ISAS - INTERNATIONAL SCHOOL FOR ADVANCED STUDIES

Xenopus laevis oocytes as a tool to study the biophysical properties of voltage gated channels

Thesis submitted for the degree of

Magister Philosophiae

CANDIDATE Caterina Virginio

SUPERVISORS Prof. Enrico Cherubini

Prof. Antonino Cattaneo

Academic Year 1992/1993

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TRIESTE
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To my husband

for his patience and understanding

INDEX

Chapter 1

Introduction

In the last few years *Xenopus laevis* oocytes have been used successfully as a translation system for foreign genetic informations to ask questions regarding the regulation of gene expression, post-translational processing and biological functions of proteins.

Mature oocytes accumulate a large quantity of the components needed to translate proteins in preparation for the initial stages of embryo development. These components can be recruited for the transcription and translation of cDNA and the translation of exogenous messenger RNAs (mRNA) species microinjected.

Gurdon and co-workers have shown that oocytes are able to translate globin from reticulocytes mRNA, with an efficiency only a few-fold lower than that of the intact reticulocyte, making the oocyte far superior than the in vitro translation system (1).

It has been further demonstrated that the oocyte carry out appropriately many types of post-translational processing, including

cleavage of precursors, glycosilation and assembly of oligomeric receptor/channel complexes (2-3-4).

For these reasons, this system has been largely used by electrophyisiologists and molecular biologists to identify and characterize the structure-function of ligand and voltage gated channels (5-6-7).

Xenopus oocytes can be injected with poly(A)$^+$RNA extracted from a given tissue or with mRNA transcribed from a cloned gene. Oocytes can be used for nuclear injections of cDNA inserted in the proper orientation downstream from an eukariotic promoter and upstream from a proper transcription termination sequence in order to have transcription, capping, polyadenilation and export of processed mRNA to the cytoplasm taken in charge by the biosyntetic machinery of the cell. These three types of approaches are useful to study the events from gene to protein at different stages of investigation.

After an incubation period that can be in the range of hours or days, the expression of ligand or voltage gated channels can be tested either with biochemical or electrophisiological methods.

Miledi and co-workers have shown that the oocyte system can be used to express and study ion channels (8-9). In fact the oocyte presents several useful characteristics:

I. The endogenous mRNA for ion channels is expected to be present in low abundance.

II. The oocyte is a very efficient translation and transcription system.

III. The oocyte cytoplasmic membrane has a very high resistance, with relatively few endogenous ion channels and thus the background of endogenous currents is low.

IV. Small amounts of expression on the range of thousands to tens of thousands new channels can be detected.

V. The ability to assay the presence of new channels electrophisiologically endows a sensitivity far greater than that obtained with biochemical methods.

1.1 Applications of *Xenopus* oocytes microinjection

There are several applications for which the *Xenopus* oocyte microinjection can be exploited as a valuable tool. I will present an overview of them followed by specific examples of experimental strategies and approaches.

1.1.1 mRNA bioassay

The microinjection of total poly(A)$^+$RNA from tissue

homogenates into *Xenopus* oocytes provides evidence for the presence of translatable mRNA encoding the desired polypeptide and offers the possibility to isolate a functional protein in a controlled and easily accessible environment.

I- The fidelity of the oocyte in mRNA translation may permit the relative "quantification" of specific mRNAs in various tissues at different stages of development and in several physiological and pathological states. The total poly(A)$^+$RNA from different tissues can be injected in oocytes. The amplitude of the currents is proportional to the number of functional channels expressed in the membrane. This is related with the amount of specific mRNA content of the tissue; therefore maps of the protein's density distribution can be formed.

II- The "transplantation" of active heterologous channel or receptor complexes into *Xenopus* oocytes in many cases generates novel membrane conductances closely resembling those of native tissues, as shown for the muscarinic acetylcholine receptor subtypes (10), the ß and the α-adrenergic receptor (11-12) and the neuronal nicotinic acetylcholine receptor (13). This attribute has been exploited by Stühmer and co-workers (14) to correlate the functional diversity of voltage gated potassium channels in mammalian brain with the existence of polymorphic family of potassium channel-forming proteins. Moreover, in a further application of this feature to the exploration of protein polymorphism, brain mRNA from mouse strains differing in

their genetically determined sensitivities to ethanol, was shown to elicit GABA-A receptors in microinjected *Xenopus* oocytes exhibiting opposite responses to externally applied ethanol (15). The production of heterologous proteins demonstrating biological activities characteristic of native molecules, offers the exceptional opportunity to evaluate the existence of multiple tissue-specific or stage-specific mRNAs encoding polymorphic protein variants.

III- The contribution of multiple heterologous subunits to the generation of native biological activities has been deduced following the microinjection of size-fractionated mRNAs. In this way, evidence implicating the involvement of multiple gene products in controlling the inactivation process of rat and rabbit sodium channels (16) and in determining the kinetic and the pharmacological properties of rat brain A-type potassium channels was derived (17).

IV- The use of the oocyte for specific mRNA bioassays has proved to be an invaluable asset to molecular cloning efforts. This tool, for the relatively rapid screening of RNA preparation, facilitates the selection of a clone from a cDNA library (fig. 1.1).

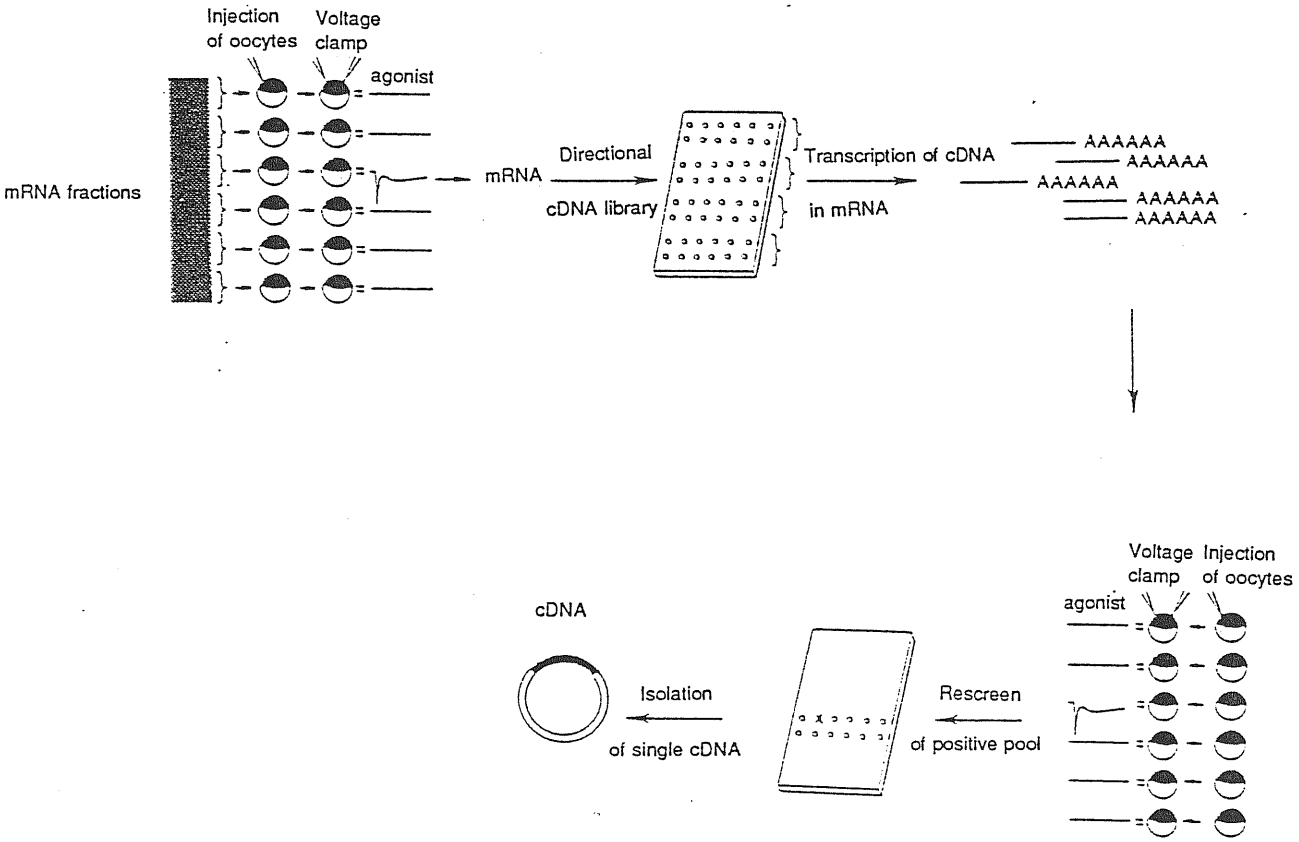

Fig. 1.1: Schematic diagram of the procedure used to select a clone from a cDNA library (adapted from ref.18).

The poly(A)$^+$RNA can be fractionated and by injecting separately in the oocytes the different fractions, it is possible to asses which fraction or fractions are needed to reconstitute the functional receptor (18). The selected fractions can be utilized to prepare a cDNA library, The latter can be screened by segregating groups of sequences, then again injecting in the oocytes the mRNAs transcribed from the different groups of sequences. The presence in an oocyte of the fully functional receptor is then the signal that the corresponding group of cDNAs contains the gene of interest. By iterating the procedure after subdivision of the positive group cDNA sequences, it is possible to arrive to the isolation of the channel's clone. Examples of application of this method are the cloning of the serotonin receptor (19), the substance K receptor (20) and thyrotropin receptor (21) in which oocyte expression provided the biological confirmation of a sequence tentatively identified by strong circumstantial evidence.

It is clear that *Xenopus* oocyte microinjection is currently providing the basis for the molecular cloning of non abundant genes for which non comparable screening paradigm is available.

1.1.2 Structure-function studies of cloned genes

Once a gene is cloned, engineered modifications in the coding sequence may allow detailed analyses of the contribution of specific polypeptide regions or even single amino acids to the biological activity

of the encoded gene products. In an early study, controlled deletions introduced into various regions of the cDNA encoding the α subunit of the nicotinic acetylcholine receptor were monitored for their effects on the induced acetylcholine response in microinjected oocytes (22). Site-directed mutagenesis of specific amino acids has subsequently been shown, via oocyte microinjection, to confer TTX sensitivity and STX insensitivity to the rat sodium channel II (23), to convert ion selectivity from cationic to anionic to the channel of the neuronal nicotinic acetylcholine receptor (24), to confer calcium channel characteristics to the sodium channel (25).

In conclusion a combination of single amino acid substitution, deletions mutagenesis and electrophisiological recordings facilitate a detailed structural analysis of the components of several channels and receptors.

1.1.3 Studies of biosyntetic pathways

The biosyntetic pathways leading to the expression of proteins in oocytes has been studied with regard to various post-translational processing events.

Using tunicamycin as a block to N-glycosilation (addition of an oligosaccharide group to an asparagine residue in a protein through a glycosidic bond), the effect of N-linked glycosilation on the expression of various receptors and channels from rat brain or chick

optic lobe poly(A)$^+$RNA was studied (2). The results of that study indicated an unequal dependence on glycosilation among different receptors. Sumikawa and Miledi (3) followed up this work by demonstrating that although N-glycosilation is not necessary for the assembly of heterologous Torpedo nicotinic acetylcholine receptors subunits, it appears to be a prerequisite for their efficient implantation into the plasma membrane.

1.1.4 Studies of regulation of gene expression

Nuclear injection of promoter-containing plasmids offers another approach to the study of transcription mechanisms in oocytes. Regulatory processes, such as transcription termination and RNA capping, polyadenilation and transport are also been succesfully approached using oocytes.

1.2 Experimental strategies

1.2.1 mRNA injection

I- Total poly(A)$^+$RNA. The injection of tissue extracted mRNA may be useful to achieve the expression of yet unisolated genes or to supplie unspecified or unknown accessory protein factors to a defined

expression system by coinjection of a cloned cDNA or synthetic mRNA.

II- Size-fractionated mRNA. The microinjection of size-fractionated poly(A)$^+$RNA serves primarily as an assay to monitor the enrichment of specific mRNA species and estimate their size. It may also provide information regarding the expected molecular weight of the encoded polypeptide, the existence of multiple mRNAs encoding polypeptides with similar activities, or the requirement for multiple non homologous components in the reconstitution of native activities.

III- Synthetic mRNA. The microinjection of synthetic mRNA transcripted from a cloned DNA allows much higher levels of expression and permits detailed analysis of a specific RNA transcripts and its protein derivatives.

1.2.2 DNA expression vectors

Nuclear microinjection of DNA expression plasmids containing coding sequences inserted downstream from an efficient eukariotic promoter may bypass the requirement for in vitro transcription of RNA. The mRNA which is succesfully produced in ovo should be better protected than its microinjected counterpart against nucleolytic degradation. This advantage, in combination with the prolonged transcription expected of the surviving vectors DNAs, facilitates production of the encoded polypeptide.

1.2.3 Coinjection

The successful isolation and expression of a cloned gene permits the subsequent coinjection of RNAs encoding specific or non specific heterologous factors capable of interacting with its gene product either to confer biological activity or to generate polymorphic catalytic or structural characteristics. Coinjection experiments may therefore be conducted with the various cloned subunits of an oligomeric molecule or with a cloned gene in conjunction with tissue-derived mRNA.

1.3 Oocyte biology

1.3.1 Structural features

An oocyte is a cell which remains, in vivo, in intimate physical contact with surrounding cellular and non cellular components. Ultrastructural analysis of oocyte sections reveals the vitelline membrane, follicle cells, theca and epithelium encompassing the oocyte (26) (fig.1.2).

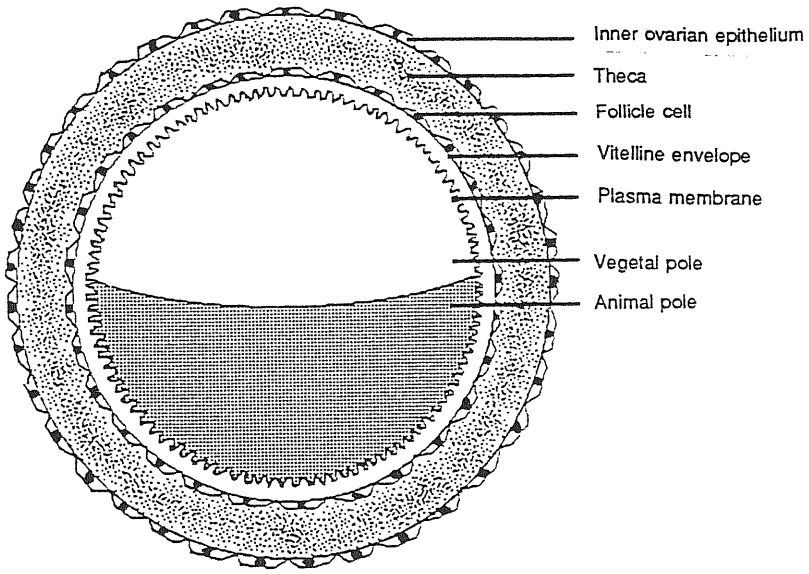

Fig. 1.2: Schematic diagram of *Xenopus laevis* oocyte and surrounding tissues. The oocyte, dissected from the ovarian envelope, is surrounded by a number of cellular and non-cellular layers. The oocyte plasma membrane is covered by a non-cellular, fibrous mat called the vitelline membrane. Several additional cellular layers flank the vitelline membrane. These include: a monolayer of follicle cells; the theca, which is a fibrous layer containing blood vessels, nerve and fibroblasts; and an inner ovarian epithelium, continuation of the ovary wall (adapted from ref. 18).

Micro- and macrovilli maintain important gap-junction connections between the oocyte and its enveloping follicle cells, providing chemical and electrical communication pathways (27).

Although at least some of the endogenous neurotransmitter receptors activities observed in oocytes have been associated with the peripheral follicle cells, denuded oocytes apparently provide the

endogenous machinery for second-messenger-mediated transduction signals induced by agonist binding to heterologous receptors (5). An additional feature of the *Xenopus* oocyte is its polarized nature. Curiously, its prominent black/white, animal/vegetal polar asymmetry, reflects a general subcellular hemispheric polarization which extends to characteristics such as the distribution of yolk platelets (28), maternal mRNA (29) and membrane receptors channels (30), as well as general cytoskeletal organization (31). In certain instances, polarized transport of nascent heterologous proteins has also been observed (31).

1.3.2 Developmental considerations

The rate of protein synthesis in oocytes and the pool of elements participating in protein synthesis are regulated in various modes throughout oogenesis and egg formation and they may be further modified through the action of proteins synthesized from heterologous mRNAs.

Immature oocytes are germ line cells that have entered meiosis and are arrested in the first prophage stage. During the first prophage stage they develop very slowly, passing through six morphologically characterized stages (32). Native oocyte mRNAs accumulate early in oogenesis and remain at a constant, steady-state level for the remainder of oocyte development (33). In the first stages of the

oogenesis, most of the oocyte polyadenylated RNA is localized in the nucleus. From there it is transported to the cytoplasm where it may be detected in fully grown oocytes (34). Within the cytoplasm of stage VI oocytes, which are commonly used for microinjection, poly(A)$^+$RNA is most concentrated in the vegetal subcortical region (35). Nucleoplasmic transport and mRNA mobilization in the oocytes are prerequisite to the successful expression of injected DNA sequences. These processes depend on the cytoskeletal elements of the oocytes (36). The stability and translational efficiency of oocytes mRNAs are modulated by RNA-binding proteins which are also developmentally regulated (37). Altogether, the complex transformation occurring throughout oogenesis underscore the importance of carefully preselecting the individual oocytes to be injected. These should be as uniform as possible in size and general appearance to ensure a similar stage of development.

Chapter 2

Maintenance of *Xenopus laevis* and oocyte injection

Procedures used for the maintenance of *Xenopus laevis* and for the removal and injection of oocytes.

2.1 Maintenance of *Xenopus laevis*

2.1.1 Environment for frogs

Xenopus laevis is a South Africa clawed frog. It is aerobic but entirely aquatic, so there is an absolute requirement for breathing air but not necessity for any land-based existence.

Twelve *Xenopus laevis* adult females were kept in a fish tank of 25x45x25 cm containing 17 l of water with a continuous tap water flow. The frogs were maintained in a constant light-dark cycle of 12 hours each. This helps to prevent the seasonal variability found with *Xenopus laevis.*

2.1.2 Feeding

The frogs were fed with granulated trout feed twice a week (5-10 g of food per frog).

2.2 Preparation of oocytes

2.2.1 Anesthesia

Frogs were anesthetized by keeping them for 15-30 minutes in water and ice at 4°C, after which the extent of anesthesia was tested. One test was to see if the frog could right itself after being placed upside down. Following anesthesia and surgical removal of the ovaries, the frog was allowed to recover in a small container of water. When the frog was completely recovered it was returned to the big tank.

2.2.2 Removal of oocytes

For the study of expression of membrane proteins, immature oocytes are needed. They were surgically extracted from frogs ovary. When the frog was fully anesthetized, it was placed on its back on ice and partially covered with ice. A small abdominal incision, about 1 cm long, stretching diagonally from lateral to medial toward the head was made (fig. 2.1).

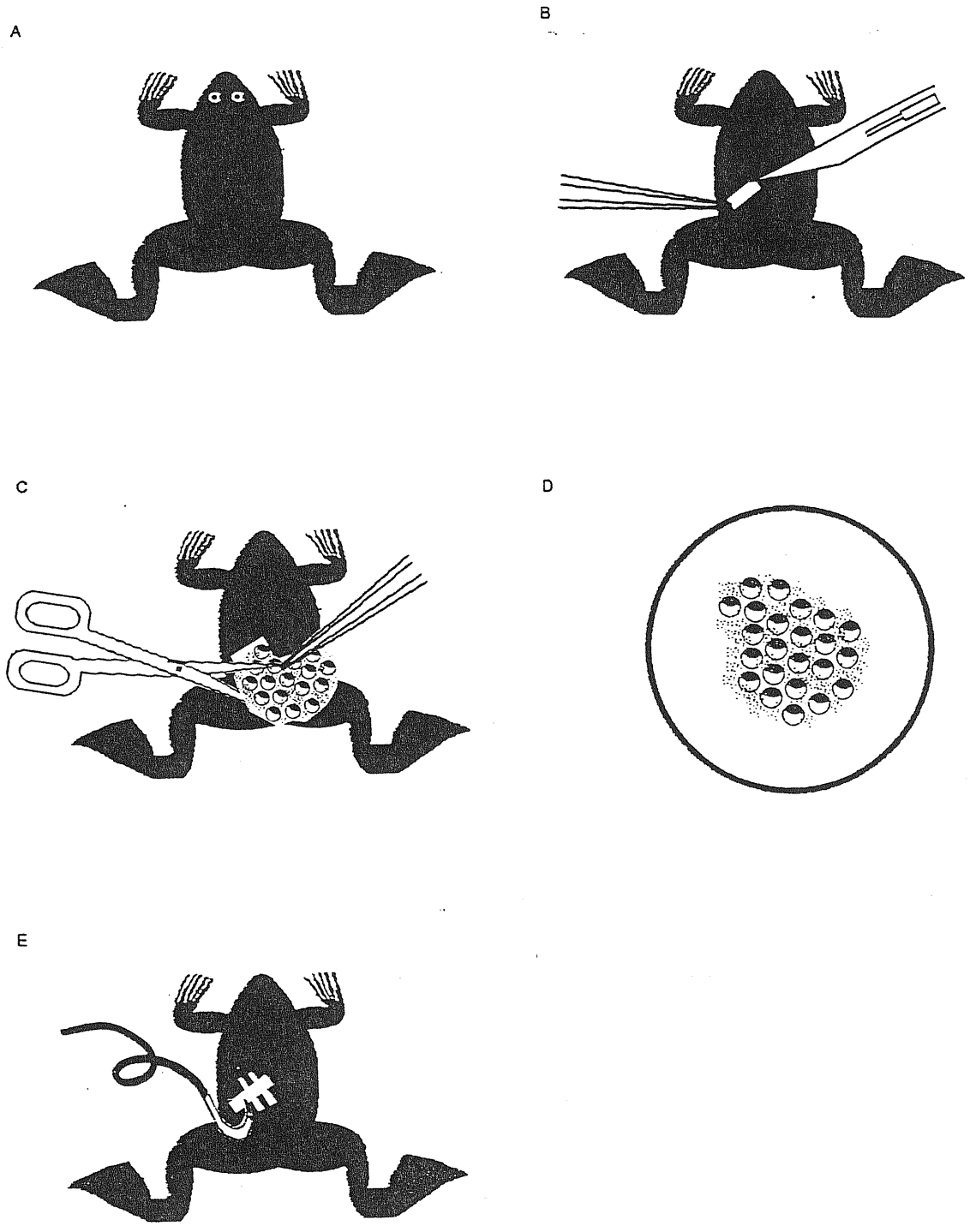

Fig. 2.1: Oocytes removal from the frog. (A) anesthetized frog (B) incision of the abdomen (C) pulling out and cutting of ovarian lobes (D) ovarian lobes in a Barth's solution containing Petri dish (E) stiching up the cut.

Because the skin is very tough it was lifted with a forceps and a scalpel was used for incision. The underlying abdominal muscle was cut and the ovaries were visible. With a pair of forceps the lobes of the ovary were pulled out and using a pair of scissors they were cut. The dissected ovarian lobes were placed in a 30 mm sterile Petri dish containing Barth's solution. After the dissection of oocytes the remaining ovary was replaced into the abdomen with the forceps and the incisions in the muscle and in the skin were separately sutured with sterile silk. The entire operation was done under clean conditions, not necessarily sterile, because of the magainin antimicrobial peptides secreted from the frog's skin (38). The same frog was used to remove oocytes multiple times.

2.2.3 Removal of follicle cells

The ovarian lobes are small sacs of epithelial cells ruptured at same ends, containing the follicles (oocytes plus follicle cells). The follicle cells were removed before injection (fig. 2.2).

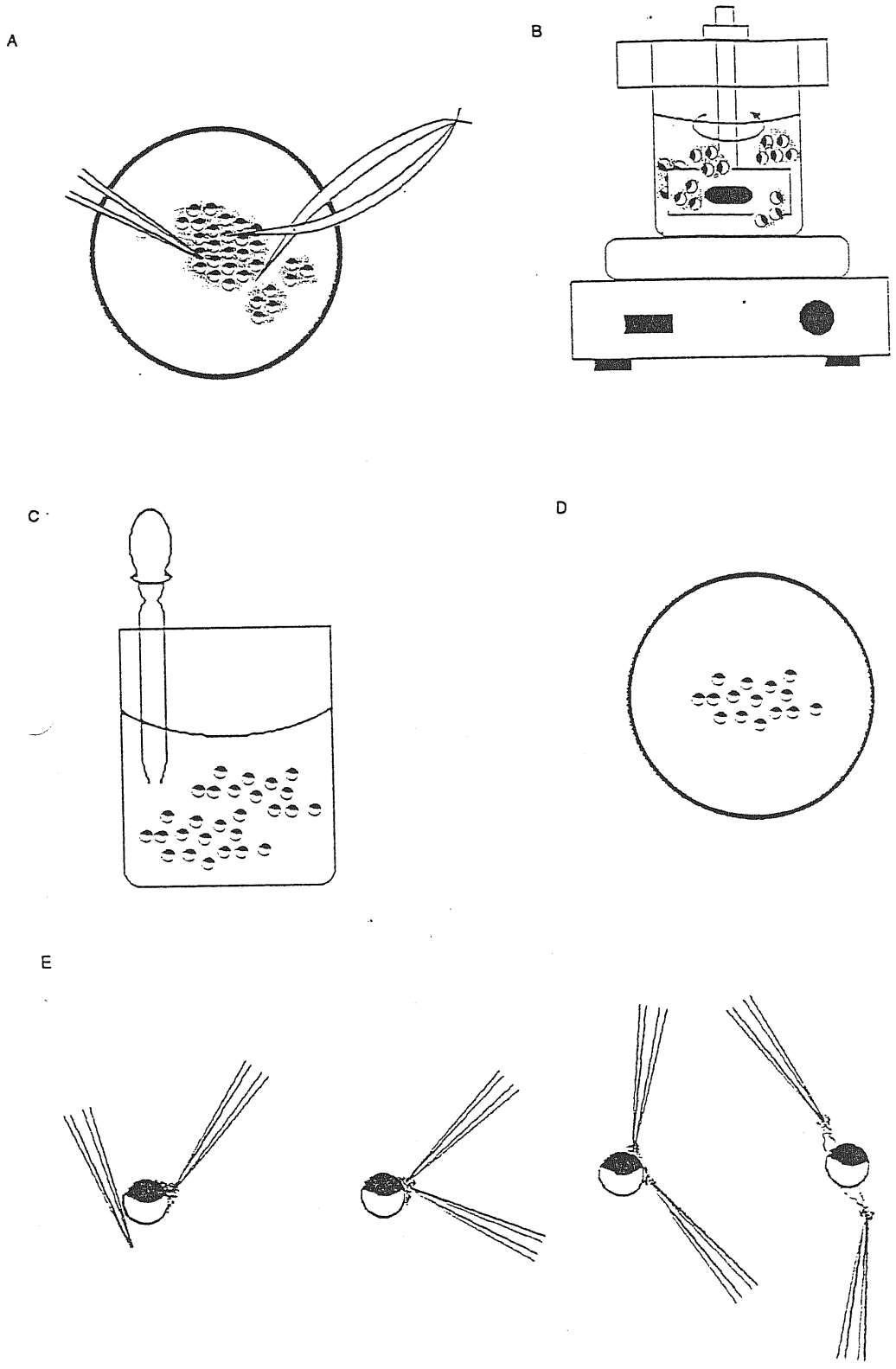

Fig. 2.2: Removal of follicle cells. (A) separation of groups of oocytes (B) incubation with collagenase (1 mg/ml) (C) washing with Barth's solution (D) separeted oocytes (E) removing of residual follicle cells with forceps.

The oocytes were separated in groups of 5-10 elements with iridectomy scissors and incubated for 60-90 minutes in Barth's solution with 1 mg/ml of collagenase (Sigma, type IA) at 20°C in a 10 ml glass beacker and continuously slowly mixed. The oocytes were handled with a wide-tipped Pasteur pipette whose tip was broken and fire polished. After the collagenase treatment, oocytes were washed extensively with Barth's solution and the residual follicle cells were removed manually using fine forceps, as shown in fig. 2.2..

2.2.4 Selection of oocytes for injection

The healthiest defolliculated oocytes were selected and transferred to a fresh dish in Barth's solution plus gentamicin 50 µg/ml (Sigma), to prevent bacterial contamination. After selection, the oocytes were maintained for a few hours or overnight before injection at 19°C, to allow any additional oocyte death to occur.

Oocytes from *Xenopus laevis* can be classified into six stages of development on the basis of anatomy (32):

-stage I: oocytes are about 50-100 µm in diameter and appear transparent

-stage II: oocytes are about 300-450 µm in diameter and appear either translucent or white depending on the extent of development

-stage III: oocytes are about 450-600 µm and can be distinguished by the appearance of pigmentation uniformly throughout

the surface

-stage IV: oocytes are 600-1000 µm with differentiated hemispheres and a very dark brown animal hemisphere

-stage V: oocytes are 1000-1200 µm with the hemispheres clearly delineated and a lightening in color of the animal hemisphere

-stage VI: oocytes are the most mature, 1200-1300 µm and can be distinguished by an impigmented equatorial band between the two hemispheres.

I have chosen stage III-IV oocytes for experiments on fast voltage gated ion channels, because the small size of these oocytes results in a faster voltage clamp of the membrane potential.

2.3 Injection of oocytes

2.3.1 Preparation of mRNA for injection

Tissue extracted mRNA was stored at -80°C in Na-acetate and ethanol. For injection it was centrifugated at 4°C in a refrigerating Eppendorf ultracentrifuge at 13000 x g for 15 minutes. The pellet was washed with absolute ethanol to remove the excess salt and again centrifugated. The mRNA pellet was resuspended at the concentration of 1 µg/µl in sterile diethylpyrocarbonate (DEPC)-treated water. Procedures for the preparation and handling of mRNA is given in section 3.

2.3.2 Cytoplasmic injection

mRNA injection in oocytes was performed with a Drummond "nanoinject variable" automatic injector (Drummond Scientific Co.,Broomall, PA, Cat. No. 3-00-203-XV) triggered by a foot pedal and attached to a three dimensional micromanipulator (Narishige, Tokio, Japan, type MX-4). The attachment was home-made. A scheme of the injection set-up is shown in fig. 2.3.

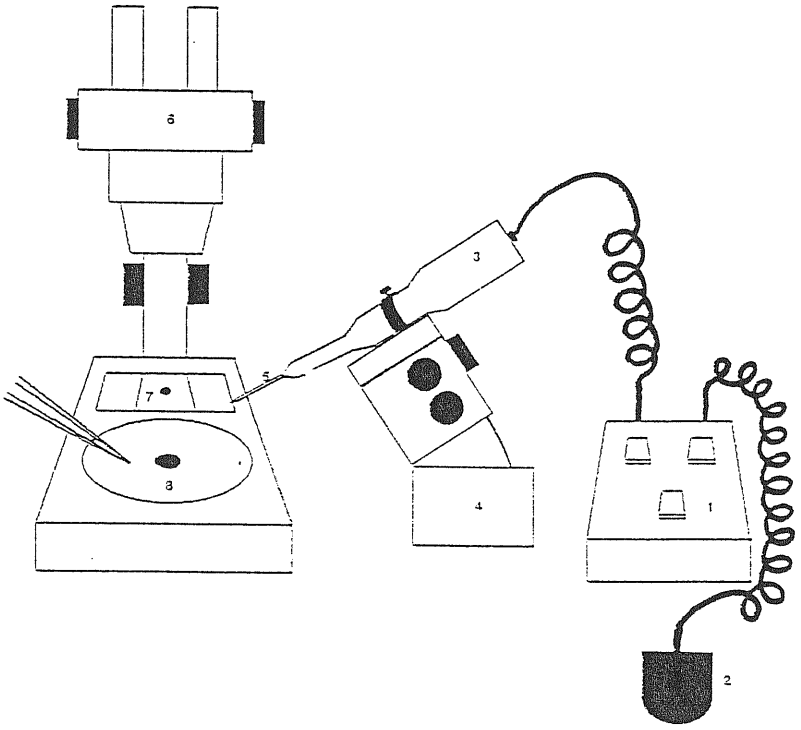

Fig. 2.3: Injection set-up. (1) Drummond automatic injector with (2) pedal control and (3) injector; (4) Narishige three dimensional micromanipulator; (5) injection neddle inserted in the injector; (6) binocular zoom stereomicroscope; (7) injection drop on Parafilm; (8) black depression slide.

Injection needles were made from Drummond glass capillaries by using a vertical pipette puller (List-Medical, 3P-A-List). After pulling, the needles were broken off at a tip diameter of 20-40 µm. The needles were back filled with light mineral oil and then inserted onto the injector. Before drawing the mRNA into the needle, the mRNA solution was centrifuged for 60 seconds at 3000 x g, to pellet debris. About 2µl of mRNA solution (1 µg/µl) were placed on a piece of Parafilm and the solution was sucked up into the pipette. This operation and the following ones were made under a binocular zoom stereomicroscope at low magnification (1 x 10-4 x 10) to ensure a wide field of view in order to control the progress of mRNA solution in the pipette during the injection.

The oocytes were placed on a black depression slide and well covered with Barth' s solution. The needle was inserted perpendicularly to the surface of the oocyte which was kept in place with forceps and held to the side opposite the needle. A volume of 50,6 nl of mRNA solution was injected and the needle was retracted straight without lateral movement in order to avoid damages to the oocyte. Standard procedures to avoid and inhibit RNAases were used such as using backed glassware, wearing sterile gloves at all stages and using DEPC-trated water.

2.3.3 Incubation of oocytes

After injection the oocytes were transferred in to microtiter plates (one oocyte/200 µl of Barth's solution with gentamicin (50 µg/µl)) at 19°C. Functional channels and receptors were detectable 2 days after the injection of mRNA.

Barth' s solution contained (in mM) NaCl: 88; KCl: 1; $MgSO_4$: 0.82; $Ca(NO_3)_2$: 0.33; $CaCl_2$: 0.41; $NaHCO_3$: 2.4; Tris-HCl: 5 (pH was adjusted to 7.4 with concentrated HCl).

Chapter 3

Preparation of mRNA for injection into *Xenopus* oocytes

There are some critical considerations in preparing the mRNA. The first and the most obvious is that the mRNA must be highly intact and essentially unmodified if it is to be translatable. The second is that the mRNA must be clean, since the oocytes can be poisoned by substances such as detergents, phenol and ethanol. Kits for the isolation of mRNA can be used. In a kit all reagents are absolutely RNAases free and the protocol is faster, easier and reliable. Before to describe the kit's procedure an introduction about the precautions used to avoid problems with RNAases, which are the main problem in working with RNA, is done.

3.1 Precautions against RNAases activity

Disposable latex sterile gloves were wore during all stages of preparation of reagents and supplies, and during isolation of mRNA,

up to and including oocytes injection. If glassware was used, it was baked at >210°C for ≥4 hr before use, the plasticware was sterile and disposable. A separate set of chemicals and tubes was reserved for the preparation of RNA. For handling chemicals only baked spatulas were used. Water was treated with 0.01% (v/v) of diethyl pyrocarbonate (DEPC, Sigma), shaken thoroughly and left overnight at room temperature, then autoclaved to destroy remaining DEPC (39).

3.2 mRNA isolation with Fast Track system

The Fast Track mRNA isolation kit (Invitrogen, San Diego, CA) was used to extract mRNA from the brain of adult rats. This kit allows isolation of poly(A)$^+$RNA directly from tissues. Briefly, 1 g of brain tissue from 30-40 days old rats was lysed in SDS buffer containing RNAase-protein degrader, incubated at 45°C and applied directly to oligo d(T)-cellulose for adsorption. The DNA and dissolved membranes, proteins and cell debris were washed off with a low salt buffer, and the mRNA was then eluted in the absence of salt. The mRNA was precipitated with 0.1 volumes of 3 M sodium-acetate and 2.5 volumes of cold absolute ethanol, stored overnight at -20°C, centrifuged at 4°C in a microcentrifuge for 15 min. The supernatant was discharged. The pellet was washed once with cold 80% ethanol

(made with DEPC-treated water) and resuspended in DEPC-treated water.

3.3 Determination of mRNA yield, purity and quality

To determine the mRNA yield the mRNA solution was diluted 50 fold by adding 10 µl of sample to 490 µl of water. To blank the spectrophotometer water was used. The diluted sample was placed into a silica cuvette, that have been soaked for 1 hr in concentrated HCl:methanol (1:1) and then washed extensively in DEPC-treated water, and the absorbance at 260 and 280 nm was read. The OD_{260}/OD_{280} ratio, which reflects the degree of contamination by proteins, should be about 2.0. An OD_{260} of 1 corresponds to approximately 40 µg/ml RNA. About 60 µg of mRNA were recovered with Fast Track mRNA isolation kit from 1 g of tissue.

In order to control the mRNA quality, 1 µg of sample was electrophoretically run on a 1.5% agarose gel in 0.5% TBE (5xTBE solution contained in g/l Tris-Base: 54; boric acid: 27.5; 20 ml EDTA 0.5 M pH=8.0 and H_2O to 1 l) and stained with ethidium bromide. As control 1 µg of good quality liver mRNA was run. Ribosomial RNA bands and a smear around these bands were the parameters for a good preparation. An example of mRNA extracted from rat brain with

the kit is shown in fig. 3.1.

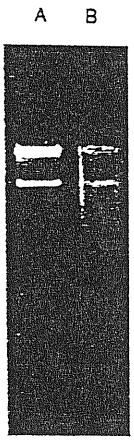

Fig. 3.1: Ethidium bromide stained gel of the mRNA extracted from rat brain with the kit. Lane A: rat liver mRNA. Lane B: rat brain mRNA.

3.4 Guanidine reprecipitation method and poly(A)$^+$RNA selection for preparation of mRNA

In spite of the good quality of the mRNA prepared with the kit, when it was injected in oocytes there was no detectable expression as monitored by two electrode voltage clamp tecnique. For this reason an alternative method was chosen. We used a modified version of the procedure described by Chirgwing and co-workers (40) devised by Cooperman and Mandel (7). This protocol involves repeated

reprecipitation of RNA from guanidine-containing solutions.

Reagents were treated with DEPC, with the exception of Tris with which DEPC reacts and sodium dodecyl sulfate which cannot be autoclaved. Corex tubes (30 ml) were rinsed thoroughly with distilled water. After drying, the tubes were siliconized by brief filling with Sigmacote (Sigma). After air drying, the tubes were soaked overnight in 0.1% DEPC. After this, the DEPC-containing water was poured off and the tubes were autoclaved.

3.4.1 Solutions for total RNA extraction

DEPC-treated water: DEPC 0.01% (v/v) was added to bidistilled water; the solution was left overnight at room temperature and autoclaved.

Sodium citrate (1 M, pH 7.0 with citric acid): DEPC 0.01% was added overnight and autoclaved.

Potassium acetate (2 M, pH 5.0 with acetic acid): DEPC treated and autoclaved.

Sodium acetate (3 M, pH 5.0 with acetic acid): DEPC treated and autoclaved.

EDTA (0.5 M, pH 8.0 with NaOH): DEPC treated and autoclaved.

NaCl (5 M): DEPC treated and autoclaved.

Guanidine thiocyanate homogenization solution (GTC): 50 g

guanidine thiocyanate; 2.5 ml 1 M sodium citrate, pH 7.0; 0.7 ml 2-mercaptoethanol. Final volume was 100 ml. The solution was heated to dissolve, then it was cooled to room temperature. pH was adjusted to 7.0 using NaOH. The solution was filtered through a 0.22 µm sterile, pyrogen free filter. 300 µl of antifoam A (Sigma) was added after filtration. The solution was stored in the dark at 4°C

Guanidine hydrochloride solution: 6 M guanidine hydrochloride was made up in DEPC water, filtered, autoclaved and stored at -20°C

Tris (1 M, pH 8.0 with HCl): it was made up with DEPC-treated water and autoclaved.

Tris (0.1 M, pH 8.0 with HCl): it was made up with DEPC-treated water. and autoclaved

Tris (1 M, pH 7.5 with HCl): it was made up with DEPC-treated water and autoclaved.

SDS (5 M): it was made up with DEPC-treated water and treated at 65°C for 1 hr.

Phenol / isoamyl alcohol / chloroform (PIC): redistilled molecular biology-grade phenol was warmed to room temperature, then heated at 60°C until it was melted. DEPC-treated water was added to saturate. The solution was equilibrated with 1 M Tris, pH 8.0, twice, then once with 0.1 M Tris, pH 8.0. If the pH of the solution was 7.5-8.0 the phenol was stored at -20°C in 1 ml aliquots, otherwise equilibration was continued. The PIC was done thawing an aliquot of phenol

solution and mixing with an equal volume of a solution made of 1 part isoamyl alcohol and 24 parts chloroform.

3.4.2 Extraction of total RNA

Tissue for RNA extraction was frozen in liquid nitrogen and kept at -80°C. The tissue was pulverized in liquid nitrogen. The fragments were quickly transferred to a 50 ml conical disposable polypropylene tube and any remaining liquid nitrogen was allowed to evaporate.

Alternatively fresh tissue was placed in the 50 ml tube. To the tube 16 ml of GTC was added and the tissue was homogenized using a homogenizer for the minimum time possible to disintegrate the tissue.

The solution was centrifuged at 10000 x g at room temperature for 10 min in a SS34 rotor in a Corex tube. The supernatant was poured into a fresh tube and the pellets were discharged.

The supernatant was added with 0.025 volumes of 1 M acetic acid (diluted in DEPC-treated water and with 0.75 volumes of absolute ethanol (at -20°C). The solution was mixed very thoroughly, and leaved at -20°C overnight.

The tube was centrifuged at 10000 x g at 4°C for 25 min. The supernatant was discharged. The following GH solution was made up: 9.5 ml of 6 M guanidine hydrochloride, 0.5 ml of 2 M potassium acetate and 0.5 ml of 0.5 M EDTA. The pellet was resuspended in a

volume of this solution equal to one-half of the original volume of GTC.

Using a 22-gauge needle and a 10 ml syringe, the solution was sheared by passing up and down 10 times in the syringe. 0.5 volumes of absolute ethanol (relative to the GH) were added. The solution was mixed, stored at -20°C for at least 3 hr and spinned down as above.

The pellet was resuspended in a volume of GH solution equal to half that used in previous step. 0.5 volumes of absolute ethanol were added. Again the solution was mixed, stored at -20°C for at least 3 hr and centrifugated. The pellet was resuspended in 490 µl of the following solution: 10 µl of 1 M Tris, pH 7.5, 10 µl of 0.5 M EDTA, 10 µl of 10% SDS and 460 µl of DEPC-treated water. The tube was warmed to 60°C for a couple of minutes to get the pellet into solution. The solution was transferred in a sterile Eppendorf tube and added with 10 µl of 5 M NaCl, with 500 µl of PIC and vortexed. It was left at room temperature for 2 min, vortexed again and spinned for 2 min in a microfuge at room temperature. The top aqueous layer was saved.

The RNA was precipitated as described in section 3.2.

3.4.3 Poly(A)$^+$RNA selection

A standard procedure was used for the isolation of mRNA from the total RNA (39). 0.5 g of oligo(dT)-cellulose were suspended in 1x sterile column loading buffer (20 mM Tris pH 7.5, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS). 500 µl of oligo (dT)-cellulose were put in a pipette

tip, plugged with sterile glass wool, treated with DEPC and autoclaved.

The column was washed with 3 column volumes of DEPC-treated water, with 3 volumes of 0.1 M NaOH, 5 mM EDTA and with DEPC-treated water until the pH of the effluent is less than 8.0. The column was then washed with 5 volumes of 1x loading buffer. The RNA was dissolved in DEPC-treated water and heated to 65°C for 5 min (heating the RNA disrupts regions of secondary structure that might involve the poly(A)$^+$ tail). The RNA solution was cooled quickly at room temperature and an equal amount of 2x loading buffer was added. The solution was applied to the column and the eluate was immediately collected in a sterile tube. The collected eluate was heated to 65°C for 5 min and reapplied to the top of the column. The column was washed with 5 volumes of 1x loading buffer and then with 5 volumes of 1x loading buffer plus 0.1 M NaCl. The poly(A)$^+$RNA was eluted from the oligo(dT)-cellulose with 2-3 volumes of sterile elution buffer (10 mM Tris pH 7.5, 1 mM EDTA, 0.05% SDS). The poly(A)$^+$RNA eluted was precipitated as described in section 3.2. The mRNA yield, purity and quality were determined as described in section 3.3. About 38 μg of mRNA were obtained from 1 g of tissue. An example of mRNA extracted from rat brain with this method is shown in fig. 3.2.

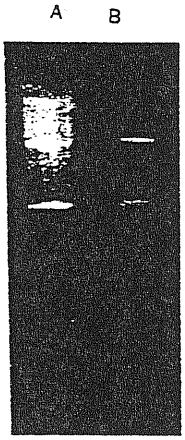

Fig. 3.2: Ethidium bromide stained gel of the mRNA extracted from rat brain with guanidine reprecipitation method and poly(A)$^+$RNA selection. Lane A: rat brain mRNA. Lane B: rat liver mRNA.

The mRNA extracted with this method after injection in *Xenopus* oocytes expressed both voltage and ligand gated channels as shown by the presence of exogenous currents (see next section).

3.5 Mixed method for mRNA isolation

We asked ourselves why the easier, faster and more efficient mRNA isolation method produced an mRNA that the oocytes were not able to translate. One possibility was that the resultant mRNA was complexed with proteins because the quality of this mRNA was good and one of the differences between the two methods was the phenol-chloroform extraction of RNA which was absent in the kit. For these

reasons the mRNA obtained with the Fast Track kit was used for a phenol-chloroform extraction as described in the last part of the section 3.4.2.

The mRNA isolated in this way was of good quality and able to induced exogenous currents in *Xenopus* oocytes.

Chapter 4

Expression of voltage gated channels in mRNA injected *Xenopus* oocytes

4.1 Two microelectrodes voltage clamp technique

The two microelectrodes voltage clamp tecnique has been generally applied to study membrane currents in *Xenopus* oocytes (41,42). For its spherical symmetry, in fact the oocytes does not present space clamp problems.

This method uses one intracellular electrode for the registration of the actual intracellular potential and one for passing the current in such a way as to maintain the membrane at the desidered holding potential. This is achieved using a feedback circuit, which is the main component of the voltage clamp system. The current needed to maintain the cell at a given potential is the measured parameter (43,44).

The current electrode clamp system used was Tec 01C amplifier (NPI Electronic GmbH, Germany). With this device the

current is measured as the current flowing through the current electrode. The intracellular potential recording electrode has such a high impedance that no current flows through it. The voltage clamp set-up uses also a potential recording electrode (reference electrode) for the bath solution. This avoids polarization errors which arise from current passing through the ground electrode. In this configuration, the transmembrane potential is taken as the difference between the intracellular potential electrode and the reference electrode (intracellular minus extracellular potential). Therefore, the small error introduced because the bath is not always exactly at ground potential, is corrected for. A schematic diagram of the voltage clamp recording apparatus is given in fig 4.1 (45).

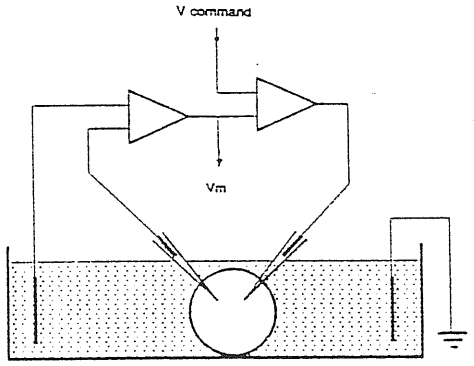

Fig. 4.1: Schematic diagram of the main component of the two electrodes voltage clamp. The output of the left amplifier-comparator (Vm) is the difference in potential between the bath and the oocyte electrodes. Vm is compared with the command potential, and any difference is injected into the oocyte through the current electrode. If Vm equals the command potential, no current is injected (from ref. 45).

The two electrode voltage clamp technique used in oocytes has developed in accordance with techniques used to measure membrane currents from small cells (43,44). The larger size of oocytes and their higher membrane capacitance, preclude exact measurements of fast occurring events. In fact the time constant for charging the membrane and so the speed of the voltage clamp depend on the electrode resistance through the relation $\tau=R \cdot C$, where R is the current electrode resistance and C is the cell capacitance. Therefore to study fast events, the cell capacitance must be reduced by using smaller oocytes (46).

4.2 Intracellular electrodes

The electrode pipettes were made from borosilicate glass capillary from Hilgemberg (Masfeld, Germany) type 1422037 (outer diameter: 2 mm; internal diameter: 1.6 mm) pulled with a two stage procedure by a vertical patch clamp puller (L/M-3P-A,List). The electrodes pipettes were back-filled with 3 M KCl. Usually the tip diameter was in the range of 1 to 5 μm and had a resistance of ≈ 1 MΩ. The electrical contact with the electrode filling solution was made through a silver wire that was chlorurated by passing current across it in a chloride containing solution. For long-term experiments, the electrode filling solution was of such an amount that the hydrostatic

pressure approximately compensates the pressure from the surface tension within the pipette. If the hydrostatic pressure in the electrode allows a large net outflow of solution, the oocytes will bulge around the site where the electrode is inserted as the oocytes accumulate KCl. If, on the contrary, there is a net inflow, the electrode resistance will increase as intracellular fluid enters the pipette. A very slight net outflow is the best choice, since this will stabilize diffusion potentials at the electrode tip.

4.3 Set-up for double electrode voltage clamp recordings

The main characteristics of the set-up were mechanical stability and electrical shield. Instruments were located on an antivibratory table that isolate the experimental set-up from mechanical vibrations. The surface of the antivibratory table was made of iron in order to fix magnetically the instruments (micromanipulators, microscope, etc.) and improved the shielding characteristics of the set-up. The set-up was covered by a Faraday cage, in order to minimize the electrical interference from the equipment located around it. The Faraday cage was mechanically isolated from the antivibratory table, thus it was supported from a second table without touching the antivibratory table. Furthermore in order to obtain an optimal shielding each single piece

of the set-up was connected to the ground. All connections to the ground were centralized in a single point, in order to avoid current loops between the instruments and the recording electrodes. The mechanical set-up used for double electrode voltage clamp recordings in oocytes is illustrated in fig. 4.2.

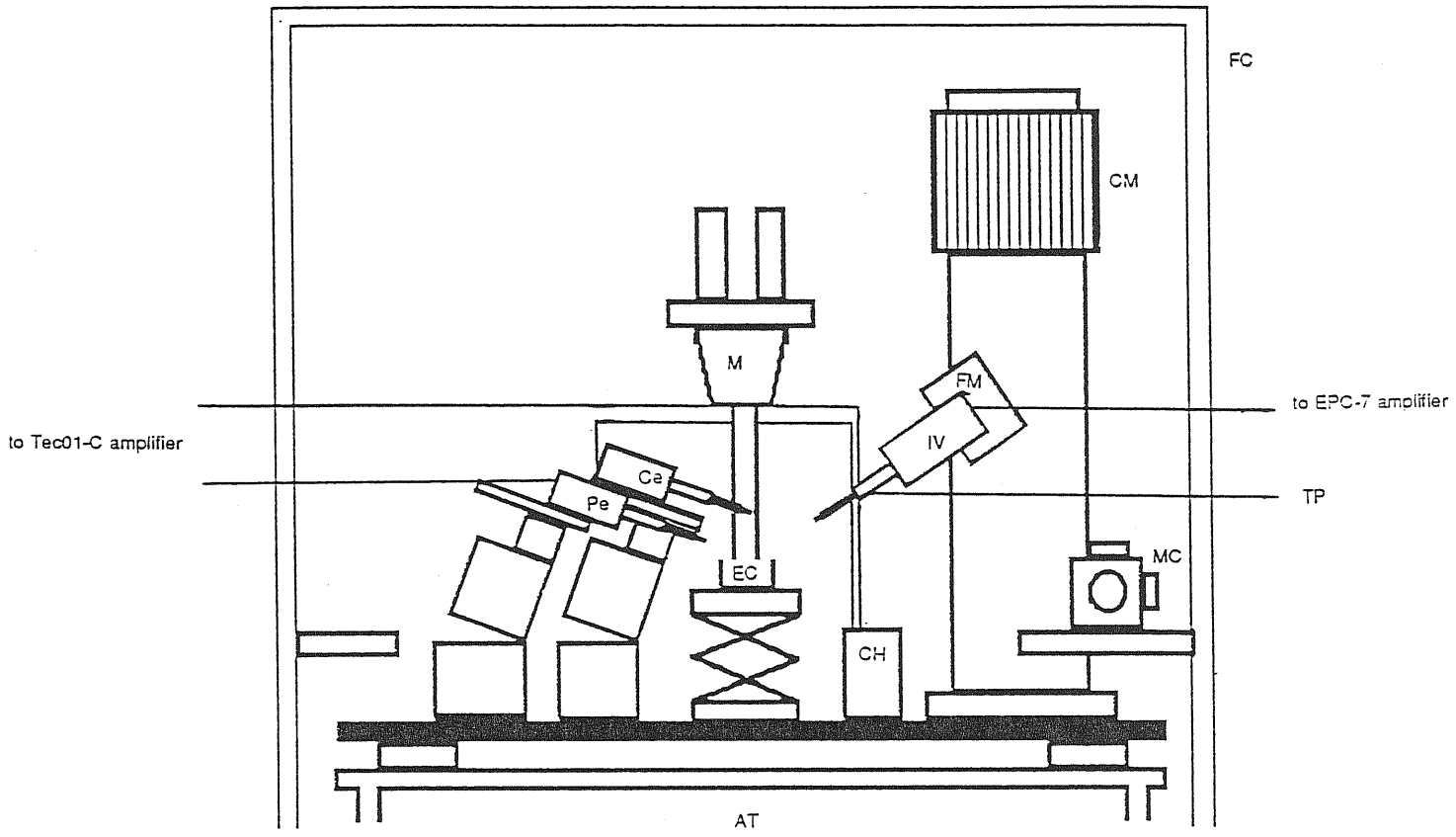

Fig. 4.2: Mechanical part of the set-up used for double electrode voltage clamp recordings (AT) antivibratory table (M) binocular zoom microscope (EC) experimental chamber (IV) current to voltage converter (Pe) potential electrode (Ce) current electrode (CH) current headstage (FM) fine micromanipulators (MC) control of micromanipulators (FC) Faraday cage (TP) tube to control the pipette pressure (CM) coarse micromanipulators.

A binocular zoom stereomicroscope (Zeiss, West Germany) was used to visualize the oocytes. The magnification used ranged from 8 to 64 x. The electrodes were positioned with coarse micromanipulators (Narishige, model MP-1, Japan) which were fixed to the antivibratory table.

Membrane currents were recorded with a standard two electrode voltage clamp amplifier (Tec 01-C, NPI) (see Fig. 4.3). The set up was coupled with a patch clamp amplifier (EPC-7, List Medical Instruments, FRG) for single channel recordings.

As shown in Fig. 4.3 the command voltage (Vcomm) for the Tec 01-C amplifier and for the EPC-7 amplifier was generated from channel 0 and 1, respectively, of a digital to analog converter (D/A) controlled by a computer. Vcomm was synchronized with the channel 3 of the D/A, in order to trigger (TRG) the oscilloscope (OSCILL). The EPC-7 amplifier, the Tec 01-C amplifier and the video tape recorder (VTR) membrane current output (Im) were connected with a home-made switch (SWITCH). The Im output of the switch was filtered (FILTER) and directly observed on the channel 1 (CH-1) of the oscilloscope, and also acquired by the channel 0 of an analog to digital converter (A/D). The Im output was also recorded, unfiltered, by the video tape recorder, via the pulse code modulator (PCM). An output signal of the membrane potential (Vm) from the EPC-7 amplifier, from the Tec 01-C amplifier and from the video tape recorder

was send to the switch and then acquired by the channel 1 of the A/D. Vm from the Tec 01-C amplifier was recorded in the VTR. Vm was directly observed on the channel 2 (CH-2) of the oscilloscope. Im and Vm from the oscilloscope were separately observed in the chart recorder (CHART REC) after choosing with a switch the one you want to observe.

The current signals were filtered at 1-10 kHz and recorded on the hard disk of a microcomputer (ATARI 1040ST). Voltage commands were given by the same microcomputer and a 16 bits digital analogue converter (M2-Lab, Instrutech, USA) using "Acquire program" written in Modula 2 by H.-Affolter (Instrutech, USA). Stored data were filtered with a low pass 8-pole Bessel filter (902LPF2, Frequency devices, Haverhill, Massachusetts) at a cut-off frequency of 1-10 kHz and then transferred to the microcomputer by a 16 bit analogue digital converter (M2-Lab, Instrutech). The sampling time for the fast occurring events was 10 kHZ. The current signal obtained during the experiment was continuously monitored on the oscilloscope (VC-6045, Hitachi, Japan).

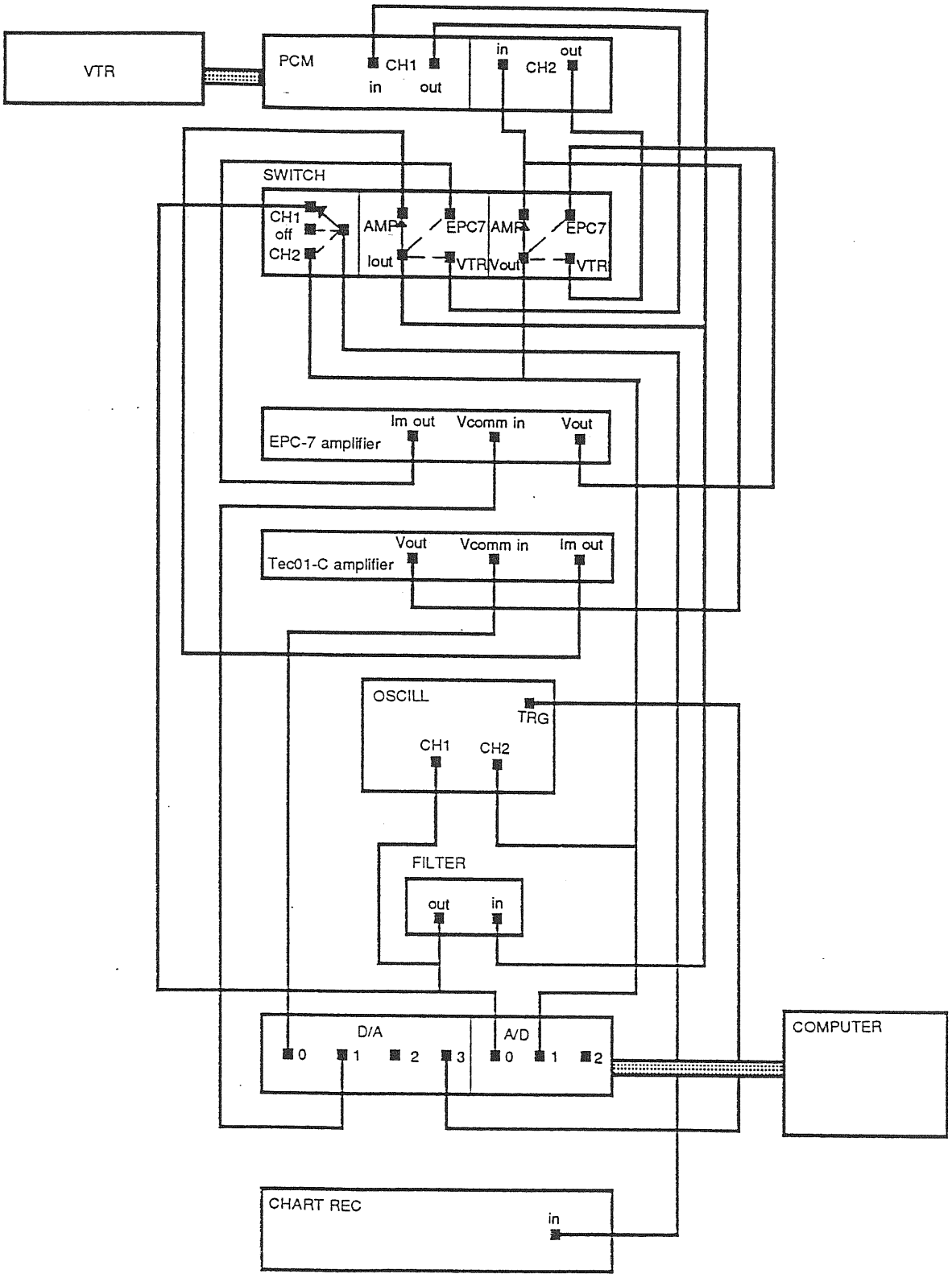

Fig. 4.3: Electronic part of the double electrode voltage clamp equipment.

Oocytes were kept in a recording chamber. The recording chamber was made from a 30 mm Petri dish modified as shown in fig. 4.4.

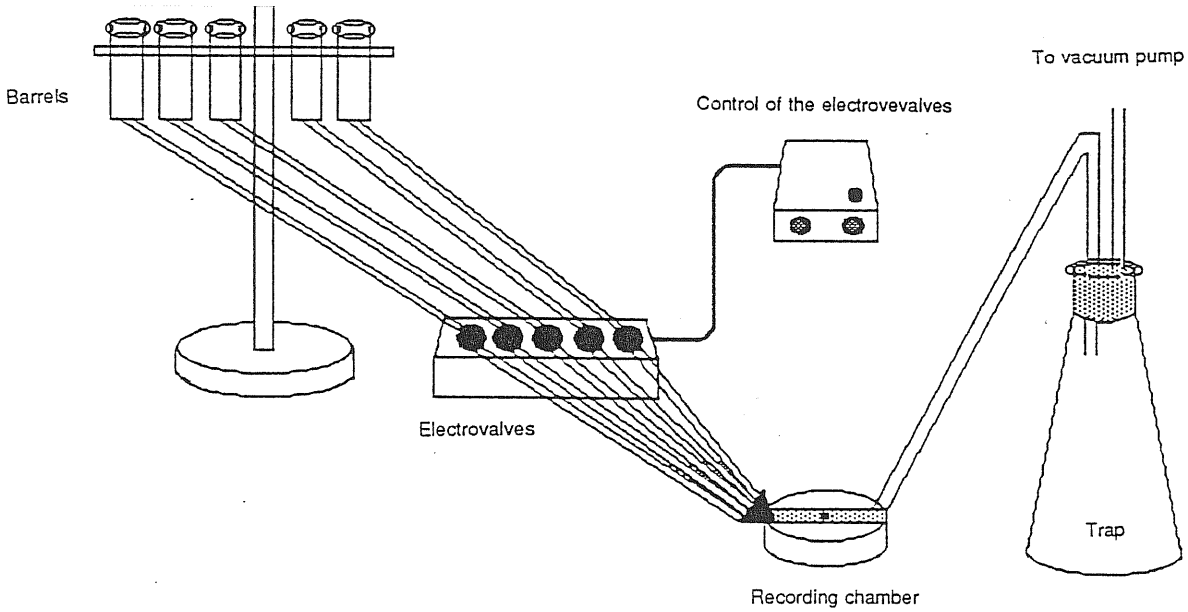

Fig. 4.4: Schematic diagram of the perfusion system.

Briefly, an insulin syringe was cut longitudinally in half and cut trasversally of the same length of the Petri dish diameter. It was placed in the dish and the two lateral compartments obtained were filled with Sylgard 186 (Dow Corning, Seneffe, Belgium). A small recess was formed with the tip of a soldering iron in the center of the syringe to maintain the oocyte in place. Oocyte was continuously superfused at a flow rate of 12 ml/min. Different solutions were applied

by gravity through 5 barrels. The barrels terminated in a Teflon tube (diameter 1.8 mm). When the Teflon tubes arrive to the chamber they were connected all together to a 3 mm diameter Teflon tube 1 cm long in order to obtain a solution flux larger than the oocyte diameter. The flow of solution through the barrels was controlled by 5 pinch solenoid electrovalves which were connected with an electrical device. This device allowed the flow of solution from only one barrel at any time. The small volume of the recording chamber (300 µl) allowed a complete exchange of the extracellular solution in 1.5 sec after the opening of the electronic valve. The outlet flow of the chamber was a teflon tube leading to a trap connected to a vacuum pump.

4.4 Voltage clamping the oocyte

All experiments were made in Normal Frog Ringer solution (in mM: NaCl: 115; KCl: 2.5;$CaCl_2$: 1.8; Hepes: 10; pH=7.2).

When the potential and the current electrodes were in the solution, the offset of the electrodes was setted to 0 mV. Then the electrodes were positioned with the manipulators as depicted in fig. 4.5.A.

The penetration of the potential electrode (fig. 4.5.B) was monitored in current clamp mode: this is associated to a shift in the membrane potential from 0 mV to -52 ± 9.7 mV (value ± standard

deviation). The penetration of the current electrode was achieved in voltage clamp mode giving a 20 mV depolarizing pulse from a holding potential of -80 mV. When the current electrode was in the bath the current consist of a very fast transient as result of the capacity and resistive component of the pipette. When the electrode was inside the cell the resistive component disappeared and only the membrane capacitance remained as shown in fig. 4.5.C.

The gain was increased slowly. Higher gain speeded up the time response of the voltage clamp but too much gain caused current oscillation. The capacitance transients were compensated with three components with different time constants. This compensation was done only when recording from fast, voltage activated channels.

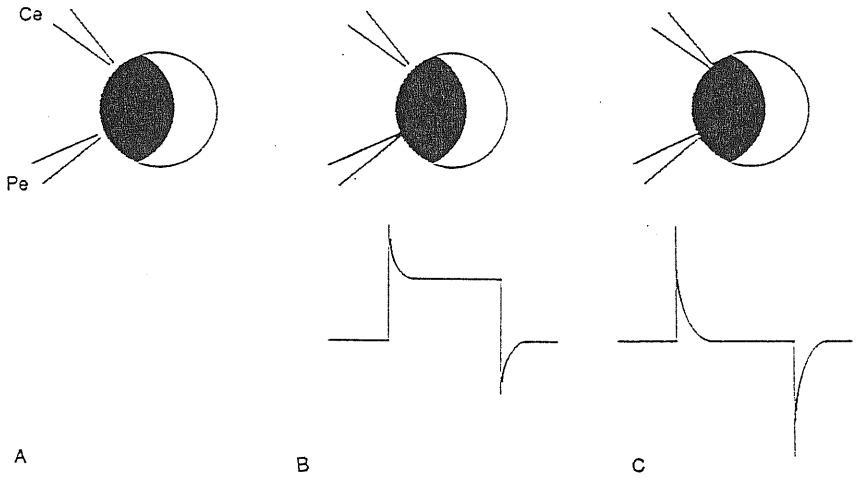

Fig. 4.5: (A) Top view of oocytes with the electrodes positioned for the penetration. (B) and (C) Current response to a 20 mV depolarizing step from a holding potential of -80 mV, before (top B) and after (top C) the penetration of the current electrode .

Chapter 5

Biophysical properties of voltage dependent sodium channels expressed in *Xenopus laevis* oocytes injected with whole rat brain or hippocampal mRNA

Voltage gated sodium channels are responsible for the fast depolarizing phase of the action potential in most electrically exitable cells (47). The physiological, pharmacological and molecular properties (48-49-50-51-52) of the sodium channels expressed in *Xenopus* oocytes injected with mRNA extracted from whole brain or with cloned cDNA have been extensively studied.

Few studies have been done in different structures of the brain. In the hippocampus, a very important structure for learning and memory, voltage gated sodium channels were studied in rat cultured hippocampal neurons (53) and in rat hippocampal astrocytes in vitro (54). The purpose of this study was to compare the biophysical properties of native voltage gated sodium channels from the whole brain to those from the hippocampus.

5.1 Sodium channels from rat brain

Sodium currents have been recorded from 19 oocytes previously (3-7 days) injected with 50 ng of mRNA extracted from whole brain (30 days old rats), using the two electrode voltage clamp technique (section 4.1). The oocytes to inject were choosen between the stage III-IV of development because their small size allowed a better control of the space clamp properties. In this way it has been possible to study fast voltage gated channels such as sodium channels.

5.1.1 Biophysical properties

5.1.1.1 Voltage dependence and reversal potential

As shown in fig. 5.1.A depolarizing voltage steps of 20 mV increment from a holding potential of -90 mV (40 ms duration), to 50 mV evoked fast inward currents. Pulses were delivered at 20 s interval. In Normal Frog Ringer extracellular solution, fast inward currents started to appear at ≈ -50 mV, then increased progressively in amplitude and decreased from ≈ 0 mV. The fast inward currents were almost null near 70 mV. These currents were completely blocked by 1 μM tetrodotoxin (TTX). All these properties suggest that the observed currents were TTX sensitive sodium currents. Current traces have been corrected for the linear leakage and capacitance transients,

using the P/4 procedure. Outward currents have been eliminated by subtracting records in 1 μM TTX. The plot of the peak current estimated from the current traces shown in fig. 5.1.A, as a function of the applied potential, is illustrated in fig. 5.1.B. The equilibrium potential for the sodium ions is given by the Nernst's equation: $E_{Na}=(R\bullet T/F)\bullet \ln([Na^+]_o/[Na^+]_i)$, where the $[Na^+]_o$ is the concentration of the extracellular sodium ions (= 115 mM) and the $[Na^+]_i$ is the concentration of the intracellular sodium ions (= 6.2 mM) (55). At 20°C the theoretical equilibrium potential for sodium ions is +73.8 mV. At membrane potentials near 70 mV, the fast inward currents were almost null. A real reversal potential was never achieved. This is probably due to the fact that the relatively rapid kinetics of the currents at positive voltages may be distorted by the time required to clamp the membrane potential. The plot of Fig. 5.1.B shows that the inward current is activated in the potential range of -50 and -40 mV and reaches a peak between -10 mV and 0 mV.

5.1.1.2 Activation and inactivation

In Fig. 5.1.C the currents during the step depolarizing pulse were normalized to the largest inward current and plotted versus the test potential. The curve is a fit to the data of the Boltzman equation: $I/Imax=1/(1+\exp((Vh-V)/k))$ where I/Imax = normalized current, V = pulse potential, Vh = voltage for half maximal activation and k is a

slope factor. Vh and k in this experiment were -25.45 ± 0.25 mV and 3.54 ± 0.18 respectively (value ± standard error). The steady state inactivation of sodium channels was obtained with a 40 ms test potential of 0 mV and the 500 ms prepulse ranged from -80 mV to 0 mV with a 1 ms interpulse of -100 mV. The holding potential was -100 mV. The inactivation started between -70 mV and -60 mV and it was complete at -20 mV. In fig. 5.1.C the currents during the test pulse were normalized as described before and plotted versus the prepulse potential. The curve is a fit to the data of the Boltzman equation: I/Imax=1/(1+exp((V-Vh)/k)) where Vh = half maximal inactivation voltage and k is a slope factor. Vh and k, in this experiment, were -44.81 ± 0.14 mV and 4.41 ± 0.12 respectively (value ± standard error).

5.1.2 Pharmacology

Sensitivity of sodium channels to TTX was assessed following exposures to different concentrations of the toxin. A TTX concentration of 1 μM completely blocked all inward currents. In Fig. 5.1.D the experiments results have been quantified and each point represent, at different TTX concentration, the peak current normalized to the current in the absence of TTX and plotted versus the Log of TTX concentration. The curve is a fit of the data to the Michaelis-Menten equation: I/Icontr=(1+([TTX]/IC_{50})^n)^-1 where I/Icontr is the normalized

peak current IC_{50} is the concentration of TTX that half the peak current and n is the Hill coefficient. The IC_{50} value was 73 ± 13.8 nM .The data points were obtained at a test pulse potential of 0 mV from an holding potential of -100 mV.

5.2 Sodium channel from rat hippocampus

Sodium currents have been recorded from 25 oocytes previously (3-7 days) injected with 50 ng of mRNA extracted from the hippocampus (30-40 days old rats), using the two electrode voltage clamp tecnique. The oocytes to inject were choosen in the developmental stages III-IV, in order to have a faster voltage clamp as explained in section 5.1.

5.2.1 Biophysical properties

5.2.1.1 Voltage dependence and reversal potential

As shown in fig. 5.2.A, depolarizing voltage steps of 20 mV increment (40 ms duration, 0.05 Hz) from a holding potential of -100 mV to 70 mV evoked fast inward currents. Fast inward currents began to rise at ≈ -50 mV reaching a maximun in amplitude at 0-10 mV in Normal Frog Ringer extracellular solution. Fast inward currents were almost null near 80 mV. 1 μM TTX completely blocked these currents. Therefore we can conclude that the fast inward currents observed in

the present experiments were, again, TTX sensitive sodium currents. Current traces have been corrected for the linear leakeage and capacitance transients, using the P/4 procedure. Currents have been eliminated by subtracting records in 1 μM TTX. The plot of the peak current as a function of the applied potential, is shown in fig. 5.2.B. The theoretical equilibrium potential for the sodium ions predicted by the Nernst's equation (section 5.1.1.1) is +73.8 mV. Also in this case, as shown in fig. 5.2.B it was impossible to reverse the current (see section 5.1.1.1). The current-voltage curve shows that the inward current is activated in the potential range from -50 mV to -40 mV and reaches a peak between 0 mV and 10 mV.

5.2.1.2 Activation and inactivation

In fig. 5.2.C, the normalized currents (respect to the largest inward current) during the step depolarizing pulse were plotted versus the test potential. The curve is a fit to the data of the Boltzman equation as described in section 5.1.1.2. The voltage for half maximal activation was -20.83 ± 0.49 mV and the slope factor was 6.82 ± 0.44 (value ± standard error). The steady state inactivation of sodium channels was obtained as described in section 5.1.1.2 but the prepulse ranged from -110 mV to -10 mV. The inactivation started between -100 mV and -90 mV and it was complete at -20 mV. In fig. 5.2.C the normalized currents were plotted versus the prepulse

potential. The data were fitted with the Boltzman equation obtaining the curve of the fig. 5.2.C. The half maximal inactivation voltage was -55.09 ± 1.10 mV and the slope factor was 12.93 ± 0.99 (value ± standard error).

5.2.2 Pharmacology

Sodium channels expressed in *Xenopus* oocytes injected with hippocampal mRNA were exposed to different concentrations of TTX in order to asses their sensitivity to the toxin. A concentration of 1 μM completely blocked all inward currents. In fig. 5.2.D the peak currents at different concentrations of TTX were normalized to the current in the absence of the toxin and plotted versus the Log of TTX concentration. The data points were fitted with the Michaelis-Menten equation as described in section 5.1.2 obtaining the curve of the graph. The IC_{50} value for TTX was 92.8 ± 18.7 nM (value ± standard error). The data points were obtained at a test pulse potential of 0 mV (40 ms duration) from a holding potential of -100 mV after subtraction from the traces of the current obtained in the presence of 1 μM TTX.

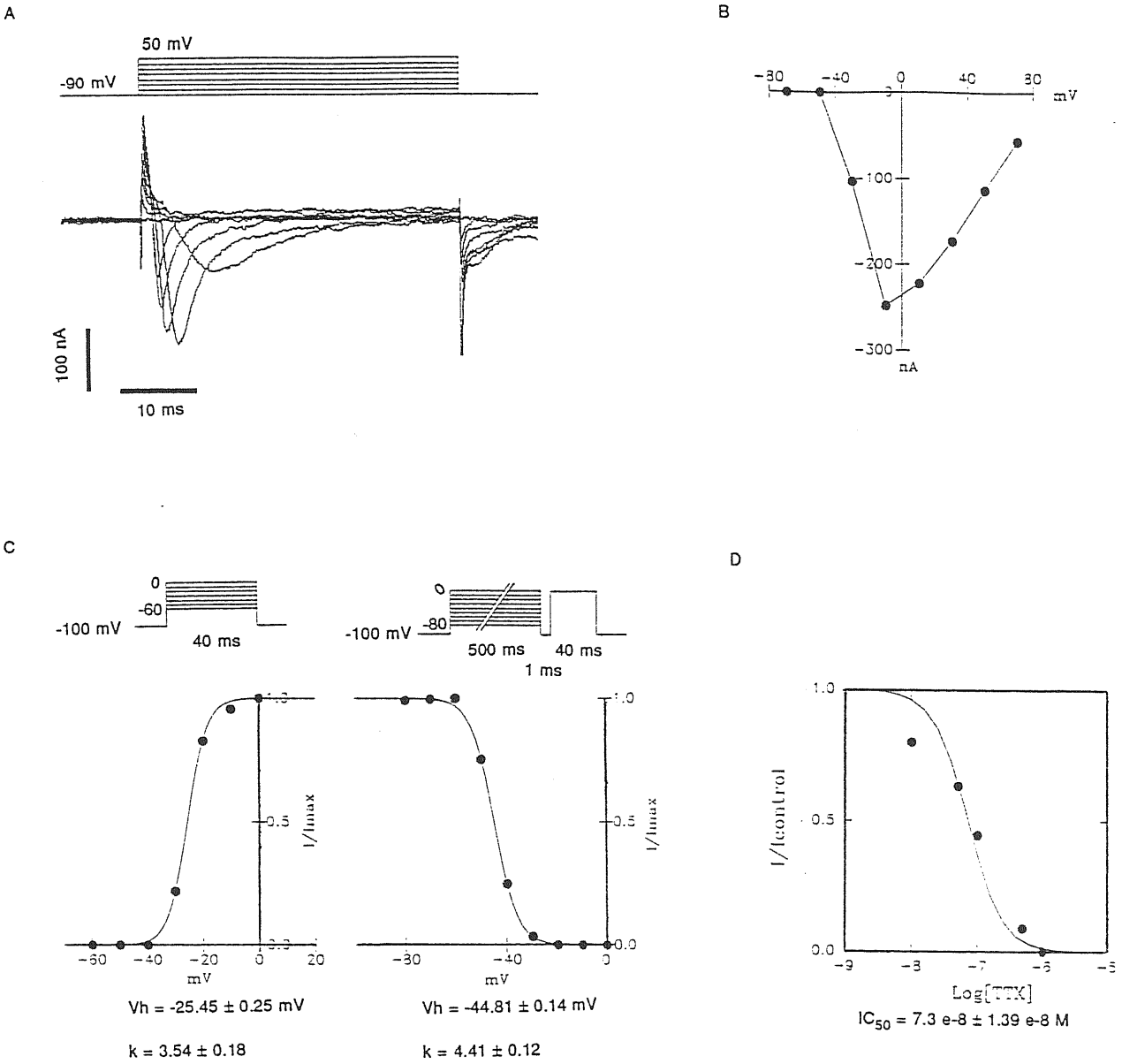

Fig. 5.1: (A) Depolarizing voltage steps (top A) evoked fast inward sodium currents in oocytes injected with rat whole brain mRNA (B) Plot of the peak current as a function of the applied potential, estimated from the current traces of fig. A (C) Activation (left) and steady state inactivation (right) curves for the sodium currents; the stimulation protocol is shown in the top of the figure (D) Sensitivity of sodium currents to TTX : plot of the normalized peak current as a function of the Log of TTX concentration. For the fitting methods see text.

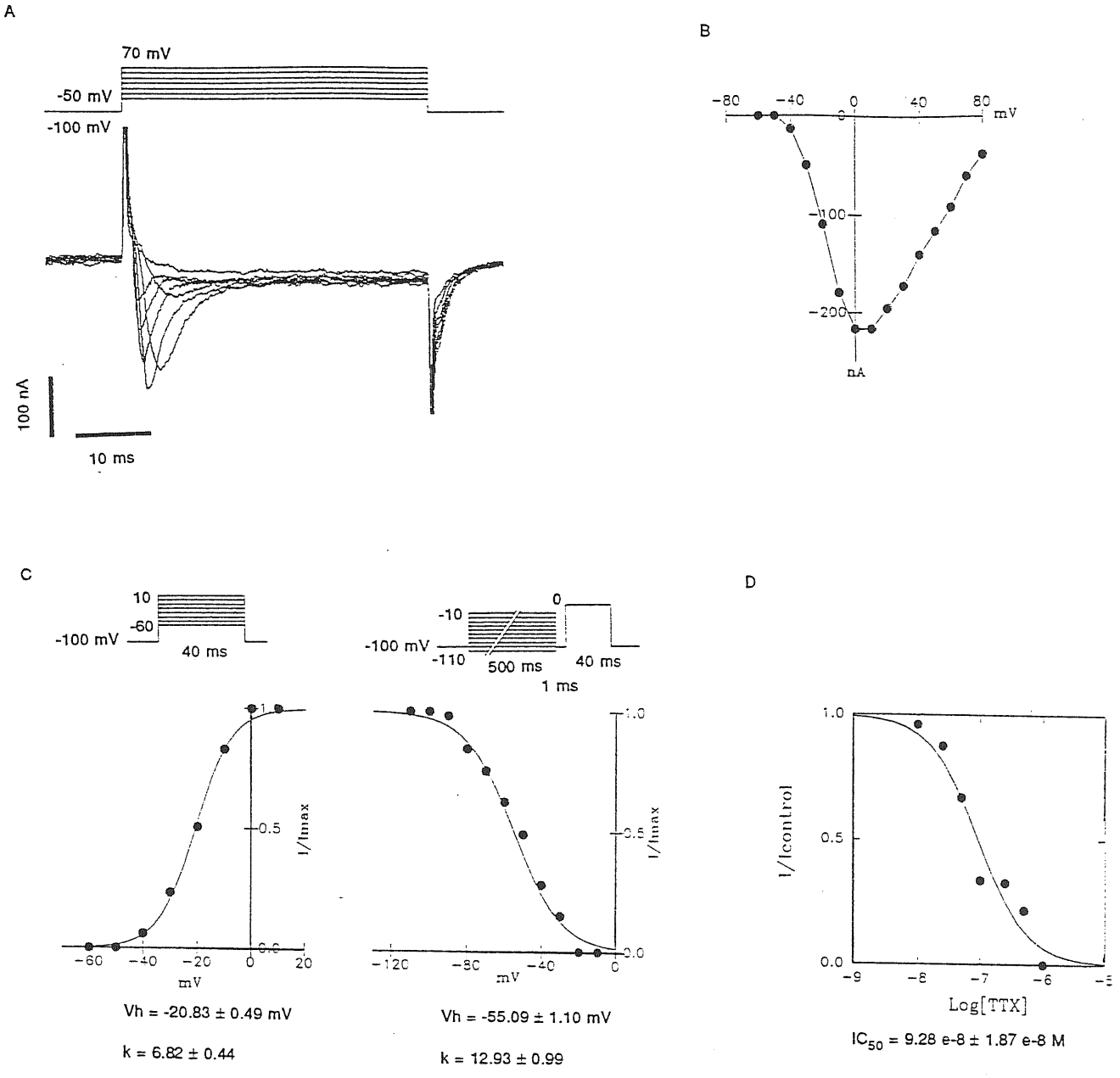

Fig. 5.2: (A) Depolarizing voltage steps (top A) evoked fast inward sodium currents in oocytes injected with rat hippocampal mRNA (B) Plot of the peak current as a function of the applied potential, estimated from the current traces of fig. A (C) Activation (left) and steady state inactivation (right) curves for the sodium currents; the stimulation protocol is shown in the top of the figure (D) Sensitivity of sodium currents to TTX : plot of the normalized peak current as a function of the Log of TTX concentration. For the fitting methods see text.

5.3 Discussion

The purpose of this work was to compare the biophysical and pharmacological properties of fast voltage gated sodium channels in *Xenopus* oocytes injected with total brain or hippocampus mRNA from adult rats.

We have found that in both cases the sodium currents have similar electrophysiological and pharmacological characteristics. In fact (1) both are activated in the potential range from -50 mV to -40 mV, (2) both reach a peak around 0 mV and (3) both inactivate completely at -20 mV. The sensitivity to TTX of sodium currents obtained with hippocampal mRNA is higher than those obtained by injection of whole brain mRNA. However these values are not significantly different.

It has been shown that many vertebrate neurons (56-57-58-59) express at least two types of sodium channels with different physiological and pharmacological properties. In particular TTX sensitive or insensitive sodium channels have been observed, the TTX insensitive channels having slower kinetics and different activation threshold. Developmental changes in the sensitivity to TTX were found to be correlated with cell size in rat dorsal root ganglion cells. The TTX sensitive sodium channels were mainly concentrated in larger diameter cells, whereas TTX insensitive sodium channels were found in smaller diameter cells (60). The same situation was found in sensory neurons

of adult frog dorsal root ganglia (61). In freshly dissociated ganglionic quail neurons TTX insensitive sodium currents were found to be present only after 12-13 days of embrionic life. (62).

Few studies concern the developmental changes in expression of sodium channels in brain and in particular in hippocampus, a very important structure for learning and memory, have been done. This will be the aim of future studies: to compare the biophysical and pharmacological properties of sodium currents expressed in *Xenopus* oocytes injected with neonatal or adult hippocampal mRNA using the same experimental approach.

A more advanced step will be to construct a cDNA library from the mRNA extracted from neonatal and adult rat hippocampus and to clone the sodium channels in order to compare sequences homology with already cloned sodium channels. Single point mutagenesis will then be performed in order to obtain sodium channel with permeation and gating properties similar to the native ones.

Aknowledgements

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References

[1] Gurdon, J.B., Lane, C.D., Woodland, H.R. and Marbaix, G. (1971) Use of frog eggs and oocytes for the study of messanger RNA and its translation in living cells. *Nature* **233**, 177-182

[2] Sumikawa, K., Parker, I. and Miledi, R. (1988) Effect of tunicamycin on the expression of functional brain neurotransmitter receptors and voltage-operated channels in *Xenopus* oocytes. *Mol. Brain Res.* **4**, 191-199

[3] Sumikawa, K., Miledi, R. (1989) Assembly and N-glycosilation of all Ach receptors subunits are required for their efficient insertion into plasma membranes. *Mol. Brain Res.* **5**, 183-192

[4] Sumikawa, K., Houghton, M., Emtage, J.S., Richards, B.M. and Barnard, E.A. (1981) Active multi subunit Ach receptor assembled by translation heterologus mRNA. *Nature* **292**, 862-864

[5] Miledi, R.,Parker, I. and Sumikawa, K. (1989) Transplanting receptors from brain into oocytes. In *Fidia Award Lecture Series* J. Smith ed., Raven Press, New York

[6] Sorek, H. and Seidman, S. (1992) *Xenopus* oocyte microinjection: from gene to protein. *Methods in enzimology* **207**, 225-265

[7] Boyle, M.B. and Kaczmarek, L.K. (1991) Electrophysiological expression of ion channels in *Xenopus* oocytes. In *Electrophysiology and microinjection* P.M. Conn ed. Academic press, **4**, 157-173

[8] Barnard, E.A., Miledi, R. and Sumikawa, K. (1982) Translation of exogenous messenger RNA coding for nicotinic acetylcholine receptor produces functional receptor in *Xenopus* oocytes. *Proc. R. Soc. London* **B215**, 241-246

[9] Gundersen, C.B., Miledi, R. and Parker, I. (1983) Voltage operated channels induced by foreign messenger RNA in *Xenopus* oocytes. *Proc. R. Soc. London* **B220**, 131-140

[10] Fakuda, K., Kubo, T., Akiba, I., Maeda, A., Mishina, M. and Numa, S. (1987) Molecular distinction between muscarinic acetylcholine receptor subtypes. *Nature* **327**, 623-625

[11] Frielle, T., Collins, S., Daniel, K.W., Caron, M.G., Lefkowitz, R.J. and Kobilka, B.K. (1987) Cloning of the cDNA for the human beta-1 adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **84**, 7920-7924

[12] Kobilka, B.K., Matsui, H., Kobilka, T.S., Yang-Feng, T.L., Francke, U., Caron, M.G., Lefkowitz, R.J. and Regan, J.W. (1987) Cloning, sequencing and expression of the gene coding for the human platelet alpha 2-adrenergic receptor. *Science* **238**, 650-656

[13] Deneris, E.S., Connolly, J., Boulter, J., Wada, E., Wada, K., Swanson, L.W., Patrick, J. and Heinemann, S. (1988) Primary structure and expression of the beta 2: a novel subunit of the neuronal nicotinic acetylcholine receptors. *Neuron* **1**, 45-54

[14] Stühmer, W., Giese, K.P., Perschke, A., Baumann, A. and Pongs, O. (1989) Molecular basis of functional diversity of voltage-gated potassium channels in mammalian brain. *EMBO J.* **8**, 3235-3244

[15] Wafford, K.A., Burnett, D.M., Dunwiddie, T.V. and Harris, R.A. (1990) Genetic differences in the ethanol sensitivity of GABAA receptors expressed in *Xenopus* oocytes. *Science* **249**, 291-293

[16] Krafte, D.S., Snutch, T.P., Leonard, J.P., Davidson, N. and Lester, H.A. (1988) Evidence for the involvment of more than one mRNA species in controlling the inactivation process of rat and rabbit brain Na channels expressed in *Xenopus* oocytes. *J. Neurosci.* **8**, 2859-2868

[17] Rudy, B., Hoger, J.H., Lester, H.A. and Davidson, N. (1988) At least two mRNA species contribute to the properties of rat brain A-type potassium channels expressed in *Xenopus* oocytes. *Neuron* **1**, 649-658

[18] Snutch, T.P. (1988) The use of *Xenopus* oocytes to probe synaptic communication. *TINS* **116**, 250-256

[19] Lubbert, H., Hoffman, B.J., Snutch, T.P., Van Dyke, T., Levin, A.Y., Hartig, P.R., Lester, M.A. and Davidson, N. (1987) cDna cloning of a serotonin 5-HT receptor by electrophysiological assays of mRNA injected *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **84**, 4332-4336

[20] Maru, Y., Nakayama, K., Tamaki, M., Marada, Y., Kuno, M. and Nakanishi, S. (1987) cDNA cloning of bovine substance-K receptor through oocyte expression system. *Nature* **329**, 836-838

[21] Parmantier, M., Libert, F., Maenhaut, C., Lefort, A., Gerard, C., Perret, J., Van Sande, J., Dumont, J.E. and Vassart, G. (1989) Molecular cloning of the thyrotropin receptor. *Science* **246**, 1620-1622

[22] Mishina, M., Tobimatsu, T., Imoto, T., Fujita, K.I., Fakuda, D., Durasaki, M., Takahashi, T., Morimoto, Y., Hirose, T., Inayama, S., Takahashi, H., Kuno, M. and Numa, S. (1985) Location of functional regions of acetylcholine receptor α-subunit by site-directed mutagenesis. *Nature* **313**, 364-369

[23] Noda, M., Suzuki, H., Numa, S. and Stühmer, W. (1989) A single point mutation confers tetrodotoxin sensitivity and saxitoxin insensitivity on the sodium channel III. *FEBS Lett.* **259**, 213-216

[24] Galzi, J.L., Devillers-Thiéry, A., Hussy, N., Bertrand, S., Changeux, J.P. and Bertrand, D. (1992) Mutations in the channel domain of a neuronal nicotinic receptor convert ion selectivity from cationic to anionic. *Nature* **359**, 500-505

[25] Heinemann, S.H., Terlau, H., Stühmer, W., Imoto, K. and Numa, S. (1992) Calcium channel characteristics conferred on the sodium channel by single mutations. *Nature* **356**, 441-443

[26] Dumont, J.N. and Brummet, A.R. (1978) Oogenesis in *Xenopus laevis* oocytes (Daudin). V. Relationship between developing oocytes and their investing follicular tissues. *J. Morphol.* **155**, 73-98

[27] Browne, C.L. and Werner W. (1984) Intracellular junctions between the follicle cells and oocytes of *Xenopus laevis*. *J. Exp. Zool.* **230**, 105-113

[28] Danilchik, M.V. and Gerhart, J.C. (1987) Differentiation of the animal-vegetal axis in *Xenopus laevis* oocytes. I. Polarized intracellular translocation of platelets establishes the yolk gradient. *Dev. Biol.* **122**, 101-112

[29] Weeks, D.L. and Melton, D.A. (1987) A maternal mRNA localized to the vegetal hemisphere in *Xenopus* eggs codes for a growth factor related to TGF-beta. *Cell* **51**, 861-867

[30] Oron, Y., Gillo, B. and Gershengorn, M.C. (1988) Differences in receptor-evoked membrane electrical responses in native and mRNA-injected *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **85**, 3820-3824

[31] Klymkowsky, M.W., Maynell, L.A. and Polson, A.G. (1987) Polar asymmetry in the organization of the cortical cytokeratin system of *Xenopus laevis* oocytes and embryos. *Development* **100**, 543-557

[32] Dumont, J.N. (1972) Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocytes development in laboratory maintained animals. *J. Morphol.* **136**, 153-180

[33] Brachet, J. (1975) Nucleocytoplasmic interactions in cell differentiation. In *Molecular biology of nucleocytoplasmic relationship* **14**, 187-201 Elsevier Scientific, Amsterdam

[34] Wakahara, M. (1981) Accumulation, spatial distribution and partial characterization of poly(A)+RNA in the developing oocytes of *Xenopus laevis*. *J. Embryol. Exp. Morphol.* **66**, 127-140

[35] Capco, D.G. and Jefferey, W.R. (1982) Transient localization of messanger RNA in *Xenopus laevis* oocytes. *Dev. Biol.* **89**, 1-12

[36] Shiokawa, K. (1983) Mobilization of maternal mRNA in amphibian eggs with special reference to the possible role of membraneous supramolecular structures. *FEBS Lett.* **151**, 179-184

[37] Richter, J.D. and Smith, L.D. (1983) Developmentally regulated RNA binding proteins during oogenesis in *Xenopus laevis*. *J. Biol. Chem.* **258**, 4864-4869

[38] Berkowitz, B.A., Bevins, C.L. and Zasloff, M.A. (1990) Magainins: a new family of membrane-active host defense peptides. *Biochem. Pharmacol.* **39**, 625-629

[39] Sambrook, Fritsch, Maniatis *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press

[40] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294-5299

[41] Sumikawa, K., Parker, I. and Miledi, R. (1989) Expression of neurotransmitter receptors and voltage-activated channels from brain mRNA injected in *Xenopus* oocytes. In *Methods in neuroscience* I, Academic Press, Inc.

[42] Kusano, K., Miledi, R. and Stinnakre, J. (1977) Acetylcholine receptors in the oocyte membrane. *Nature* **270**, 739-741

[43] Smith, T.G., Lecar, J., Redmann, S.J. and Gage, P.W. (eds) (1985) Voltage and patch clamping with microelectrodes. *American Physiological Society* Bethesda, Maryland, The Williams and Wilkins company, Baltimore

[44] Standen, N.B., Green, P.T.A. and Whitaker, M.J. (eds.) (1987) Microelectrode techniques. *The Plymouth Workshop Handbook* The company of biologists Ltd., Cambridge

[45] Stühmer, W. (1992) Electrophysiological recording from *Xenopus* oocytes. *Methods in enzymology* **207**, 319-339

[46] Krafte, D.S. and Lester, H.A. (1992) Use of stage II-III *Xenopus* oocytes to study voltage-dependent sodium channels. *Methods in enzymology* **207**, 339-345

[47] Hille, B. (1991) *Ionic channels of exitable mambranes.* Sunderland, Massachusetts, Sinauer Associates Inc (eds)

[48] Sumikawa, K., Parker, I. and Miledi, R. (1984) Partial purification and functional expression of brain mRNAs coding for neurotransmitter receptors and voltage-operated channels. *Proc. Natl. Acad. Sci. USA* **81**, 7994-7998

[49] Patton, D.E. and Goldin, A.L. (1991) A voltage-dependent gating transition induces use-dependent block by tetrodotoxin of rat IIA sodium channel expressed in *Xenopus* oocytes. *Neuron* **7**, 637-647

[50] Gershon, E., Weigl, L., Lotan, I., Schreibmayer, W. and Dascal, N. (1992) Protein kinase A reduces voltage-dependent Na^+ current in *Xenopus* oocytes. *The Journal of neuroscience* **12**, 3743-3752

[51] Zhou, J., Potts, J.F., Trimmer, J.S., Agnew, W.S. and Sigworth, F.J. (1991) Multiple gating modes and the effect of modulating factors on the μl sodium channel. *Neuron* **7**, 775-785

[52] Heinemann, S.H., Terlau, H. and Imoto, K. (1992) Molecular basis for pharmacological differences between brain and cardiac sodium channels. *Pflügers Arch.* **422**, 90-92

[53] Van den Berg, R.J., Kok, P. and Woskuyl, R.A. (1993) Valproate and sodium currents in cultured hippocampal neurons. *Exp. Brain. Res.* **93**, 279-287

[54] Sontheimer, H., Ransom, B.R. and Waxman, S.G. (1992) Different Na^+ currents in P0- and P7-derived hippocampal astrocytes in vitro: evidence for a switch in Na^+ channel expression in vivo. *Brain research* **597**, 24-29

[55] Barish, M.E. (1983) A transient calcium-dependent chloride current in the *Xenopus* oocyte. *Journal of Physiology* **342**, 309-325

[56] Yoshida, S., Matsuda, Y. and Samejima, A. (1978) Tetrodotoxin-resistant and calcium components of action potentials in dorsal root ganglion cells of the adult mouse. *Journal of Neurophysiology* **41**, 1096-1106

[57] Fukuda, J. and Kamayama, M. (1980) Tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels in tissue-cultured spinal ganglion neurons from adult mammals. *Brain Research* **182**, 191-197

[58] Baccaglini, P. and Cooper, E. (1982) Electrophysiological studies of new born rat nodose neurons in cell culture. *Journal of Physiology* **324**, 429-439

[59] Heyer, E. and MacDonald, R.L. (1982) Calcium and Sodium-dependent action potential of mouse spinal cord and dorsal root ganglion neurons in cell culture. *Journal of Neurophysiology* **47**, 641-655

[60] Ogata, N. and Tatabayashi, H. (1992) Ontogenic development of the TTX-sensitive and TTX-insensitive Na^+ channels in neurons of the rat dorsal root ganglia. *Developmental brain research* **65**, 93-100

[61] Campbell, D.T. (1992) Large and small vertebrate sensory neurons express Na^+ and K^+ channels subtypes. *Proc. Natl. Acad. Sci. USA* **89**, 9569-9573

[62] Schlichter, R., Bader, C.R. and Bernheim, L. (1991) Development of anomalous rectification (Ih) and of a tetrodotoxin-resistant sodium current in embryonic quail neurons. *Journal of Physiology* **442**, 127-145