peptide binds to a different GPCR, it activates the arachidonic acid pathway, which leads to an activation of the same K^+ channels (Piomelli, et.al., 1987) resulting in a narrowed spike and reduced transmitter release. It is possible that, in crayfish, DF_2, through the actions of a GPCR similar to the 5-HT receptor in *Aplysia*, causes an increase in transmitter release by an inhibition of K^+ conductance. This might involve cAMP/PKA as well as PKC as it does in *Aplysia* (Sugita, et.al., 1992; Sugita, et.al., 1997). There is no evidence of the involvement of CaMKII and PKG in the modulation of K^+ currents in *Aplysia*, and there is no reason to propose that there would be in crayfish. Nonetheless, there is not sufficient evidence to rule out the possibility that more than one mechanism may be involved. For example, there could be presynaptic modulation of K^+ channels through PKA and PKC, and postsynaptic activation of CaMKII, PKG and NOS/NO as discussed above. These systems may be activated simultaneously through different receptors for DF_2, a presynaptic GPCR which

modulates K^+ currents, and a postsynaptic peptide-gated Ca^{2+} channel which initiates NO production. However, other schemes are possible.

4.4.3 Synapsin Pathway

Most of the vesicles in the nerve terminal are bound to actin filaments associated with an extension of the endoplasmic reticulum (ER) via a protein called synapsin I, or a synapsin-like protein (Stefani, et.al., 1997; Llinas, et.al., 1991; Benfenati, et.al., 1992). Microinjection of mRNA coding for rat synapsin I into the crayfish neurons results in increased EPSP amplitude during repetitive stimulation (Dearborn, et.al., 1998) indicating that synapsin I can be involved in modulating transmitter output in crayfish. This protein facilitates the binding of the vesicles to the ER by binding the vesicle on one end, the –COOH (tail) end, and the actin filament on the other end, the $-NH_2$ (head) end (Stefani, et.al., 1997; Llinas, et.al., 1991). This tethering of synaptic vesicles makes them unavailable for transmitter release. Those vesicles that are available for release become associated with discrete areas of the nerve terminal membrane called active zones. It is at these active zones that neurotransmitter exocytosis occurs (Nichols, et.al., 1992). Once associated with these zones, the vesicles await a trigger to begin exocytosis. This trigger is calcium, which initiates the release of neurotransmitter (Nichols, et.al., 1992; Hille, 1992; Zucker, 1974). It can be implied, therefore, that anything that causes an increase in the number of vesicles available for release by decreasing the number of vesicles associated with the actin filaments of the ER, and/or anything that increases the concentration of free intracellular Ca^{2+} at the active zones, will increase the amount of transmitter released.

In order to increase the number of vesicles available for exocytosis, they must first be liberated from their storage area on the actin filaments of the ER extensions. To release the vesicles from this sequestration, synapsin I, or a synapsin-like protein, must be phosphorylated and, therefore, rendered inactive (Hille, 1992; Nichols, et.al., 1992). On synapsin I, there are two regions where phosphorylation must occur to completely release the vesicle. Failure to phosphorylate both sites on synapsin I will result in either failure of liberation of the vesicles, or a reduced effectiveness of those partially liberated vesicles. The first region of phosphorylation is the head end. This will release the vesicle-synapsin complex from the actin filament. Once this phosphorylation occurs, the phosphorylation of the tail end allows the vesicle to dissociate from the synapsin. Then the vesicle is free to dock to the active zone and to release its contents during exocytosis. Studies using the giant synapse of the squid stellate ganglion (Llinas, et.al., 1991), rat forebrain (Benfenati, et.al., 1992) and bovine forebrain (Stefani, et.al., 1997) revealed that CaMKII is responsible for phosphorylation of the tail and of synapsin I. Inhibition of CaMKII in the crayfish NMJ resulted in a partial inhibition of the neuromodulation by DF_2 (Noronha and Mercier, 1995). This observation might be explained by a decrease in the level of tail phosphorylation of a synapsin-like protein, which would prevent the peptide from increasing the number of vesicles available for release. In the bovine forebrain, phosphorylation of the head end of synapsin I is mediated by at least two kinases, another isoform of CaMK called CaMKI, and PKA (Stefani, et.al., 1997). If a similar scheme occurs in crayfish, inhibition of PKA by Rp-cAMPS (Weston, et.al., 1997) would reduce the level of head phosphorylation, which would inhibit the release of the synapsin-like protein from the actin filaments of the ER and eventually decrease

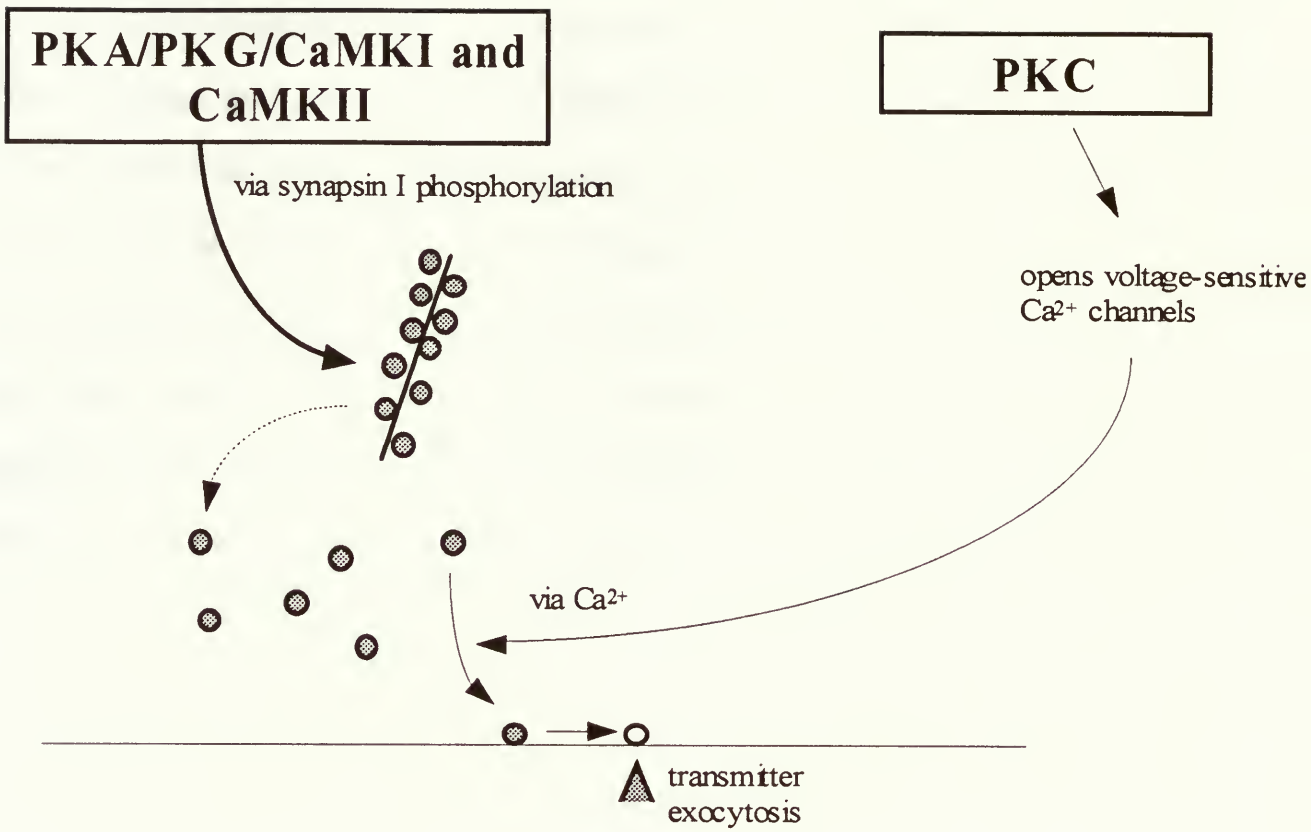
transmitter release. The inhibition of a portion of DF_2's effect seen when inhibiting PKG with Rp-8-pCPT-cGMPS (Figs. 3-6 and 3-10) and the increase in EPSP amplitude seen in the presence of the PKG agonist 8-pCPT-cGMP (Figs. 3-3,4,5,7,8 and 10) could be explained in two ways. It is possible that PKG *also* phosphorylates a PKA-sensitive site on synapsin, or PKG phosphorylates some other site. There is no direct evidence to support either hypothesis.

Since CaMKII, PKA and PKG are involved in the neuromodulatory pathway of DF_2 and each of these kinases is involved in phosphorylation of synapsin I in other systems, it is likely that part of the peptide's effect on transmitter output at crayfish neuromuscular junctions involves synapsin I or a synapsin-like protein (Fig. 4-3).

Increasing the number of docked synaptic vesicles available for exocytosis would increase the amount of transmitter released. In the presence of an elevated level of intracellular Ca^{2+}, release would be further increased. It is possible that, as in many other systems (Nishizuka, 1986), PKC can augment the intracellular Ca^{2+} concentration by phosphorylating the voltage-sensitive Ca^{2+} channels, thereby increasing the activity-dependent Ca^{2+} conductance. This phosphorylation of the voltage-sensitive Ca^{2+} channels has also been shown to be dependent upon PKA in the frog heart (Hartzell, et.al., 1991). Nishizuka (1986) states that PKA and PKC can phosphorylate the same targets, such as voltage–sensitive Ca^{2+} channels. Another possibility is that there is cross-talk between the cAMP/PKA system and the PKC system. For example, in *Aplysia* pleural ganglionic neurons, the PKA pathway influences the PKC pathway and the reverse is also true (Sugita, et.al., 1992; Sugita, 1997). This phenomenon of cross-talk could also explain the reports of Hartzell (1991) and Flaadt (1993) that PKA does not

FIGURE 4-3: ***Possible Targets for the Kinases in Modulation – Involvement of a Synapsin-Like Protein***

Synapsin I, or a synapsin-like protein, is responsible for sequestering synaptic vesicles by binding them to actin filaments of extensions of the ER. In order to liberate the vesicles from these filaments, first PKA, PKG and/or CaMKI must phosphorylate the head ($-NH_2$) region of synapsin releasing the synapsin-synaptic vesicle complex from the actin. Simultaneous inhibition of PKA and PKG prevents the complex from dissociating from the actin completely inhibiting modulation. Next, CaMKII releases the vesicles from the synapsin allowing the binding of the vesicle to the active zone, readying it for release. Inhibition of CaMKII prevents the dissociation of the vesicle from synapsin, slowing the diffusion to the active zone and reducing the vesicle's ability to dock at the active zones, slowing release. PKC increases the Ca^{2+} current through voltage-sensitive Ca^{2+} channels increasing the intracellular calcium concentration which results in an increased probability of release.


PKA/PKG/CaMKI and CaMKII
PKC
via synapsin I phosphorylation
opens voltage-sensitive Ca2+ channels
via Ca2+
transmitter exocytosis

directly phosphorylate the Ca^{2+} channels, but rather the activation of the PKA system initiates the PKC-mediated phosphorylation.

Cross-talk between PKA and PKC could also help to explain the results seen in Fig. 3-10 where simultaneous inhibition of PKA and PKG completely blocks DF_2's effect. It appears that not only is activation of PKA and/or PKG sufficient for neuromodulation, but activation of both kinases is also necessary. This implies that the mechanism outlined in Fig. 4-1 may not be as simple as it appears. The left portion (involving PKA and PKG) and the right portion (involving PKC) probably also influence one another. Further investigation into the specific targets of each kinase and the possibilities of cross-talk in this preparation should provide further elucidation of the mechanisms of neuromodulation and further insight into synaptic release.

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