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International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

Collaborative Research Programme

TERMINAL EVALUATION REPORT

Part I

Title of Project
Ionic channels in plant cells: Molecular basis for plant improvement in semi-arid regions
Keywords: Plant cells. Cell membrane. Ionic channels. Biophysics. Modulation of ionic channels. Ion transport in plants.
UNIDO contract # 91/052 **ICGEB ref.#:** CRP/ARG90-02R
Project initiation: 22 May 1991 **Project termination:** 22 May 1994/January 1995
Principal Investigator's name: Prof. Francisco J. Barrantes.
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Abstract:

The main objective of this project was to characterize ionic channels in plant cells with the aim of contributing to the understanding of the molecular mechanisms involved; it was also hoped to thereby learn about the control of ionic and water permeability in plants of importance in semi-arid regions. Towards this end ionic channels in simple model systems were studied. It is expected that the knowledge derived from these studies will be of use in the selection and manipulation of vegetation in semi-arid zones such as cover a large part of Argentina and other South American territories, and in designing appropriate pharmacological agents for their control.

During this project we 1) completed the installation of and improved on the existing electrophysiological set-up with the funds provided by the ICGEB; 2) developed an appropriate simple model system, i.e. cytoplasmic vesicles derived from the tonoplast from the characean algae Chara contraria; 3) worked out methods for the mechanical obtention of the cytoplasmic droplets from Chara internodal cells; 4) carried out patch-clamp studies using this simple model system, addressing our attention to the main ionic channel present in these vesicles, a K^+ channel of large conductance ("Maxi- K^+ channel"). Permeability, conductance, and blockade properties of this K^+ channel were initially characterized. The effect of temperature was studied next at the single-channel level. Eyring's transition state theory was applied to the thermodynamic analysis of conductance and the kinetics of opening and closure of the K^+ channel, for the first time for a plant ion channel. 5) On the molecular biology side, we started with the design and synthesis of oligonucleotides for the screening of the Maxi-K^+ channel. 6) Finally, we carried out the initial characterization of functional alterations in ion channels by pharmacological agents widely used as pesticides in plant therapeutics. We used the acetylcholine receptor (AChR) channel from mouse muscle as a model system for toxicity studies, with the purpose of further extending our observations to the *Chara* K^+ channel. It should be mentioned right from the outset that this progress has been achieved in spite of the exodus of human resources that has occurred during this period.

Objectives/Methodology

Research on the control of ionic permeability in plant cells can have practical implications in agriculture, water of course being a limiting factor in plant productivity throughout arid and semi-arid regions of the world. Some hormones increase the efficiency of water utilization in plants by their ability to cause stomatal closure. Little is known, however, about the action of plant hormones at the molecular level. Our understanding of such molecular details can have important bearing on the design, selection and manipulation of plants in arid and semi-arid regions. The current project was intended to contribute towards this goal by using a combination of molecular biology and biophysical methods.

There are specialized organs and tissues that appear to be targets of the plant hormones possibly involved in the regulation of ionic transport. Such is the case with the foliar stomatic apparatus and the root apex, sites where a marked ionic exchange takes place. Although the physiology of the stomatic phenomena has been widely studied in intact epidermal tissue (Jarvis and Mansfield [1980] Plant Cell Environ. 3, 279), functional stomatic cells (Ogawa [1981] Plant Sci. Lett. 22, 103; Behl and Hartung [1986] Planta 168, 360), and in guardian cell protoplasts (Schnabl et al. [1978] Planta 143, 33; Sato [1985] Plant Cell Physiol. 26, 805), little is known about the molecular mechanisms underlying these physiological processes. K^+ and H^+ fluxes have been reported to occur in epidermal and mesophyllic guardian cells.

There is evidence on the occurrence of ionic channels in cytoplasmic vesicles form characean algae (Luhring [1986] Protoplasma 133, 19). These cells offer a distinct advantage for studying ionic channels in comparison to e.g. stomatic cells of higher plants, given the ease with which specimens can be obtained from the algae. Powerful biophysical techniques such as the patch-clamp method (Sakmann and Neher [1984] Ann. Rev. Physiol. 46, 455) used in this project (object of various reviews by one of us (Barrantes [1993] In: Protein-Lipid Interactions, p.231; [1993] FASEB J. 7,1460) can be applied to the characterization of ionic channels in the model system, but are seldom reproducible in cells from higher plants. The algae system therefore proved to be a suitable model to characterize plant ionic channels with molecular precision, as can be concluded from Ph.D. thesis work by one of the participants of the project (Zanello, 1994, Univ. Nac. del Sur).

Results
(compare against the set objectives)

a) First year.

During the initial phase of this project we a) defined a simple model system, i.e. cytoplasmic vesicles from the characean algae Chara contraria, b) perfected methods for their mechanical obtention from internodal cells and c) began our patch-clamp studies using this simple model system, addressing our attention to the characterization of the main ionic channel present in these vesicles, a K^+ channel of large conductance. Cell-sized vesicles devoid of cell wall were obtained from the giant internodal cells (Sakano and Tazawa [1986] Protoplasma 131, 247) of the algae Chara contraria and studied by means of the patch-clamp technique. The K^+ channel present in this membrane exhibits a high conductance (100 pS in symmetric 100 mM KCl), voltage-dependent behavior, Ca^{2+} sensitivity, and marked selectivity for K^+ over Na^+. We came up with the first reports, at the level of single-channel recording in a plant ionic channel, of the permeability blockade exerted by two typical K^+ channel inhibitors, the ions Cs^+ and tetraethylammonium (TEA^+). Blockade by Cs^+ was found to be concentration- and voltage-dependent. TEA^+ affected both inward and outward K^+ currents when applied to either side of the membrane. The effects are similar to those exerted by these ions on certain types of K^+ channels in animal cells, indicating possible structural homologies between the channel protein present in algae and those found in a wide range of living organisms. In addition, the effects are indicative of the existence of binding sites for TEA^+ near both ends of the channel vestibules, thus suggesting structural asymmetry between these two domains. These studies were presented in preliminary form in two local scientific meetings and a full paper has also appeared (Zanello and Barrantes [1992], Plant Sci. 86, 49).

b) Second year.

i) On the *electrophysiology* side we continued with the characterization of the Maxi-K^+ channel present in cytoplasmic droplets obtained from the giant internodal cells of the algae Chara contraria and studied it by means of the patch-clamp technique.

We also characterized the effect of temperature on the activity of the channel of Chara cytoplasmic droplets by means of the patch-clamp technique (Zanello et al. [1993] Biophys.J. 64, A328; Zanello and Barrantes [1994] Plant Cell Physiol. 35, 243). The activity of the channel was recorded in inside-out patches over a range of temperatures (3°C to 25°C). Channel conductance increased as temperature augmented, with a Q_{10} value of 1.2, whereas the probability of channel opening (P_O) decreased from about 0.4 to 0.1 (at -80 mV). This could be explained by the combined effect of a reduction in the mean open duration and an increase in the closed times, with Q_{10} values of 0.7 and 1.1-1.6 respectively. The *Chara* K^+channel thus exhibits a higher probability of being in the closed state at higher temperatures. Eyring's transition state theory was applied to the thermodynamic analysis of conductance and the kinetics of opening and closure of the K^+ channel. A four-states linear kinetic model was proposed for the Chara K^+channel after simulation and analysis of single-channel data at different temperatures. The values obtained for the changes in activation enthalpy and entropy were critically compared with, and found to be similar to, those reported for voltage-dependent K^+ channels in animal cells. The relative insensitivity of channel conductance to temperature, with an activation enthalpy of about 2.4 $kcal.mol^{-1}$, suggests that ions traverse the pore by diffusion. Channel closure appears to have higher energetic requirements, with an activation enthalpy of 6.4 $kcal.mol^{-1}$ at 15°C. The negative values obtained for the entropic changes in K^+ conductance and channel closure indicate that both processes are accompanied by an increase in the order of the system as the temperature increases.

The existence of subconductance states was also confirmed by means of the patch-clamp technique in the Chara K^+ channel. Multiple subconductance states were detected at different values of membrane voltage and temperature. Most of the channel openings (90%) occur in the highest conductance level, while the open channel probability in minor subconductance states was very low (< 0.1) (Zanello et al. [1992] Ann. Meet. Arg. Biophys. Soc.)

ii) On the *molecular biology* side, we began the design and synthesis of oligonucleotides for the screening of the Maxi-K^+ channel described above in biophysical and physiological terms.

Only two plant potassium channel sequences have been reported, both from Arabidopsis thaliana. These two sequences, ***akt1*** and ***kat1***, are very similar and also show homology with the Drosphila channel ***eag***:

```
eag     mpggrrglvapqntfleniirrsnsqpdssfllanaqivdfpivycnesfckisgynrae  60
giim    mrggallcgqvqdeieqlsresshfslstgilpslgarsnrrvklrrfvvspydhkyriw  60
akt     mrggallcgqvqdeieqlsresshfslstgilpslgarsnrrvklrrfvvspydhkyriw  60
kat     msiswtrnfferfcveeynidtikqssflsadllpslgarinqstklrkhiispfnpryr  60

eag     vmqkscryvcgfmygeltdketvgrleytlenqqqdqfeillykknnlqcgcalsqfgka  120
giim    eaflvvlvvytawvspfefgflrkprp-------------------------------  87
akt     eaflvvlvvytawvspfefgflrkprp-------------------------------  87
kat     awemwlvllviysawicpfqfafitykk-------------------------------  88

eag     qtqetplwlllqvapirnerdlvvlfllтfrditalkqpidsedtkgvlglskfaklars  180
giim    ------------------------------------------------------------  87
akt     ------------------------------------------------------------  87
kat     ------------------------------------------------------------  88

eag     vtrsrqfsahlptlkdptkqsnlahmmslsadimpqyrqeapktpphillhycafkaiwd  240
giim    ------------------------------------------------------------  87
akt     ------------------------------------------------------------  87
kat     ------------------------------------------------------------  88

eag     wvilcltfytaimvpynvafknktsedvSLLVVDSIVdviffidivlnfh----------  290
giim    ---------------------------PLSITDNIVnaffaidiimtff----------  109
akt     ---------------------------PLSITDNIVnaffaidiimtff----------  109
kat     -----------------------------------daifiidnivngffaidiiltff  111
eag     TTFVGPGGE-VVSDPKVIRMNYLKSWFIIDLLSCLPYDVfnafdrdedgigslfsalkvv  349
giim    VGYLDKSTYLIVDDRKQIAFKYLRSWFLLDLVSTIPSEAamrissqsyglfnmlrlwrlr  169
akt     VGYLDKSTYLIVDDRKQIAFKYLRSWFLLDLVSTIPSEAamrissqsyglfnmlrlwrlr  169
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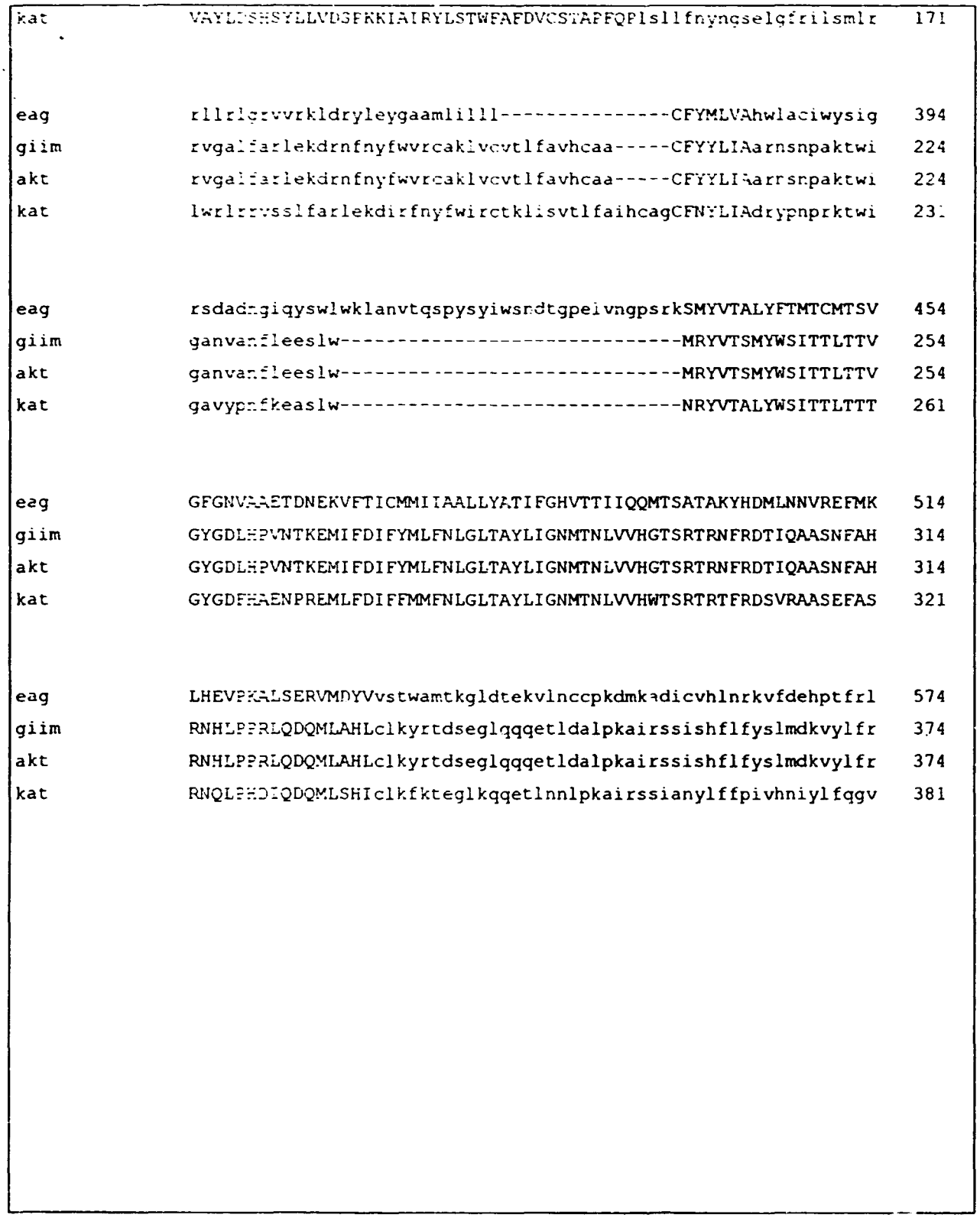

kat VAYLDSHSYLLVDSFKKIAIRYLSTWFAFDVCSTAPFQPlsllfnyngselgfrilsmlr 171
eag rllrlgrvvrkldryleygaamlillll----------CFYMLVAhwlaciwysig 394
giim rvgalfarlekdrnfnyfwvrcaklvcvtlfavhcaa-----CFYYLIAarnsnpaktwi 224
akt rvgalfarlekdrnfnyfwvrcaklvcvtlfavhcaa-----CFYYLIAarrsnpaktwi 224
kat lwrlrrvsslfarlekdirfnyfwircтklisvtlfaihcagCFNYLIAdrypnprktwi 231
eag rsdadngiqyswlwklanvtqspysyiwsndtgpelvngpsrkSMYVTALYFTMTCMTSV 454
giim ganvanfleeslw----------MRYVTSMYWSITTLTTV 254
akt ganvanfleeslw----------MRYVTSMYWSITTLTTV 254
kat gavypnfkeaslw----------NRYVTALYWSITTLTTT 261
eag GFGNVAAETDNEKVFTICMMIIAALLYATIFGHVTTIIQQMTSATAKYHDMLNNVREFMK 514
giim GYGDLHPVNTKEMIFDIFYMLFNLGLTAYLIGNMTNLVVHGTSRTRNFRDTIQAASNFAH 314
akt GYGDLHPVNTKEMIFDIFYMLFNLGLTAYLIGNMTNLVVHGTSRTRNFRDTIQAASNFAH 314
kat GYGDFHAENPREMLFDIFFMMFNLGLTAYLIGNMTNLVVHWTSRTRTFRDSVRAASEFAS 321
eag LHEVPKALSERVMDYVvstwamtkgldtekvlnccpkdmkadicvhlnrkvfdehptfrl 574
giim RNHLPPRLQDQMLAHLclkyrtdseglqqqetldalpkairssishflfyslmdkvylfr 374
akt RNHLPPRLQDQMLAHLclkyrtdseglqqqetldalpkairssishflfyslmdkvylfr 374
kat RNQLPHDIQDQMLSHIclkfkteglkqqetlnnlpkairssianylffpivhniylfqgv 381

```
eag     asdgclralamhfmmshsapgdllyhtgesidslcfivtgsleviqddevvailgkgdvf  634
giim    gvsndllfqlvsemkaeyfppkedvilqneaptdfyilvngtadlvdvdtgtesivrevk  434
akt     gvsndllfqlvsemkaeyfppkedvilqneaptdfyilvngtadlvdvdtgtesivrevk  434
kat     srnflfqlvsdidaeyfppkediilqneaptdlyilvsgavdftvyvdqhdqfqgkavig  441

eag     gdqfwkdsavgqsaanvraltycdlhaikrdkllevldfysafansfarnlvitynlrhr  694
giim    agdiigeigvlcyrpqlftvrtkrlcqllrmnrttflniiqanvgdgtiimnnllqhlke  494
akt     agdiigeigvlcyrpqlftvrtkrlcqllrmnrttflniiqanvgdgtiimnnllqhlke  494
kat     etfgevgvlyyrpqpftvrttelsqilrisrtslmsamhahaddgrvimnnlfmklrgqq  501

eag     lifrrvadvkrekelaerrknepqlpqnqdhlvrkifskfrrtpqvqagskelvggsgqs  754
giim    mndpvmtnvlleienmlargkmdlplnlcfaaireddlllhqllkrgldpnesdnngrtp  554
akt     mndpvmtnvlleienmlargkmdlplnlcfaaireddlllhqllkrgldpnesdnngrtp  554
kat     siaiddsntsghenrdfksmgweewrdsrkdgygldvtnptsdtalmdaihkedtemvkk  561

eag     dvekgdgevertkvlpkapklqasqatlarqdtideggevdssppsrdsrvviegaavss  814
giim    lhiaaskgtlncvlllleyhadpncrdaegsvplweamveghekvvkvllehgstidagd  614
akt     lhiaaskgtlncvlllleyhadpncrdaegsvplweamveghekvvkvllehgstidagd  614
kat     ilkeqkierakverssssetagrsyandsskkdpycsssnqiikpckreekrvtihmmses  621

eag     atvgpsppvattssaaagagvsggpgsggtvvaivtkadrnlalererqiemassratts  874
giim    vghfactaaeqgnlkllkeivlhggdvtrprrtgtsalhtavceeniemvkylleqgadv  674
akt     vghfactaaeqgnlkllkeivlhggdvtrprrtgtsalhtavceeniemvkylleqgadv  674
kat     kngklillpssieellrlasekfggcnttkitnadnaeiddldviwdgdhlyfssn----  677

eag     dtydtglretpptlaqrdlvatvldmkvdvrlelqrmqqrigriedllgelvkrlapgas  934
giim    nkqdmhgwtprdlaeqqghedikalfreklherrvhietsssvpilktgirflgrftsep  734
akt     nkqdmhgwtprdlaeqqghedikalfreklherrvhietsssvpilktgirflgrftsep  734
kat     ------------------------------------------------------------  677
```

```
eag     sggnapdnssgqttpgdeicagcgaggggtpttqapptsavtspvdtvitisspgasgsg  994
giim    nirpasrevsfriretrarrktnnfdnslfgilanqsvpknglatvdegrtgnpvrvtis  794
akt     nirpasrevsfriretrarrktnnfdnslfgilanqsvpknglatvdegrtgnpvrvtis  794
kat     ------------------------------------------------------------  677

eag     sgtgagagsavagaggaqlldpgatvvssaggnglgplmlkkrrsksgkapappeqtlas 1054
giim    caekddiagklvlllefqgvarigfqqvwyccyqsyeqrqqcrd----------------  838
akt     caekddiagklvlllefqgvarigfqqvwyccyqsyeqrqqcrd----------------  838
kat     ------------------------------------------------------------  677

eag     tagtataapagvagsgmtssapasadqqqqhqsaadqspttpgaellhlrlleedftaaq 1114
giim    ------------------------------------------------------------  838
akt     ------------------------------------------------------------  838
kat     ------------------------------------------------------------  677

eag     lpstssggaggggsgsgatpttppptiaggsgsgtptsttatttptgsgtatrgkldfl 1174
giim    ------------------------------------------------------------  838
akt     ------------------------------------------------------------  838
kat     ------------------------------------------------------------  677
```

An alignment of the more conserved regions of these three sequences was made and three candidate stretches of sequence selected for the synthesis of oligonucleotide primers. The oligonucleotides were designed to be degenerate to cover all possible sequence variations at these positions: this makes it more likely that they will amplify any related potassium channel sequence, but also increases the likelihood of artefactual amplifications. Unfortunately, there is always a trade-off between these two factors. The three regions of interest and the oligonucleotide probes that we have synthesised are shown below.

Region 1 ***S2***

```
akt1  LSITDNIVNAFFAIDIIMTFFVGYLD
kat1  IFIIDNIVNGFFAIDIILTFFVAYLD
```

eag LLVVDSIVDVIFFIDIVLNFRTTFVG

Oligonucleotide *K1* Sense

ATTTTTGCNATAGATATAAT based on the amino-acids FFAIDII:

T C C C C TG I V

T C

Region 2 S3

aktl IAFKYLRSWFLLDLVSTIP

katl IAIRYLSTWFAFDVCSTAP

eag IRMNYLKSWFIIDLLSCLP

Oligonucleotide *K2* Sense

ATAGCNATNAANTACCTNAANACNTGGTT from amino acids: IAFKYLRSWF

C T G T G T IR ST

T MN K

Oligonucleotide *K3* Antisense

ACATCNAANACAAACCANGCNCTNAGAT from a.acids LDLLFWSRLY

GG T GGG G T G V FA TS

T II K

Region 3 S5

aktl TSMYWSITTLTTVGYGDLHP

katl TALYWSITTLTTTGYGDFHA

eag TALYFTMTCMTSVGFGNVAA

Oligonucleotide *K4* Antisense

CCAAANCCNACNGANGTNAGNCANGTNTA from a. acids GYGVNTLTTI

GT GT T T GT F TS MC M

The above probes were obtained in the following concentrations:

K1	470 µg/ml (70 µM)
K2	260 µg/ml (26 µM)
K3	640 µg/ml (64 µM)
K4	560 µg/ml (56 µM)

Based on the sequence of the kat1 gene (Anderson et al [1992], PNAS 89, 3736) these primers should produce products of:

K1 and K3 = 125 bp
K1 and K4 = 491 bp
K2 and K4 = 407 bp.

c) Third year.

During the last period we commenced studies on the effect of toxic substances such as herbicides and insecticides on ion channels. The objective of this part of the project was to contribute to the understanding of certain alterations in the ionic permeability properties of the cellular membrane due to the action of chemical agents widely used as pesticides and herbicides, and having known neurotoxic effects. We attempted to carry out a comparative study of the action of these toxic agents on model ionic channels of the plant and the animal cell.

It has been assumed that several herbicides such as Dichlobenil, Simazine, Paraquat, etc. that are effective in the control of Chara in laboratory assays, act through their partition in and disruption of the membrane bilayer. There is a marked increase in cell permeability common to most of the treatments with these compounds, however, which could be accounted for equally well by ion transport mechanisms mediated by ionic channels, as measured by radioactive tracer efflux assays with minerals, amino acids, or sugars (Shimabukuru and Hoffer [1992] Plant Physiol. 98, 1415). More interestingly, the normal efflux of cations appears to be reduced upon herbicide treatment. In the case of pesticides, binding and biophysical assays have demonstrated that certain pirethroid compounds of

ample use in plant therapeutics have toxic effects on different types of ion channels (Chalmers and Osborne [1986] Pestic. Biochem. Physiol. 26, 139; Sherby et al. [1986] Pestic. Biochem. Physiol. 26, 107). Similarly, organophosphates such as Paraoxon, Dichlorvos (DDVP), tetraethylpyrophosphate (TEPP) have also been shown to exert their toxic effects at the plasma membrane level, apparently by altering its electrical properties (Tattersall [1990] Br. J. Pharmacol. 101, 349; Filbert et al. [1992] Brain Res. Bull. 28, 473). There are no reports in the literature on studies of the pathological state of ion channels in plant cells as studied by the patch-clamp technique. The latter technique was employed in order to allow us to describe the state of the channels upon treatment with the toxic compound referred to above.

Our first attempt involved the study of the possible interaction of Parathion and the nicotinic AChR by means of fluorescence techniques, and the characterization of its effect as agonist or antagonist of the receptor. Parathion caused a diminution in fluorescence intensity of the weak agonist DansCol (dansilaminoethyl-trimethylammonium), in the presence of the non competitive antagonist PCP (phencyclidine), similar to the effect caused by the agonist suberyldicholine. Ethydium bromide fluorescence was incremented by the addition of Parathion at a concentration of 0.1-2 μM, similarly to the effect caused by carbamylcholine. These results suggested that Parathion acts as an agonist of the AChR at low concentrations (Caldironi et al. [1993] Ann. Meet. Arg. Soc. Neurochem.). Patch-clamp studies on the single-channel activity of the AChR channel of the mouse muscle type, expressed in the BC3H-1 clonal cell line, corroborated agonist properties of the organophosphorus compound (Caldironi et al. [1994] Ann. Meet. Arg. Soc. Biochem.).

Due to the fact that 1) the amount of nucleic acid material that can be obtained from Chara is relatively small and 2) the possibility of exploring the occurrence of a K^+ channel in a higher plant, we decided to switch to Sedum paquiphylum, a Crassulacean species native from South and Central America, adapted to arid and semiarid environments.

A method for the extraction of total RNA from Sedum leaves cells was implemented following the technique reported by De Vries et al. (1988) In: Plant Molecular Manual B6: 1-13). Due to the fact that plant material contains relatively high levels of RNase activity,

normally located in the vacuoles, special attention must be paid to these enzymes during the RNA extraction procedure. The method we have adopted is based on the obtention of a fine frozen powder by grinding the plant material in liquid N_2, and rapidly transferring the powder to a mixture of phenol and extraction buffer at 90°C. The use of these extreme temperatures have been reported to prevent RNA degradation.

Mesophyll cells constitute an appropriate system for the study of ionic and water transport through the membranes (plasmalemma and tonoplast), and would contribute to our research on ion channels, extending our characterization of the Chara K^+ channel to the level of a higher plant. We also developed a method for the rapid isolation of S. paquiphylum mesophyll cell protoplasts, that consists of a brief incubation of the tissue in an enzymatic medium composed by a mixture of cellulase and pectolyase. The protoplasts obtained by this technique proved to be suitable for patch-clamp recordings of whole-cell currents (preliminary results) and for future experiments dealing with the modulation of plant ionic currents by plant hormones.

By using the Polymerase Chain Reaction (PCR) and the oligonucleotide probes that we designed and synthesized on the basis of available literature data (see above), we plan to search for the gene encoding a K^+ channel of Sedum and, eventually to extend our investigations to the Maxi-K^+ channel of Chara. The latter has been the most extensively studied plant ion channel from the point of view of its function, but its structure still remains virtually unexplored.

Difficulties encountered during the project.

In our original application, the proposed research team consisted of the following personnel:

F. J. Barrantes, G. Amodeo, L. P. Zanello, A.M. Roccamo, E. Mata and M. Martínez

Lic. Gabriela Amodeo found a position abroad since she was unable to obtain a fellowship from the Argentinean Scientific and Technological Research Council (CONICET). We were therefore unable to count on her collaboration.

After one and a half years working in our group as support personnel while finishing his university studies, electronic engineer Mr. Eduardo Mata, whose role was to provide hardware support to the research team, was eventually given a position by the CONICET. However, as a consequence of the meagre salary, Mr. Mata did not take up the position and left his research activities for a position in industry.

Ms. Monica Martinez, an excellent software expert graduated in Computer Sciences from the local university, left the group for a position in the petrochemical industry, where she earns far more than we could afford to pay her.

During the second year of the project, we expected to count on the collaboration of Dr. Cecilia Bouzat, who had just obtained her Ph.D. working on ionic channels under the supervision of Dr. Barrantes. In spite of her very active production, however, the Argentinean Research Council (CONICET) continued its closed door politics for entry into the research career, and Dr. Bouzat was also obliged to find a position abroad. She has now returned, but her expertise in patch-clamp techniques would have been appreciated for a more rapid development of the project.

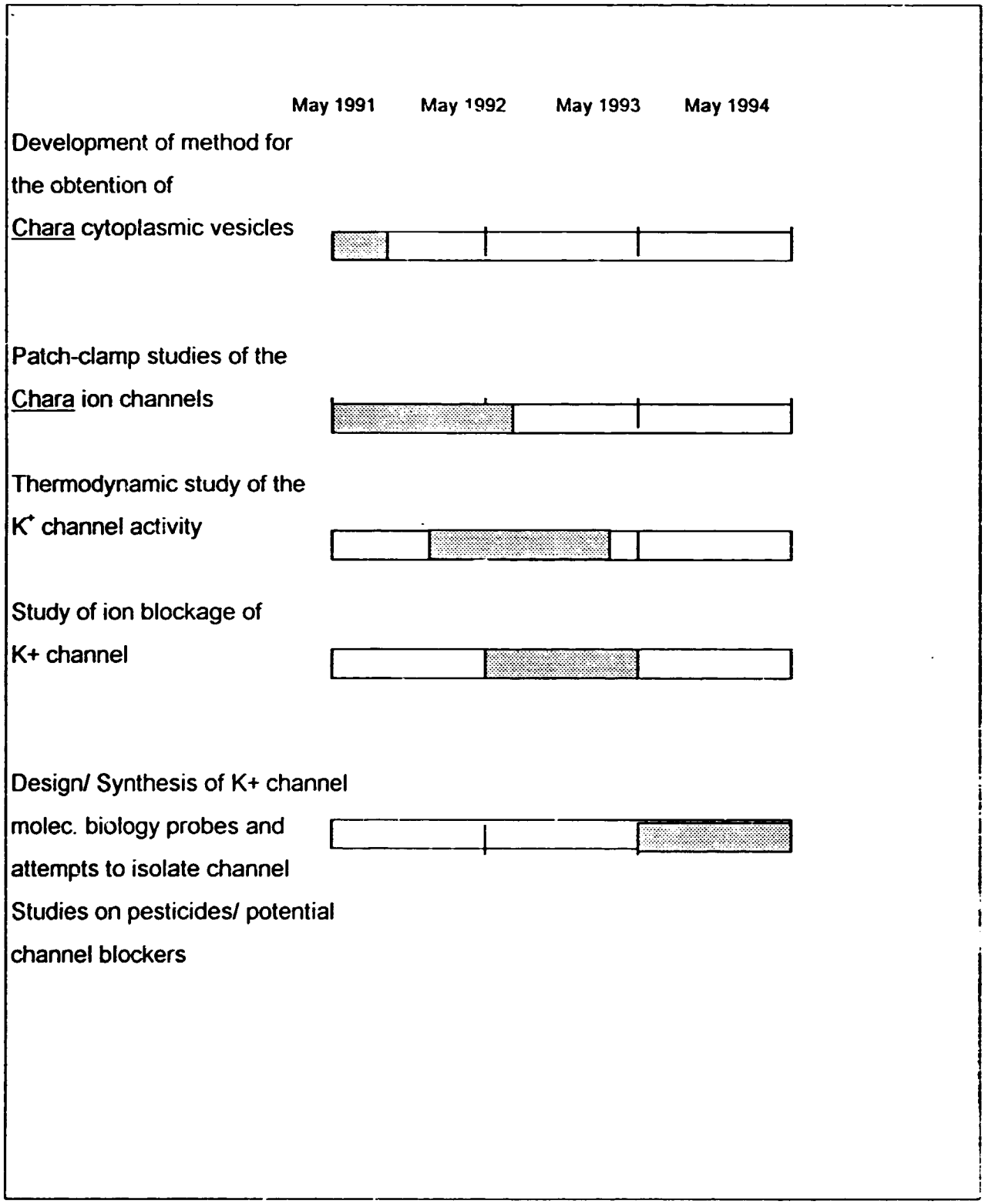
It was also announced that we expected an engineering student to join the team to collaborate in software development and data analysis. This was accomplished, and Mr. Mario Alcaraz actively collaborated during the second period of the project.

Finally, we also stated in our previous report that if we were able to incorporate a molecular biologist into the team, some of the objectives would be tackled with a combined molecular biology-biophysical approach. Although it has not been possible to hire such a person, we were able to start with the molecular biology approach as detailed below. We have counted on the help of Dr. Adrian Wolstenholme, of the Department of Biochemistry, University of Bath, U.K., for the oligonucleotide synthesis.

As previously mentioned, the exodus of human resources during the development of this project has meant that only one assistant researcher, Laura P. Zanello, has been actively involved in the practical aspects apart from the principal investigator. Dr. Zanello completed her Ph.D. studies under the supervision of Dr. Barrantes, and her Ph.D. thesis achieved the highest grade.

Work plan and time schedule

(originally envisaged)



Work plan and time schedule

(actual)

Project landmarks:

- Definition of a simple model system for the study of plant ion channels by means of the patch-clamp technique: isolation of cytoplasmic vesicles from the characean algae Chara contraria.

- Improvement of the existing electrophysiological set-up with the funds provided by the ICGEB.

- Basic characterization of the electric activity of the Maxi-K^+ channel present in the membrane of cytoplasmic vesicles at the single-channel level. Description of channel conductance, selectivity, duration of open and closed states, burst activity, blockade by Cs^+ and tetraethylammonium.

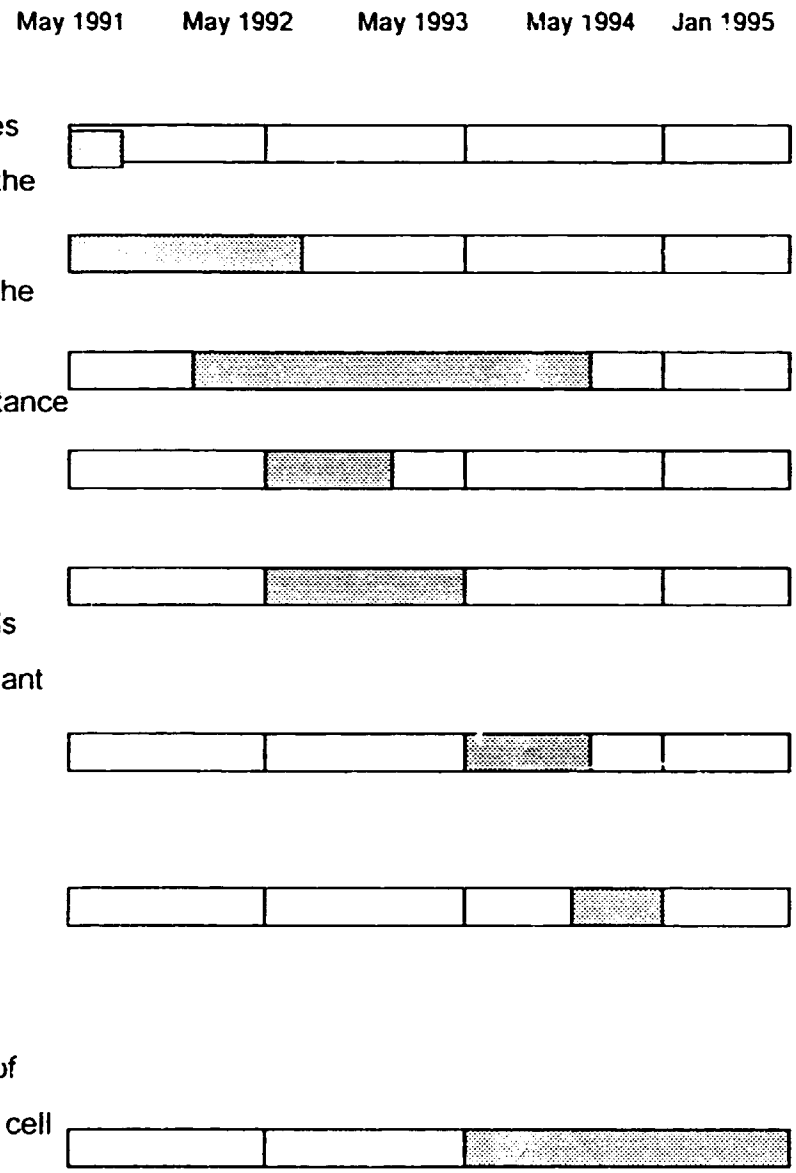
- Thermodynamic analysis of the temperature sensitivity of single-channel conductance and kinetics. Proposal of a kinetic model for the Chara K^+ channel.- Description of subconductance states in the Chara K^+ channel.

- Design and synthesis of oligonucleotides for the screening of the Maxi-K^+ channel.

- Implementation of a method for total RNA extraction from plant tissue of the specie Sedum paquiphylum.

- Initial characterization of the effect of the pesticide Parathion on the activity of model ionic channels of the animal and the plant cell. single-channel activity of the AChR channel, by fluorescence and electrophysiological techniques.

Duration of individual tasks:



Meetings, Symposia, etc. attended by the personnel involved in the project:

June 11-12, 1991 L.P. Zanello and F.J. Barrantes attended the First Argentinean Symposium on Plant Biotechnology, Vaquerías, Córdoba, Argentina, where a communication was presented (see Publications).

Dec. 5-6, 1991 L.P. Zanello and F.J. Barrantes attended the Symposium "Frontiers in Ion Channel Research", in the International School of the Centro de Estudios Cientificos de Santiago, Santiago, Chile.

Dec. 12-13, 1991 L.P. Zanello attended the XX Annual Meeting of the Argentinian Biophysical Society at La Plata, Argentina, where she presented a communication (see Publications).

Feb. 1993. C. Bouzat and F.J. Barrantes attended the 35th. Annual Meeting of the Biophysical Society in Houston, Texas.

Nov. 16-18, 1992 E. Aztiria, M. Alcaraz, C. Bouzat, L.P. Zanello and F.J. Barrantes attended the XXI Annual Meeting of the Argentinian Biophysical Society in Huerta Grande, Córdoba, Arg., where a communication was presented (see Publications).

Feb. 14-18, 1993 L.P. Zanello and F.J. Barrantes attended the 37th. Annual Meeting of the Biophysical Society in Washington, D.C., where a communication was presented (see Publications).

Oct. 27-29, 1993 L.M. Antonucci, E. Aztiria, L.P. Zanello and F.J. Barrantes attended the VII Annual Meeting of the Argentinian Society for Neurochemistry in Villa Giardino, Córdoba, Arg., where a communication was presented (see Publications).

Oct. 26-29, 1994 L.M. Antonucci, E. Aztiria, H. Caldironi, A.M. Roccamo, L.P. Zanello and F.J. Barrantes attended the XXX Annual Meeting of the Argentinian Society of Biochemistry Cataratas del Iguazú, Misiones, Arg., where a communication was presented (see Publications).

Training of personnel involved in the project:

Nov. 10-17, 1991 E.M. Aztiria participated in the International Workshop "Structure and Engineering of Proteins (with special emphasis on membrane-associated and channel-

forming proteins)", organized by F.J. Barrantes in the Instituto de Bioquímica, Universidad Nacional del Sur, Bahía Blanca, Arg.

Sept. 1991- Feb. 1992 L.P. Zanello visited the Laboratory of Prof. Ramón Latorre at the Universidad de Chile, Santiago, Chile. During her stay, L.P. Zanello was instructed on whole-cell recording techniques and some methods for the analysis of single-channel patch-clamp recordings.

Aug. 10-23, 1992 L.P. Zanello participated in the training course "Intensive course for cloning and expression of eucaryotic genes", at the Institute of Genetic Engineering and Molecular Biology, Universidad de Buenos Aires, Buenos Aires.

Dec. 7-18, 1992 L.P. Zanello participated in the course "P· :ch-clamp recording techniques and monitoring of intracellular calcium", at the Laboratory of Biophysics, International Center of Physics, Universidad Nacional de Colombia, Santa Fe de Bogotá, Colombia.

Sept. 5-18, 1993 A. M. Roccamo participated in the course "Antibody modification by genetic engineering: Production of fragments of antibodies in bacteria". Center of Genetic Engineering and Biotechnology, La Havana, Cuba.

Aug. 9-28, 1993 E.M. Aztiria participated in the training course: "Intensive course for cloning and expression of eukaryotic genes", at the Institute of Genetic Engineering and Molecular Biology, Universidad de Buenos Aires, Buenos Aires.

Other activities:

Apr. 5-8, 1993 The personnel involved in the project were memberes of the teaching staff in the international course "Frontiers in Cellular and Molecular Neurophysiology", at the Instituto de Investigaciones Bioquímicas.

Oct. 4-20, 1994 The personnel involved in the project participated in the international course "Expression in heterologous cellular systems and evaluation of functional properties of ion channel forming proteins", organized by F.J. Barrantes, at the Instituto de Investigaciones Bioquímicas.

L.P. Zanello has at present a Teaching Assistant position in the Chair of General Biology, Department of Biology and Biochemistry, Universidad Nacional del Sur. She collaborated in teaching at the postgraduate course "Ultraestructura y Función Celular (Cellular Ultraestructure and Function)" organized by Prof. M. Prado Figueroa, Dept. of Biology and Biochemistry, Universidad Nacional del Sur.

C. Bouzat has at present a Teaching Assistant position in the Chair of Pathological Biochemistry, Department of Biology and Biochemistry, Universidad Nacional del Sur. She has also collaborated in teaching at the postgraduate course 'Ultraestructura y Función Celular (Cellular Ultraestructure and Function)" organized by Prof. M. Prado Figueroa, Dept. of Biology and Biochemistry, Universidad Nacional del Sur.

NETWORKING

PUBLICATIONS

The investigations carried out during this project resulted in two full length publications (Zanello and Barrantes, 1992, 1994), one abstract published in the Biophysical Journal (Zanello et al., 1993) from a work presented in the Annual Meeting of the Biophysical Society in USA, and six communications to local meetings (listed in Publications). L.P. Zanello has recently completed her Ph.D. thesis under the supervision of F.J. Barrantes, obtaining the highest grade.

- Zanello, L. P. and Barrantes, F. J. Canales de potasio en la membrana de un alga carófita y su uso potencial como modelo para el estudio de la acción de agentes farmacológicos y toxicológicos. (K^+ channels in the membrane of a carophyte algae and their potential as model systems for the study of pharmacological and toxic agents). Simposio Argentino de Biotecnología Vegetal, Vaquerias, Cordoba, June 1991.

- Zanello, L. P. and Barrantes, F.J. Efecto de la temperatura sobre la actividad del canal de K^+ de *Chara* (Effect of temperature on the activity of the K^+ channel from *Chara*). Ann. Meet. Soc. Argentina de Biofisica, La Plata, December 12-13, 1991.

- Zanello, L.P., Alcaraz, M. and Barrantes, F.J. Múltiples estados de subconductancia en el canal de K^+ de *Chara contraria*. (Multiple subconductance states in the K^+ channel from *Chara contraria*). Ann. Meet. Soc. Argentina de Biofísica, Huerta Grande, Córdoba, Argentina, November 16-18, 1992.

- Zanello, L.P. and Barrantes, F.J. Termodinámica de un canal de K^+ en célula vegetal. (Thermodynamics of a K^+ channel in the plant cell). Ann. Meet. Soc. Argentina de Biofísica, Huerta Grande, Córdoba, Argentina, November 16-18, 1992.

- Zanello, L. P. and Barrantes, F.J. Blockade of the K^+ channel of *Chara contraria* by Cs+ and tetraethylammonium resembles that of K^+ channels in animal cells. ***Plant Sci.*, *86***, 49-58, 1992.

- Zanello, L.P., Aztiria, E. and Barrantes, F.J. Thermal sensitivity of a plant voltage-gated channel and a ligand-gated neurotransmitter receptor channel. ***Biophys. J. 64***, A328, 1993.

- Caldironi, H.A., Antonucci, L.M. and Barrantes, F J. Interacción entre el compuesto organofosforado Parathion y el receptor de acetilcolina nicotínico detectada por técnicas de fluorescencia (Interaction between the organophosphorus compound Parathion and the nicotonic acetylcholine receptor detected by fluorescence techniques). Ann. Meet. Soc. Arg. Neuroquímica, Villa Giardino, Córdoba, Argentina, October 27-29 1993.

L.P. Zanello. Ph.D. thesis "Characterization of a K^+ channel of the plant cell by means of the patch-clamp technique and its possible analogies with K^+ channels of the animal cell", Universidad Nacional del Sur, Argentina, 1994.

- Zanello, L. P. and Barrantes, F.J. The effect of temperature on the K^+ channel of *Chara*. A thermodynamic analysis. ***Plant Cell Physiol.*, *35*,** 243-255, 1994.

- Caldironi, H.A., Aztiria, E.M., Antonucci, L.M. and Barrantes, F.J. El compuesto organofosforado Parathion exhibe propiedades agonistas sobre el receptor de acetilcolina nicotínico (The organophosphorus compound Parathion exhibits agonist properties on the nicotinic acetylcholine receptor). Ann. Meet. Soc. Arg. de Bioquímica, Cataratas del Iguazú, Misiones, Argentina, October 26-29, 1994.

Part 4

STATEMENT OF EXPENDITURES

To be filled by ICGEB		To be filled by the Affiliated Centre	
Budgets as per original proposal		**Summary of expenditures** *	
1) Capital equipment	US$	1) Capital equipment	US$ 27,285.47
2) consumables	US$	2) consumables	US$ 22,004.42
3) training	US$	3) training	US$ 14,104.73
4) literature	US$	4) literature	US$ 1,869.74
5) miscellaneous	US$	5) miscellaneous	US$ .....
TOTAL GRANT	**US$.....**	**TOTAL**	**US$ 65,264.36**

Please itemize the following budget categories (if applicable)

Capital equipment

- Large-scale iquid-nitrogen storage container with alarm.
- 4-pen plotter.
- 486 personal computer with printer and accessories for patch-clamp data acquisition.
- Thermal cycler for PCR experiments.
- Hewlett-Packard 6-pen plotter for patch-clamp analysis system.
- Macintosh Centris 650 single-channel patch-clamp analysis system, to be used in conjunction with new EPC-9 amplifier.

Training (provide names, duration of training, host laboratory).

- Dr. Laura Zanéllo, with Prof. Dr. Ramón Latorre. Laboratory of Electrophysiology. Dept. Biology, Univ. Chile, Santiago, Chile. Five month training.
- Lic. Ana M. Roccamo. Postgraduate course on Antibody modification by genetic engineering: production of fragments in bacteria. La Havana, Cuba. Two weeks.
- Lic. Eugenio Aztiria took intensive course on cloning and expression of eukaryotic genes, at INGEBI, Buenos Aires. Two weeks.
- Lic. L. Zanello participated in international course on Patch-clamp recording techniques and monitoring of intracellualr calcium. Bogota, Colombia. Two weeks.

Literature

- Suscription to Reference Update for keeping abreast of the literature.
- Various books on molecular biolgy, channels, thermodynamics of receptors, etc.
- L. Zanello suscribed to the Japanese Soc. Plant Cell. Physiology and its journal.

* Please do not send invoices, receipts etc.; these should be kept by the Affiliated Centre for future reference and sent to ICGEB upon request.