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Hydrazines are toxic compounds which have numerous military and industrial applications including their use in missile propellants and advanced aircraft such as the F-16 and space vehicles. Because of the recurrent exposure due to routine storage use and disposal of these compounds, understanding their toxic effects on the nervous system is important in the aerospace field. Toxic exposure to hydrazine can result in status epilepticus and eventual respiratory collapse. Acute hydrazine exposure can produce repeated tonic-clonic seizures in animals and man. This project has continued to direct its effort in understanding the molecular mechanism by which hydrazines may produce their neuronal excitatory effects. We have continued and expanded our investigation of the effects of hydrazine on specific electrophysiological properties on identified neurons in the invertebrate Hermissenda Crassicornis and have expanded these studies to investigate the effects of hydrazine on isolated neurons in culture. Our studies have documented that hydrazines increase neuronal excitability in the LP-1 neuron of this nudibranch mollusc. Studies have been directed at further establishing the technical capability of investigating the effects of hydrazines on the rate of sustained repetitive firing. It has been shown that

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**Medical College of Virginia  
Virginia Commonwealth University**

January 10, 1992

Dr. Genevieve Haddad  
AFOSR/NL  
Building 410  
Bolling AFB, DC 20332-6448

**RE: AFOSR 87-0235 Invention Report**

Dear Dr. Haddad:

In this reporting period 1-5-88/31-5-89 there have been no inventions made on  
AFOSR 87-0235.

Sincerely,

Robert J. DeLorenzo, M.D., Ph.D., M.P.H.  
George B. Bliley III Professor of Neurology  
Chairman of Neurology  
Professor, Departments of Pharmacology and Biochemistry  
Director, Molecular Neuroscience Research Facility

Herbert B. Chermide III  
Director, Sponsored Programs Administration

RJD:cc

**STATUS REPORT**

**Title: THE EFFECTS OF HYDRAZINES ON NEURONAL EXCITABILITY**

**Grant Number: AFOSR-87-0235**

**Robert J. DeLorenzo, M.D., Ph.D., M.P.H. - Principal Investigator**

**Project Period: (01 May 88 TO 31 May 89)**

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**ABSTRACT**

Hydrazines are toxic compounds which have numerous military and industrial applications including their use in missile propellants and advanced aircraft such as the F-16 and space vehicles. Because of the recurrent exposure due to routine storage use and disposal of these compounds, understanding their toxic effects on the nervous system is important in the aerospace field. Toxic exposure to hydrazine can result in status epilepticus and eventual respiratory collapse. Acute hydrazine exposure can produce repeated tonic-clonic seizures in animals and man. This project has continued to direct its effort in understanding the molecular mechanism by which hydrazines may produce their neuronal excitatory effects. We have continued and expanded our investigation of the effects of hydrazine on specific electrophysiological properties on identified neurons in the invertebrate Hermissenda Crassicornis and have expanded these studies to investigate the effects of hydrazine on isolated neurons in culture. Our studies have documented that hydrazines increase neuronal excitability in the LP-1 neuron of this nudibranch mollusc. Studies have been directed at further establishing the technical capability of investigating the effects of hydrazines on the rate of sustained repetitive firing. It has been shown that hydrazines increase the rate of sustained repetitive firing in this system. Studies have also been initiated to elucidate the molecular mechanism mediating the effects of hydrazine on increased neuronal firing in isolated neurons. Initial studies indicate that hydrazines have selective effects on calcium currents studies under voltage clamp techniques. It has also been determined that specific anticonvulsant drugs may have potential benefit in blocking the excitable effects of hydrazine on neuronal activity. The project has attained the initial specific aims and preliminary goals developed for this phase of the project period.

### RESEARCH OBJECTIVES

The overall major objective of this study is to determine the mechanisms by which hydrazines increase neuronal excitation and cause seizure activity. We have expanded studies to examine the effects of hydrazines on specific ion conductances in neurons in the nudibranch mollusc, Hermisenda Crassicornis. These studies are also directed at obtaining an insight into the effects of these toxic compounds on neuronal excitability and seizure discharge. The central hypothesis that is being tested in this project is that hydrazines increase neuronal firing by altering specific membrane currents.

During this year of the project, we have made substantial progress in obtaining our short-term goals. The project has almost completed characterizing the effects of hydrazine on increased neuronal firing and examined specific ion currents in the identified LP-1 neuron. Thus, we have been successful in characterizing the effects of hydrazine on sustained repetitive firing (SRF) and spike frequency adaptation (SFA) in Hermisenda LP-1 neurons. This accomplishes the major short-term goal of our project.

Studies have also been continued to characterize specific membrane currents in the LP-1 Hermisenda neuron. The currents that are being investigated include  $I_A$ ,  $I_C$ ,  $I_{Ca}$ ,  $I_{Na}$ ,  $I_K$ . These studies are being conducted with two electrode voltage clamp techniques. Following the characterization and identification of these currents, studies will next be initiated to determine the effects of hydrazines on specific ion currents under the voltage clamp technique. Hydrazine has been found to effect  $I_{Ca}$ .

Initial experiments have also been directed at accomplishing the longer-term goals of the project by developing significant preliminary data and feasibility study information. We have investigated the effects of specific anticonvulsants on the excitability of the LP-1 neuron in

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Hermisenda. We have determined the conditions by which the benzodiazepines and other neuroleptic compounds can be utilized to decrease repetitive firing and alter other parameters of these cells. These experiments include the development of specific techniques for the intracellular micro-injection of these compounds or for direct bath application. The solubility of these compounds and the use of appropriate controlled bathing medium have been studied and we now are capable of testing these agents on LP-1 physiology in the presence and absence of hydrazine. These studies will allow us to conduct the determination of the effects of these compounds on hydrazine-produced effects in this neuron. Initial studies to investigate the effects of hydrazines on calcium regulated ion conductances in identified neurons have also been initiated. Potassium currents are major regulators of neuronal excitability and, if they are involved in the toxic effects, we have developed the technology to determine this in the second and third years of this study. The calcium regulated potassium currents are indirectly regulated by the effect of hydrazine on calcium currents.

#### RESEARCH STATUS REPORT

During this project period we have made significant progress in obtaining the specific aims outlined in the original proposal. The program research is on target and we have completed the initial research and characterization of our system to allow us to continue with the more involved specific aims of the longer term goals. Recent developments in our laboratory have allowed us to study this in spinal cord and hippocampal neurons in culture. We plan to obtain data in the next year to study the effects of hydrazines in vertebrate neurons in culture. In addition, we will test the possibility of developing a hippocampal slice preparation to study the effects of hydrazine on neuronal excitability in this more intact neuronal preparation.

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**Effects of Hydrazine on Spike Frequency Adaptation in Hermisenda Neurons**

Spike frequency adaptation (SFA) is a cellular mechanism that is utilized by cells to reduce the frequency of firing when a constant current stimulus is applied to the cell. This process has been well investigated as a mechanism for regulating seizure activity. It has been shown that anticonvulsant drugs that enhance SFA are potent anticonvulsants against generalized tonic-clonic seizures, the type of seizures produced by toxic hydrazine exposure. Thus, modulation of SFA may be an important mechanism by which anticonvulsants produce their effects against generalized tonic-clonic seizures. In addition, since hydrazines induce tonic-clonic seizures, it is reasonable to investigate their effects on SFA in identified neurons. Alterations in SFA firing could have the opposite effect of the anticonvulsant drugs and account for the convulsant effects of hydrazine in man and animals.

During this project period we have expanded our studies on the effects of hydrazines on spike frequency adaptation (Fig. 1). Figure 1 illustrates the response of a RP11 cell to a 4 nA depolarizing current stimulus of 10 seconds duration. These studies expand our studies performed on the LP-1 neuron in Hermisenda. This research utilizes both intracellular recording and voltage clamp techniques. Numerous experiments were performed by impaling the LP-1 and RP11 neurons with a single microelectrode. In these experiments constant current is applied to the cell and spike trains are produced in response to stimulation. These spike trains are recorded using a chart recorder or computer data system.

We have determined that in the presence of hydrazine, there is a significant effect on spike frequency adaptation. Hydrazine produced a reproducible reduction of spike frequency adaptation. These studies document that hydrazine limits spike frequency adaptation not only in the LP-1 neuron but also in RP11 and other neurons. The effects of hydrazine clearly

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document that this compound can block the cells ability to turn off repetitive spiking. Thus, a neuron exposed to hydrazine becomes excitable. Hydrazine produced the same effects on this neuron as several known convulsants. The reversibility of the hydrazine effects on SFA indicates that the concentrations used are not simply killing or injuring the cell resulting in increased neuronal excitability. These experiments have been performed under conditions where the hydrazine effect is not lethal to the cell and the cell can make a complete electrophysiological recovery. These experimental results have been conducted with numerous appropriate controlled experiments. This research now establishes that hydrazines can behave as other convulsant drugs and produce increased neuronal firing. We have also demonstrated in our laboratory that anticonvulsant drugs such as Diazepam, Medazepam, and Phenytoin can block sustained repetitive firing and enhance SFA. These compounds dramatically enhance SFA. Experiments are currently underway to determine if these compounds can block the specific excitatory effects of hydrazine on the LP-1 neuron.

These experiments are technically difficult to perform because they require the mixing of both hydrazines and other drugs in the bathing solution. This must be done under conditions to maintain the solubility of the anticonvulsant drugs while simultaneously applying hydrazine. Solubilizing both of these compounds in appropriate concentrations in artificial sea water has been technically difficult, but we have now produced preliminary data that suggests that we have been successful in developing this technique. Thus, we are now investigating the effects of anticonvulsant drugs in blocking the effects of hydrazine on repetitive firing and spike frequency adaptation. We have established the effects of specific anticonvulsant drugs on sustained repetitive firing in identified neurons in Hermisenda. These experiments document that we now are ready to initiate the next phase of this research and determine which

compounds can regulate the effects of hydrazine on neuronal excitability.

In summary, we have accomplished the major specific aims of this first project period. We have established that hydrazine clearly reduces SFA. The effect of hydrazine was clearly reversible and was shown to effect SFA in the same manner as other convulsant drugs. We have also conducted the initial experiments in this invertebrate model to test the effect of anticonvulsant drugs on the effects of hydrazine on this system. We have documented that anticonvulsant drugs can regulate SFA and are now initiating experiments to determine their action on hydrazine's excitable effect on excitability.

#### **Characterization of Membrane Currents in Identified Hermissenda Neurons**

During the first year of this project we have also been successful in characterizing specific membrane currents in identified Hermissenda neurons. We have also investigated spike frequency adaptation and sustained repetitive firing in other identified neurons besides LP-1. These studies are important because specific neurons have variable excitatory properties. Thus, it is important to determine whether the effects of hydrazine seen on the LP-1 neuron are representative of other neurons in this preparation. We have obtained data that clearly documents our preliminary findings that we can measure and characterize the currents  $I_A$ ,  $I_{Ca}$ . We have measured these currents successfully in the LP-1 neurons in our laboratory. We have also investigated other specific ion conductances including  $I_K$ ,  $I_{Na}$ ,  $I_C$ . These currents are clearly present in the LP-1 neuron and we have obtained initial evidence that establishes that we can isolate these currents under voltage clamp procedures.

The  $I_A$  current is called the "A" current. The A current is an outward voltage-dependent potassium current which is blocked by three millimolar 4-aminopyridine. Thus, we have been

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successful in evaluating this current in a sodium-free medium that contains cobalt or cadmium to block the inward flow of sodium or calcium. This also inhibits the  $I_C$  current. Thus, under conditions where we can block most other ion flows, we can isolate the  $I_A$  current. In addition, the A current has been shown in the LP-1 neuron to be inactivated at resting potential. Thus, this current can be measured in a two-pulse paradigm in which the membrane potential is a pre-step to a hyperpolarizing voltage.

The A current is interesting because it exhibits both calcium dependence as well as voltage dependent activation. It is our hypothesis that the A current may be an excellent candidate in mediating some of the effects of hydrazine on neuronal excitability. Now that we have established the initial parameters of these currents in the LP-1 and other neurons in Hermisenda ganglia, we are ready to initiate the second phase of our studies looking at the effects of hydrazines on this specific current.

We have also made considerable progress in characterizing the inward calcium current,  $I_{Ca}$ . The calcium current is measured as peak inward current in a sodium-free sea water solution containing three millimolar, 4-aminopyridine and TEAE (100 millimolar) which blocks both the  $I_A$  and  $I_K$  currents. Barium is utilized to replace calcium as the current carrier to eliminate the effects of calcium on the potassium currents. We have been successful in clamping LP-1 and RP11 neurons and holding voltage of approximately -50 millivolts. Test pulses were given to elicit the calcium current for durations of 500 milliseconds. We have determined that the  $I_{Ca}$  exhibits calcium dependent inactivation in the LP-1 and RP11 neurons. (Fig. 2). The studies initiated in this first year of research have demonstrated that we now have established parameters to study this current and the effect of hydrazine on this isolated calcium current.

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We have been successful in establishing and characterizing extensively two specific ion currents and have begun studies on sodium and other currents. It is essential to rigorously establish the parameters of these currents in the identified neuron so that careful analysis of the effects of hydrazine can be determined. It is important to exclude artifactual effects secondary to changes in cell environment. Thus, we are being extremely careful to rigorously define our electrophysiological system before characterizing the effects of hydrazine. We have accomplished the initial goals of our project and are now ready to initiate the hydrazine studies on these identified currents. We plan to expand these studies to investigate other currents especially if hydrazine does not effect  $I_A$  or  $I_{Ca}$  currents.

#### **Effects of Hydrazine on $I_{Ca}$**

A major advance during this year of our research progress has been that we have been able to study the effects of hydrazine on isolated ion currents under voltage clamp techniques. Figure 2 illustrates an inward current ( $I_{Ca}$ ) in an RP11 neuron in response to a 400 msec command pulse from -60mV to 0 mv. Dr. Forman's techniques are now very well standardized and we can routinely measure  $I_{Ca}$  currents in LP-1 and RP11 neurons in the Hermisenda ganglia. We have initiated for the first time experiments studying the effect of hydrazine on these isolated calcium currents. This is very difficult to perform because we have to do appropriate control conditions to be sure the hydrazine is directly effecting the current and not the recording apparatus in the bath. Figure 2 demonstrates the effects of hydrazine on the  $I_{Ca}$ . Fifteen minutes following the perfusion of the neuron with hydrazine, the  $I_{Ca}$  is dramatically reduced. These results provide the first demonstration of the direct effect of hydrazine on ion currents.

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Several investigators invertebrate systems as well as invertebrate neurons have demonstrated that an increase in calcium causes a diminution in spike frequency in large mollusc neurons. Most neurons can shut off the spiking that occurs during spontaneous spiking. This is called spike frequency adaptation. If calcium increases in the neuron, the cell can shut off the spontaneous firing. Thus, if calcium entry is inhibited, you would block the ability of the cell to shut off spontaneous firing. This literature when applied to the experimental results obtained in this study indicate that a possible mechanism by which hydrazine causes increased sustained repetitive firing could be by reducing calcium entry. This will in turn effect the excitability of the neuron and effect spike frequency adaptation. If this is the case in RP11 cells, then the reduction in  $I_{Ca}$  may account for or contribute to the possible effects of hydrazine in the reduction of spike frequency adaptation or the cause of sustained repetitive firing shown in Figure 1. These results are exciting to us in that they may provide some of the first molecular evidence for how hydrazine may cause seizures in man.

The studies undertaken in this second year have provided important evidence for studying the effects of hydrazine on isolated ion currents. The finding that hydrazine can limit or decrease the  $I_{Ca}$  is an important finding. We are currently vigorously testing all the control conditions so that we can publish this important finding and document its' reliability. This should be accomplished during the third year of the project.

#### **Phasing of Research Projects**

During this research effort, we have made exceptional progress in our ability to bring together necessary personnel and equipment to initiate these studies. We have successfully documented the effects of hydrazine on sustained frequency adaptation and began experiments

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to characterize this effect on specific ion currents. We have also successfully developed the procedures for studying the interaction between hydrazine and other known anticonvulsant and convulsant drugs. We have accomplished the major objectives planned for this part of the project. In addition, we have also established the technical base to extend this research into the investigation of other specific ion currents and carefully characterize the effects of hydrazine on these currents in the LP-1 and RP11 neurons and other cells in this mollusc. These studies will be important in providing the first electrophysiological data on the effects of hydrazine on ion currents. We have also demonstrated for the first time that hydrazine inhibits  $I_{Ca}$  in voltage clamp studies.

Although these studies are performed on an invertebrate system, it is also essential that this research be extended to vertebrate neurons. We have successfully developed the technology to prepare and maintain vertebrate culture neurons isolated from fetal hippocampus and spinal cord. We are initiating studies to perform and evaluate sustained frequency adaptation, sustained repetitive firing, and specific ion currents in these vertebrate cells. We have included this research in our long term goals and hope that within the second or third year of this project to be able to extend our studies in the mollusc system to the vertebrate neurons in culture. This is a significant advance in our research effort and has been brought about by the rapid progress that we have made on this project. These studies will significantly expand our objectives and may provide important comparisons between invertebrate and vertebrate neurons. The demonstration of the effect of hydrazine on these cells with rigorous electrophysiological studies may provide the initial framework for understanding the excitable effect of this compound on neuronal function.

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#### **Professional Personnel Engaged in this Study**

Dr. Robert J. DeLorenzo has acted as the *Principal Investigator* in studying the effects of hydrazines on neuronal excitability. In addition, Dr. Robin Forman has participated as a co-investigator. Dr. Forman has been responsible for specific electrophysiological experiments and for assisting in the development of the studies on the anticonvulsant effects on isolated LP-1 and RP11 neurons. Dr. William Taft has also been involved in assisting with the anticonvulsant and convulsant drug studies on isolated neurons. We have also initiated a collaborative interactions of Dr. Sompong Sombati and Dr. William Anderson who have developed the neuronal cells in culture. Dr. Sombati, in collaboration with Dr. Forman and Dr. DeLorenzo, has initiated studies on these vertebrate neurons in culture and has begun characterization of specific ion currents in these cells. This collaboration has developed rapidly and it is presumed that in the second and third years of this project, Dr. Sombati, in collaboration with Dr. Forman and Dr. DeLorenzo, will expand our studies to include the effects of hydrazine on these cultured vertebrate neurons. Ben Churn, Graduate student in the Department of Pharmacology, has also assisted in some of the basic biochemical studies related to this project. In addition, Bruce Puryear, a medical student at the Medical College of Virginia, has also been involved in research on this project.

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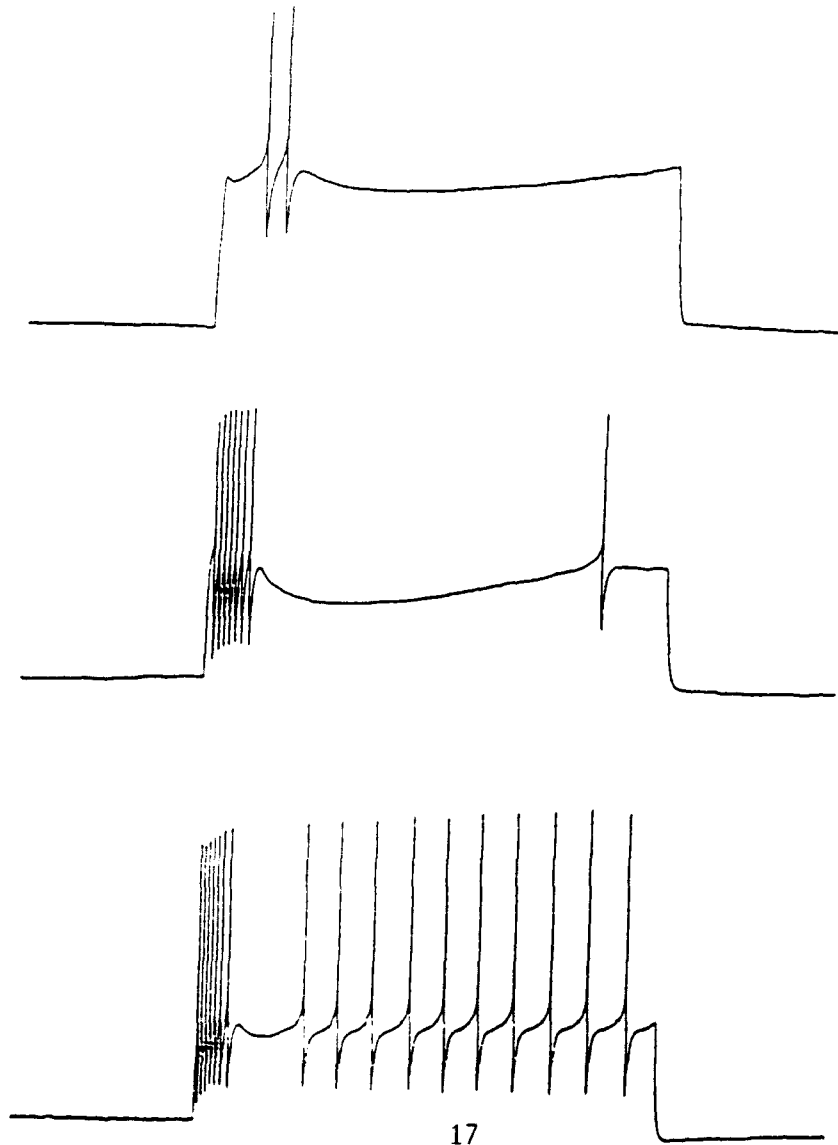
### **Inventions and Patents**

No specific inventions or patents have been developed from this research period.

### **Summary**

During the second year of this research project, we have made considerable progress in directing our efforts at studying the effects of hydrazine on neuronal excitability and on individual ion currents. The initial studies have been exciting and have led to research that will provide the first documentation of the effects of this compound on isolated neurons. In addition, the ability to incorporate experiments into this project that will also allow us to investigate the effects of hydrazine on vertebrate neurons considerably expands the scope of our initial goals. Development of this technology in our laboratory over the last year will greatly enhance the application and feasibility of these studies. We plan to continue studies in vertebrate neurons during the third year of the project. We have also been successful in characterizing specific ion currents and their sensitivity to specific anticonvulsant and convulsant drugs. These studies will allow us now to more rigorously investigate mechanisms that may block the toxic effects of hydrazines on the nervous system.

Fig. 1: Response of a RP11 cell to a 4 nA depolarizing current stimulus of 10 sec duration. Upper panel: Control baseline before hydrazine application. Resting potential during this trace was  $-65\text{mV}$ . Middle panel: Membrane potential held at  $-50\text{mV}$  by injection of 2.0 nA depolarizing current. Lower panel: 10 min following bath application of 10mM hydrazine. RP =  $-57\text{mV}$ . This figure documents that the application of hydrazine causes a reduction in spike frequency adaptation or an increase in sustained repetitive firing. This figure also demonstrates that the effect of hydrazine is not merely due to a change in resting potential of the cell since we can control the resting potential as shown in this figure and does not effect the ability of hydrazine to increase cell firing. Since sustained repetitive firing is believed to be a correlate of epilepsy, the ability of hydrazine to increase sustained repetitive firing suggests that this may be a mechanism by which it acts as a convulsant in animals and man.



DeLorenzo, R. J.

Fig. 2: Inward calcium ( $I_{Ca}$ ) current in RP11 cells. The  $I_{Ca}$  current is in response to a 400 msec command pulse from  $-60\text{mV}$  to  $0\text{mv}$ . Upper panel: Control before hydrazine application. Lower panel: 15 min following bath application of hydrazine. Bath contained  $0\text{Na}^+$ ,  $100\text{mM TEA}$ ,  $3\text{mM 4AP}$  in Hermisenda saline. The osmolarity was adjusted with TMA. Other preliminary data indicates that application of hydrazine may decrease  $I_{Ca}$  in LP-1 and other neurons in addition to the RP11 cell.

