A study on transduction and transmission in catfish ampullary electroreceptor organs

Een studie naar transductie- en transmissieprocessen in ampullaire electroreceptororganen van de meerval

(Met een samenvatting in het Nederlands)

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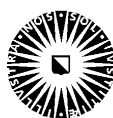

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Introduction and summary of the chapters

1. Electroreception

Electroreception is the ability of animal species to detect weak electric fields. It is mediated by a sensory system that occurs in some aquatic vertebrates, is useful for prey- and predator detection, orientation in space, electrolocation and communication. Electroreception as a sensory system was first recognized by Lissmann (Lissmann, 1958; Lissmann and Machin, 1958). He performed a series of classical behavioral experiments on the weakly electric fish *Gymnarchus niloticus,* and found that the fish' electric organ produces weakly electric discharges that can be detected by specialized sensory organs. Since then electroreception has been demonstrated in dogfish (Dijkgraaf and Kalmijn, 1966), catfish (Dijkgraaf, 1968) and a great variety of other fish species and amphibians (Bullock and Heiligenberg, 1986). Also, the primitive, egg laying mammal *Platypus* can sense electric fields with its bill (Proske *et al*., 1993).

There are two kinds of electroreception, crudely indicated by the terms "active" and "passive" which are distinguished by the characteristics of the adequate stimulus as well as by the morphology of the electroreceptor organs.

Active electroreception employs tuberous, or phasic electroreceptor organs. These are burried in the skin of the animal and separated from the outer world by layers of cells. This means that the electrical stimulus is transferred capacitively to the electroreceptor cells. From electricity theory we know that capacitive transfer only occurs with AC stimuli of sufficiently high frequencies. Tuberous electroreceptor organs are sensitive to high frequency stimuli of more than about 100 Hz.

This thesis will deal with passive electroreception, which is mediated by ampullary or tonic electroreceptor organs. An illustration of an ampullary electroreceptor organ is given in figure 1. It consists of a cavity in the skin, in which the sensory cells reside. The sensory cells make galvanic contact with the environment thus enabling the electrical stimulus current to pass the apical cell membrane. This mode of operation makes the organs sensitive to low frequency stimuli of 0.001 to 50 Hz (Andrianov *et al*., 1996; Bretschneider *et al*., 1985). Ampullary electroreceptor organs can be found in weakly electric as well as non-electric fish, in freshwater species as well as in marine species. In marine species (sharks and rays), the organs are called ampullae of Lorenzini after their discoverer. Ampullae of Lorenzini are morphologically distinct from (fresh water) micro ampullae, because of their long ampullary ducts. Two freshwater species are used for our studies: the North American catfish brown bullhead, or *Ictalurus sp*, and the Asian tropical catfish *Kryptopterus bicirrhis*. Especially the latter has a most remarkable feature: it is transparent. This makes it very suitable for research purposes, as will be apparent from later chapters.

2. Signal transduction

This thesis is about the transduction mechanism of catfish ampullary electroreceptor organs. The current hypothesis on the signal transduction in electroreceptor cells exists since its formulation by Bennett and Clusin (Bennett and Clusin, 1979). They argued that external electrical stimuli pass the apical membrane nearly unattenuated. Thus the stimulus current directly induces a change of membrane

potential at the basolateral face. This in turn modulates neurotransmitter release at the synapse. The neurotransmitter opens ion channels at the postsynaptic nerve terminal, and depolarizes the afferent nerve fiber. The depolarization of the afferent nerve fiber alters the spike frequency, which is relevant information for the catfish central nervous system.

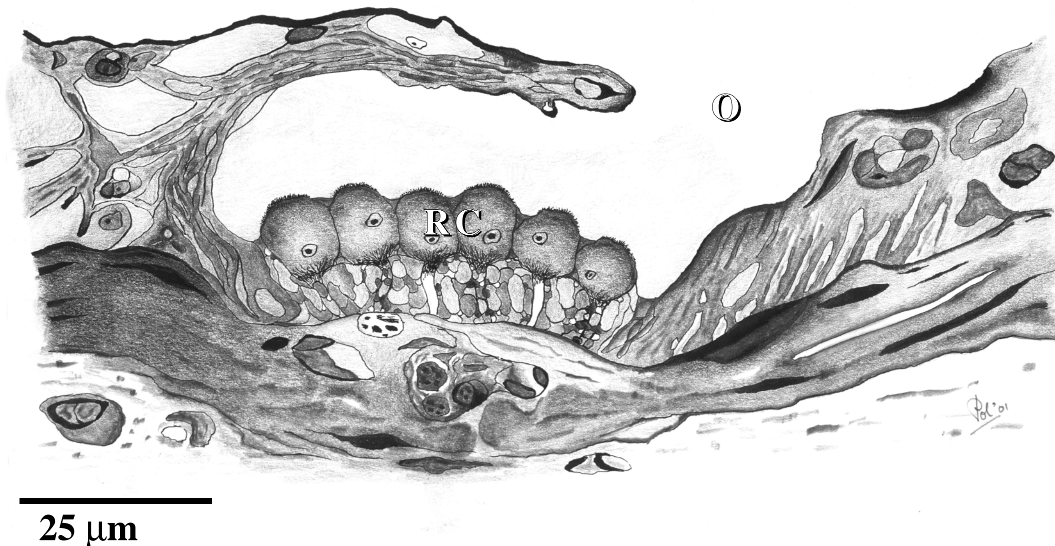


Figure 1. Drawing of a section of an ampullary electroreceptor, based on the EM work by Wachtel and Szamier (Wachtel and Szamier, 1969). The round electroreceptor cells (RC) have microvilli on their apical membranes. They are clustered on the bottom of the ampulla, and are exposed to the environment through the ampullary opening (O). Scaling bar = 25 µm

The charm of this theory is that it explains two important aspects of the functioning of the electroreceptor organs: the spontaneous activity, and the up and down modulation thereof. Spontaneous activity is the presence of spikes in the afferent nerve fiber even in the absence of an electrical stimulus. This feature is easily implemented in the model. By assuming a slight, permanent depolarization of the electroreceptor cells, continuous neurotransmitter release can be established. This continuous neurotransmitter release permanently excites the afferent nerve fiber thus evoking spontaneous activity. A scheme of the model is depicted in figure 2.

3. This thesis

The model as presented above leaves some questions still open, and fails to explain some more recent findings. For example; which ion channels are involved in the stimulus transduction? The spontaneous activity and the sensitivity are uncorrelated (Bretschneider and Peters, 1992), which is unlikely if they have the same origin. Why are there so many (+/- 20) receptor cells connected to only one afferent nerve fiber; is convergence essential for the sensitivity?

The subsequent chapters describe experiments that aim at completing and adapting the current model on signal transduction in the electroreceptor organ.

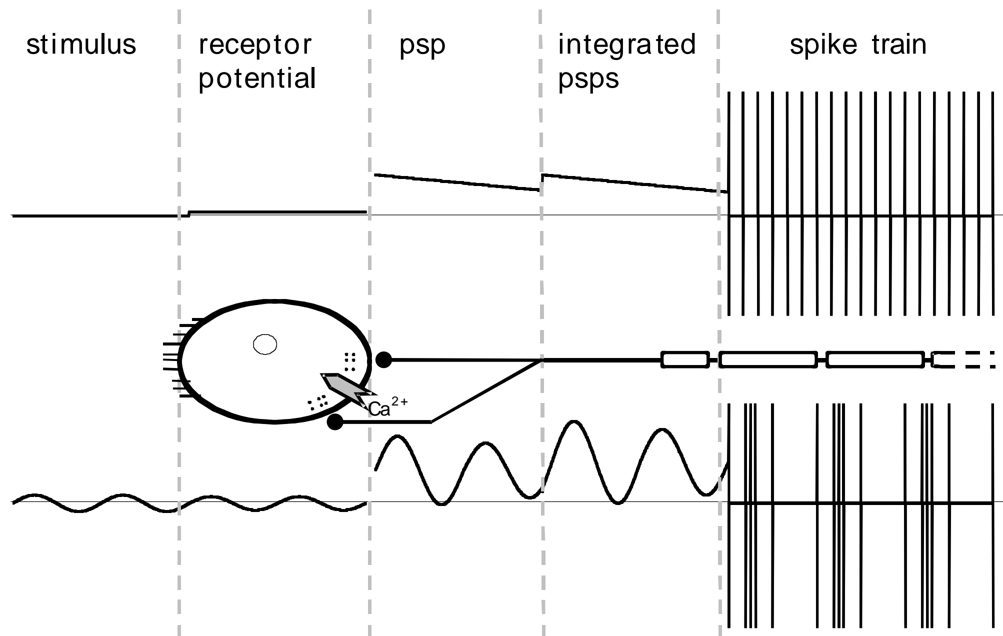


Figure 2. Schematic model of the electroreceptor organ functioning according to Bennett and coworkers (Bennett and Clusin, 1979). The upper trace illustrates the potentials in the different parts of the system when no current is applied. Although the stimulus strength is 0, the receptor cell is slightly depolarized. The depolarization of the receptor cells causes neurotransmitter release, and the post synaptic membrane depolarizes. The synapse is believed to function as an amplifier. These post synaptic potentials generate action potentials further down the dendrite where the myelin sheath appears. In the lower trace, a sinusoidal stimulus is applied. The sinusoidal stimulus is slightly attenuated by the apical membrane when it enters the receptor cells, where it modulates the neurotransmitter release. The post synaptic potential is larger than the stimulus because of the synaptic amplification, and action potentials are generated. The stimulus waveform is reflected in the spike frequency.

3.1. Ion channels

In the basolateral membrane of the electroreceptor cells voltage sensitive calcium channels should be present, because there is a glutamatergic synapse (Heijmen *et al.*, 1994). In such synapses the release of neurotransmitter is dependent on the

voltage dependent influx of calcium ions. The model also infers ion channels in the apical membrane. As explained, the signal transduction is not capacitive which implies that there must be a galvanic current passing the apical membrane in order to transduce the stimulus. In biological systems, electrical currents are often carried by ions and ion channels. Pharmacological experiments showed that a great variety of specific ion channel blockers affect the sensitivity of the electroreceptors. (Andrianov *et al.*, 1992b; Bennett and Obara, 1986; Lu and Fishman, 1995a; Lu and Fishman, 1995b; Peters *et al.*, 1989; Sugawara and Obara, 1984b). At present, the type and characteristics of the ion channels present in the cell membrane of electroreceptor cells have not been determined. In chapter one, patch clamp experiments are described that are designed to investigate the nature of the currents and ion channels in the electroreceptor cell membrane. In chapter two calcium currents evoked by electrical stimuli are described. Ratiometric Fura-2 measurements are applied for this. It was found that an electrical stimulus generated a calcium influx reflecting the stimulus waveform. This calcium influx could be manipulated by blocking sodium and potassium channels. The conclusion is that the sodium and potassium channels provide the apical conductance that allows the membrane potential to depolarize and activate the presynaptic calcium channels.

3.2. Spontaneous activity
The afferent nerve of the electroreceptor organs is spontaneously active. In the Bennett & Clusin model, the electroreceptor cells continuously release neurotransmitter. This neurotransmitter release induces the spontaneous activity of the afferent nerve. However, since then experiments showed that there is no correlation between spontaneous activity and the modulation of the spike frequency caused by an electrical stimulus (Bretschneider *et al.*, 1980; Teunis *et al.*, 1989). This leads to the formulation of an alternative model speculating that the modulation and the spontaneous activity have different origins (Bretschneider and Peters, 1992).
In chapter three, we pharmacologically separate the receptor cells from the afferent nerve fiber using the neurotoxin tetanus toxin, while recording the spontaneous spike activity extracellularly. In this way we find that the electroreceptor cells' neurotransmitter release regulates the modulation of the spike frequency, but is not required for the generation of spontaneous spike activity of the afferent nerve fiber. This means that the nerve must be able to generate the spontaneous activity on its own. A proposed mechanism for this low frequency spontaneous spike activity is implemented in the mathematical model presented in chapter four.

3.3. The afferent nerve fiber
The dendritic tree of the afferent nerve fiber in the electroreceptor organ has a complex structure, which we think is related to its functioning. In chapter four, an immunocytochemical staining shows heavy branching of the nerve cell. Each cell in the ampulla has multiple synapses with the afferent nerve fiber. This implies that input of about 16 receptor cells, with approximately a total of 45 synapses, modulates the activity of a single afferent nerve fiber. It seems a superfluous use of resources, but this might be essential to reach the impressive sensitivity of the electroreceptor organ. Convergence between electroreceptor organs improves the sensitivity, indicating that it may be an important mechanism in the receptor

functioning (Peters *et al.*, 1997a; Peters and Mast, 1983). Alternatively, since the electroreceptor organs are fairly exposed in the outer skin, the cells may be suffering from a fast turn over. This would mean that the electroreceptor organs need enough spare resources to be able to remain functional when mechanically damaged. In chapter five, the geometry of the dendritic tree is used in a numerical model in order to predict the behavior of the neuron with complete or partial innervation. According to the findings in chapter three the spontaneous spike activity is an intrinsic property of the nerve fiber itself. Phenomenologically, the numerical model behaves quite like the intact organ. In the model convergence leads to improved sensitivity, as was found experimentally (Peters *et al.*, 1997a; Peters and Mast, 1983). We conclude that a large number of electroreceptor cells in the organ are required for the impressive sensitivity of the system.

Chapter one

Membrane currents in the electroreceptor cells of the glass catfish *Kryptopterus bicirrhis*.

Preface

In this chapter attempts to patch clamp electroreceptor cells of the glass catfish *Kryptopterus bicirrhis* are described. This publication is not meant to discourage people to try and patch clamp the electroreceptor cells, nor do we claim that this is impossible. There are still a number of untried methods, and some may work. However, here we describe a variety of methods that do not appear to work, so as to prevent future futile attempts and to inform on the probable causes of the lack of success. This chapter also motivates the alternative approaches described in the rest of this thesis. There are fortunately almost always alternative methods an science leading to the same general goal, albeit along more circuitous paths than those envisaged in the first head-on attempts. Let us first look at the problems of the latter approach.

1. Introduction

The notion that the membranes of ampullary electroreceptor cells have ion channels seems obvious. The electroreceptor cells communicate with an afferent nerve through a glutamatergic synapse (Andrianov *et al*., 1994a; Andrianov *et al*., 1992a). Hence, at the very least, presynaptic voltage dependent calcium (Ca) channels should be present to modulate neurotransmitter release. Further empirical evidence for involvement of ion channels in electroreception comes from several types of experiments. First of all, the ion composition of the surrounding water is very important for the transduction system (Bauswein, 1977; Roth, 1971; Roth, 1982; Schouten and Bretschneider, 1980; Zhadan and Zhadan, 1975). Application of ion channel blockers confirmed these findings and suggested the presence of Ca, K, Na and Cl(Ca) channels (Lu and Fishman, 1995a; Lu and Fishman, 1995b; Peters *et al*., 1989; Zwart *et al*., 1988). Additionally, it was found that a K-pump, Na/Ca-pump and the Na/K-pump are vital for the functioning of the organ (Lu and Fishman, 1995a; Sugawara and Obara, 1984a). All of these experiments are performed by recording the output of the whole organ while changing ion compositions of the media, or adding channel blockers. However, in 1986 Bennett and Obara already proposed that "intracellular recording from receptor cells is required to verify many of the hypotheses concerning receptor function and establish the properties of the apical and basal membranes" (Bennett and Obara, 1986). Until now, this has not been done.

As it happens, electroreceptor cells are small cells that are electrically insulated from each other and their environment with tight junctions (Wachtel and Szamier, 1969). Inserting an intracellular electrode leads to an electrical shunt along the hydration mantle of the electrode. In large cells such as neurons or syncytia such as intestine epithelia, it is no problem, because the large membrane surface compensates for the current. In the tiny, isolated electroreceptor cells however, the shunt will result in a collapse of the membrane potential and reliable recordings can no longer be made (Lassen and Rasmussen, 1978; Van Dongen and Bretschneider, 1984). That is why we think the quest for ion channels in electroreceptor cells is better pursued with the patch clamp technique.

2. Experimental procedures

When we first saw the glass catfish *Kryptopterus bicirhhis*, we thought that patch clamping the electroreceptor cells could never be an enormous problem. We were faced with a transparent preparation, and under a microscope at 400x magnification, the electroreceptor organs and cells are clearly visible, see figure 3. The ampullae have openings of variable diameter, but it is very rare that the opening is so small that a patch electrode cannot enter through this opening. Therefore it seemed logical to try and patch clamp the electroreceptor cells just as they were, *in vivo*.

2.1. Methods for the in vivo experiments

The fish were kept in small groups in 100 liter tanks, containing Cu-free tap water at about 25°C. Before the experiments the fish were anesthetized by adding Saffan (Pitman-Moore, Harefield, UK)(100 μl/100ml) to the water in a small dish. The drug is a mixture of Alphaxalone (0.9%) and Alphadolone (0.3%) and entered the blood

circulation probably through the gills. It is a fast way of tranquilizing the animals: within 15 minutes the reflexes have completely disappeared. Saffan immobilizes the gill movements and thus impairs the breathing of the animals. Artificial breathing was employed in some cases if the fish were to recover after the experiments.
The fish were put in a dish which was placed on the stage of an upright microscope (Olympus BX50W1) with long working distance water immersion objectives. Electrodes were made from borosilicate glass capillaries (1.5 mm o.d., 0.86 mm i.d.) with filament, using a List L/M-3P-A upright pipette puller (List electronics, Darmstadt. Germany). For recordings an Axopatch 200B amplifier was used, with an CV 203BU headstage. Data were acquired and analyzed with PClamp6 software.

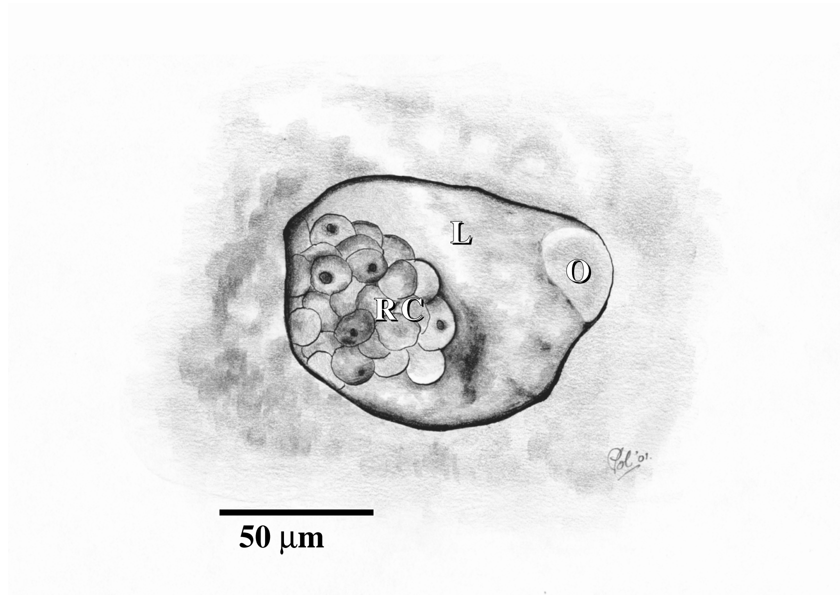


Figure 3.
Drawing of the electroreceptor organ after photographs made in our lab. RC = electroreceptor cells, L= ampullary lumen, O = opening of the ampulla. Scaling bar is 50 µm.

2.1.1. Results of the in vivo experiments
In vivo patch clamping of animal cells has some disadvantages, of which the most severe is that the animals are in motion continuously. This motion is caused by the heartbeat and ventilation movements. In our case, the breathing (gill movements) was stopped by the application of the anaesthetic Saffan. This is a disadvantage because the lack of ventilation impairs the health of the animal. On the other hand, fish have substantial gas exchange through the skin and thus can stay in good condition for a long time, even without artificial breathing. The heartbeat of the fish remained despite the anesthetics but generated only minor mechanical interference.
On commencing the experiments we found that it is very hard to form a gigaseal. There are several reasons for that. First of all the apical membrane contains microvilli which interfere with the establishment of a gigaseal. With patch clamping the electrode should be placed on a patch of membrane that is as smooth as possible in order to form the not yet fully explained bond between the slightly charged glass of

the electrode and the slightly charged cell membrane. A microvillus can get physically in the way, allowing some leakage.
Apart from the microvilli, there is most likely a large glycocalyx on the apical membrane of the receptor cells. This too makes the formation of a gigaseal more difficult.
Additionally, Szamier and Wachtel found that the ampulla is filled with a jelly, containing very fine fibris and some granules (Wachtel and Szamier, 1969). When entering an electroreceptor organ with a patch electrode, we did not see an effect in the magnitude of the response to a test pulse, the capacative current did not alter in magnitide or shape, nor did we observe a dc offset. The jelly may be too diffuse to generate such an effect. However it is very well possible that the fine fibers and granules stick to the patch electrode due to, thus preventing seal formation.
In order to cope with these problems, we varied the experimental procedures in several ways. Electrodes in a variety of shapes and sizes were manufactured. The shape of the tip of an electrode is very important for the gigaseal formation. Because of the microvilli and the glycocalyx, we first tried sharp electrodes, with a small tip diameter. We thought we would be able to put them on the cell surface just between the microvilli. Later we tried electrodes with larger tip diametres, blunt electrodes, with and without heat polishing the tip.
In effect we have never been able to establish a gigaseal *in vivo*.
There is one final thing that may interfere with the gigaseal formation. Normally, the fish swim in fresh water, therefore to obtain information with an on-cell patch, one has to fill the electrode with fresh water. Since the gigaseal probably depends on charges on the surfaces of the cell and of the electrode, the ionic content of the surrounding medium may play a part. There is no experimental evidence for this, but the finding that the presence of CsCl influences the seal formation (Hamill *et al.*, 1981), indicates that such factors are potentially important.

2.2. In situ recording
We decided to cancel the *in vivo* work and try to approach the electroreceptor cells from the basolateral side. This side does not contain microvilli and most likely no thick glycocalyx. By approaching the receptor cells from the basolateral side, much of the problems specific for the apical side can be by-passed. A drawback of this approach is that it can only be done by dissecting a piece of fish skin. Because the electroreceptor as such remains intact we refer to this method as *in situ* recording.

2.2.1. Methods for in situ recording
The fish were kept under the same circumstances as described before. Preceding the experiments, the fish were given a lethal dose Saffan (200μl/100 ml). After 20-30 minutes when all reflexes had disappeared, a piece of skin of about 25 mm^2 was dissected from the flank of the fish. This piece of skin was mounted on a recording chamber. The recording chamber was designed after an example by Furue and Yoshii (1997) (Furue and Yoshii, 1997). A similar setup has been designed in our lab for extracellular recordings in *Ictalurus* (Andrianov *et al.*, 1992a; Andrianov *et al.*, 1992b). A method which is also used in chapter 3 of this thesis. The skin patch was placed with the basolateral side facing up, with the edges extending over the side of the recording chamber. The edges of the preparation were then fixated by a small

silicon ring. This way the basolateral side of the skin patch could be irrigated with saline, whereas the apical side could be irrigated with fresh water.

2.2.2. Results of the in situ method

We expected that with this setup the polarity of the skin (fresh water vs saline) could be maintained, and that the skin patch would remain healthy. However, in the skin of *Kryptopterus* multiple exits of the lateral line system are present. In preparing the skin patch as thin as possible, it is very difficult to avoid including some of the lateral line exits. The lateral line exits are holes in the preparation, which lead to mixing of the apically and basolaterally administered fluids. The mixing has severe osmotic effects, and fast degeneration of the skin patch. Furthermore, any damage to the skin patch often results in a fast clouding of the preparation. This makes it impossible to recognize the different cell types.

Another difficulty with this technique is that the cells that need to be recorded are embedded in other cell types. Touching the cells or cell-debris must be avoided because it can clog the electrode. In general there are two solutions to this problem: One is often referred to as the "blind method" described by Blanton et al. (Blanton *et al*., 1989). Here pressure is applied in the recording electrode, while moving through the preparation. This way the cells and cell-debris are "blown away" by the flow of fluid at the tip of the electrode preventing clogging. The approach of a target cell can be monitored electrophysiologically, by applying small voltage steps. Once the electrode is close to a target cell, the pressure is released and a giga-seal can be made.

The other option is referred to as the "cleaning" method (Edwards *et al*., 1989), where one has to take care that the target cell is near the surface of the slice, so visual control is possible. A refinement of these methods can be found in the book by Sakmann and Neher "Single channel recording" (second edition) (Sakmann and Neher, 1995). Both these methods require relatively soft (brain) tissue in order to remove the debris, and the irrelevant cells. As it happens, the skin of *Kryptopterus* is an efficient organ. Fresh water fish have, more than anything, the problem of maintaining their internal ion balance. Therefore the skin is tight and high resistant (up to 50 $k\Omega*cm^2$ in mormyrid fishes (Bennett, 1971), and 6 $M\Omega$ in the canal of the ampullea of Lorenzini (Waltman, 1966)). In order to establish these features, the skin cells are tightly packed. Therefore we found it impossible to use air pressure to remove the surrounding cells without rupture of the entire preparation. After a number of serious attempts we decided to abort the *in situ* method on account of lack of success.

2.3. Hatching cells

Previously, an important discovery was made by F. Bretschneider (personal communication). When the fin preparation was made, part of it was kept in saline buffer at 4 °C. Due to this treatment, the electroreceptor cells specifically detached from the skin piece, and left the ampulla through the ampullary opening. This phenomenon occurs because of the temperature drop. At 4 °C, the ATP-ases are impaired in their function. This is a trick that is often used in biochemical studies to control for ATP-dependent processes. In our case, at low temperatures, the NaK-ATP-ase stops working. Therefore the cells experience an osmotic shock, swell and

die. When the skin piece is kept in the refrigerator long enough, all cells types undergo this fate, but in the first hour or so, the electroreceptor cells are specifically struck by this treatment. The specificity is most likely due to the fact that the receptor cells are the least protected of all cell types. As they are situated on the surface of the skin, they have to conduct electrical currents and consequently allow ongoing ionic exchange with the extracellular fluid. When the electroreceptor cells swell, they detach from the skin and drift out of the ampulla. We call this process the hatching of the cells. If the preparation remains undisturbed, the hatched cells stay in clusters on the outside of the ampullary opening. The advantage of this method is that the cells loose their microvilli and glycocalyx. This leaves us with dissociated, smooth cells in a saline buffer, which are ideal for patching.
A drawback is that this process is highly destructive for the cells. The cytoskeleton of the cells gets disrupted, and most likely the cell's organelles, as illustrated in figure 4. By the time a cell gets detached from the tissue and hatches from the ampulla, most of the internal structure has gone. Even so, if ion channels are still present in the cell membrane, we may be able to record from them and determine their kinetic properties.

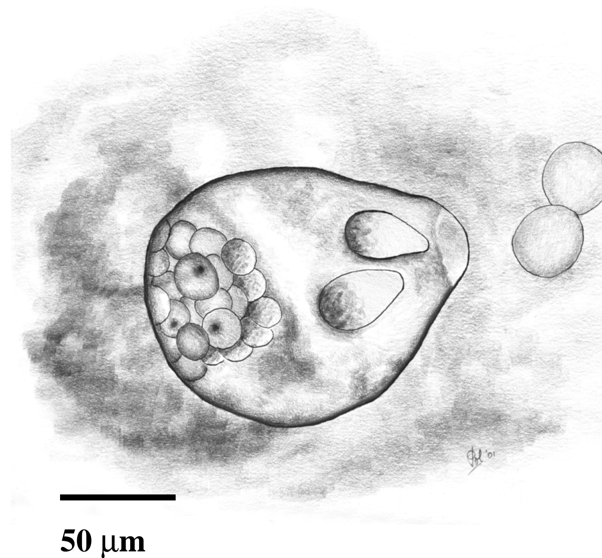


Figure 4. Drawing of hatching cells after pictures made in our lab. The cells that are hatching have the cytoskeleton confined to only a part of the cell. The cells that have hatched have poor contrast: the cytoskeleton has deteriorated. Scaling bar is 50 µm.

2.4. Methods for the hatching cell experiments

For the experiments the fish were anesthetized with a lethal OD Saffan (200 µl/100ml tap water). After 20-30 minutes when the reflexes had disappeared, the anal fin was dissected and put in a petri dish containing saline (in mM 120 NaCl, 3 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and pH 7.4). The fin preparation was incubated in the saline at 4 °C for 1-1.5 hr. After this period, the electroreceptor cells hatched from the receptor organs and could be approached with a electrode. The electrode resistances in saline were between 7 and 12 MΩ, tips were fire polished. Equipment used for the recordings is as described before.

2.4.1. Single-channel recording

Indeed, after some attempts we were able to obtain stable gigaseals from the isolated cells. As mentioned above, the cells thus obtained are very fragile and much care needs to be taken when approaching them with a patch electrode. The holding potential (V_h) was set to +70 mV. We expect that the treatment inactivates the membrane ATP-ases of the receptor cells. Therefore the membrane potential will diminish and drop to a value close to zero. Keeping V_h at +70 mV ensures that the potential difference across the membrane is around the normal resting membrane potential (V_{rest}) or even slightly hyperpolarized.

In the end, we recorded a single channel. The recorded activity is depicted in figure 5. In total the recording lasted approximately 30 seconds, then the cell detached from the electrode. Its downward deflections correspond to an outward current, suggesting that the channel conducts either potassium or chloride. We conclude that we have recorded a chloride channel. Chloride channels conduct outward currents, and they can be active at hyperpolarized conditions in order to stabilize the membrane potential (Hille, 1991).

Delighted by this promising recording, we set to work to repeat the recording and find out more of the characteristics of the ion channels on the electroreceptor cells. However, in the next 168 attempts where a stable gigaseal was realized we never saw an ion channel again.

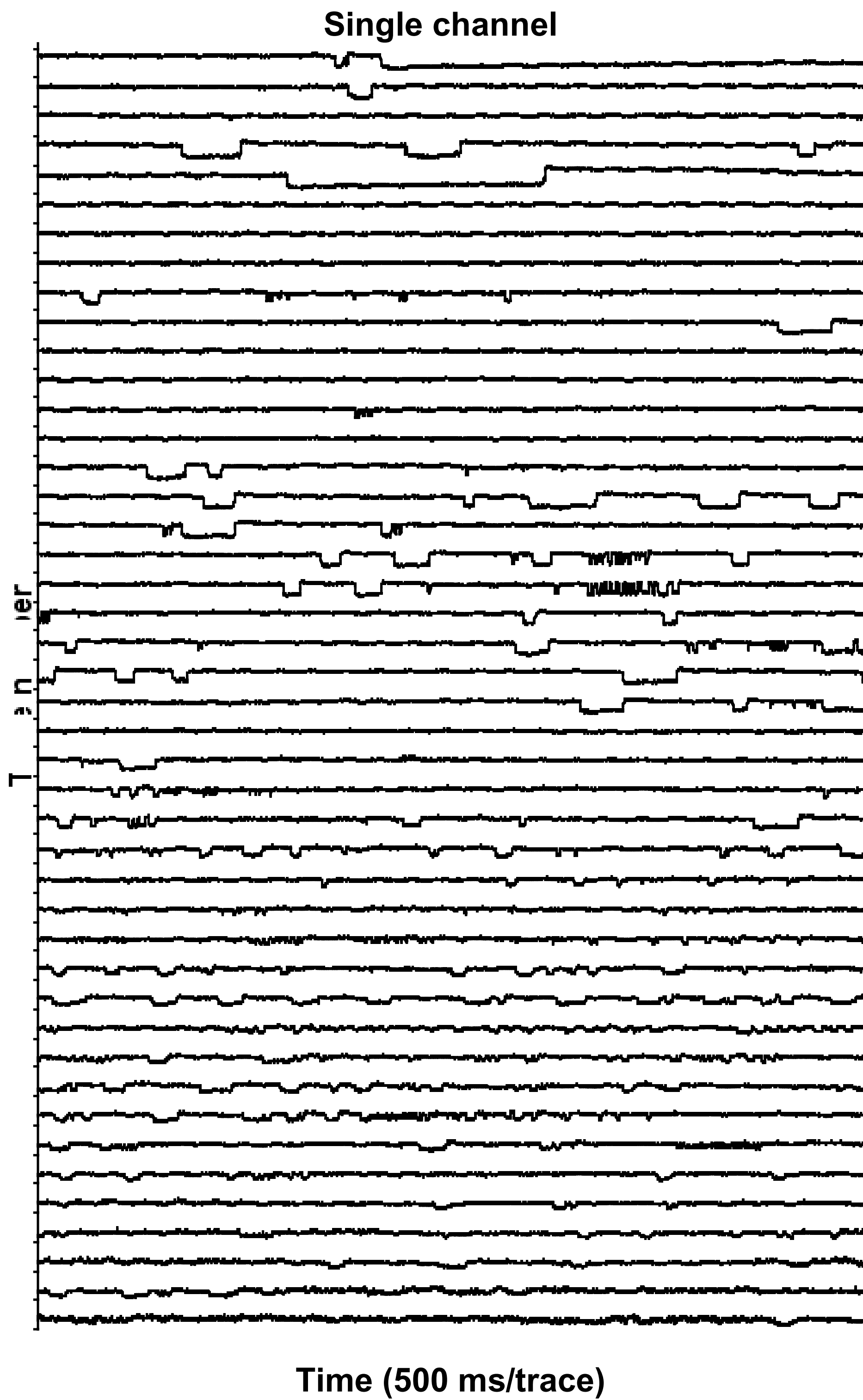


Figure 5. Cell attached single channel recording of an electroreceptor cell. Methods are described in the text.

2.5. Perforated patch clamp

At this point we were able to establish gigaseals routinely in the electroreceptor cells. Apparently the number of ion channels in our cells was very small. Therefore the next step was to perform whole cell recordings. In whole cell recording, the patch electrode is filled with saline buffer resembling intracellular medium. Once a gigaseal is established, the patch of cell membrane separating the cell from the electrode is removed by light suction at the electrode. Because of this, the entire membrane current of the cell is measured rather than the current through the patch only. As mentioned above, the cells obtained by our hatching method are very vulnerable. Gaining electrical access to the cells by light suction proved to be impossible, because the cells ruptured at the slightest mechanical strain. There is however a method to avoid this stressing of the cells and still gain electrical access to the cells. That method is called perforated patch clamp. A review on perforated patch clamp was written in 1991 by James Rea et al (Rae *et al*., 1991).

In the perforated patch clamp method, the membrane patch is permeabilized with the use of Nystatin or Amphotericin B. These antibiotics form pores in the lipid bilayer of the cell membrane with a Stokes-Einstein radius of 4Å.The pores are large enough to conduct monovalent ions (Na^+, K^+, and Cl^-) but not di- or polyvalent ions (Ca^{2+}), and nonelectrolytes larger than glucose (Hille, 1991; Rae *et al*., 1991; Sakmann and Neher, 1995).

2.5.1. Methods for perforated patch clamp

Nystatine (Sigma Chemical Co. St Louis) was dissolved in dimethyl sulfoxide (DMSO) (3 mg/100µl). This solution was added to 1 ml of intracellular medium (in mM: 10 NaCl, 140 KCl, 0.5 MgCl2, 10 ethylene glycol-bis(β-aminoethyl ether) (EGTA), 1 CaCl2, 10 mM Hepes, pH7.4). Subsequently, the mixture was either filtered with a 0.22 µm millipore filter (Millex-GS, Millipore S.A., France) or spinned for 5 min at 30000 rpm to get rid of remains of participate. The Nystatine solution was kept at 4 °C and used within one hour after preparation.

Glass electrodes were made as described above. Electrode resistances were about 7 MΩ. The tips of the electrodes were pre-filled with Nystatin-free intracellular medium, by dipping the back in a drop of intracellular medium. The rest of the electrode is back filled with Nystatine containing medium. This way, a gigaseal can be made before the Nystatine reached the cell membrane, and pores were inserted. After establishing a gigaseal it took 5-20 minutes before a decrease in resistance was observed. The total resistance then dropped from 1 - 10GΩ to 50 – 250 MΩ. This gave a sufficiently low access resistance to start recording. The equipment for data aquisition and analysis is described above.

2.5.2. Results of perforated patch clamp

In total we performed over 200 experiments of this type. Often, we didn't see an obvious non linearity in the I-V curves of our recordings. However some (8) recordings showed noteworthy responses. Four of those recordings are depicted in figure 6. The figure shows the original data traces of the recordings. The two insets are the I-V curves 1 ms after the onset of the stimulus (onset component) and 1 ms before the end of the stimulus (sustained component). As can be seen in all current traces, the cells respond to a depolarizing voltage step with a slow rise in inward

current. At the end of the stimulus, the current drops to zero immediately, sometimes showing a small tail current. The slow inward current means there is an activating component present in the currents, that is not due to passive (Ohmic) conductance or membrane capacitance. The I-V curves all show non-linearity.
These characteristics strongly indicate the presence of voltage gated ion channels. Further interpretation is difficult because of the large differences between the individual recordings. The magnitude of the response for example varies greatly. This is probably not due to normal variation in the receptor cells, but to variation in the preparations. Furthermore, the activation time constants (τ) vary greatly among the

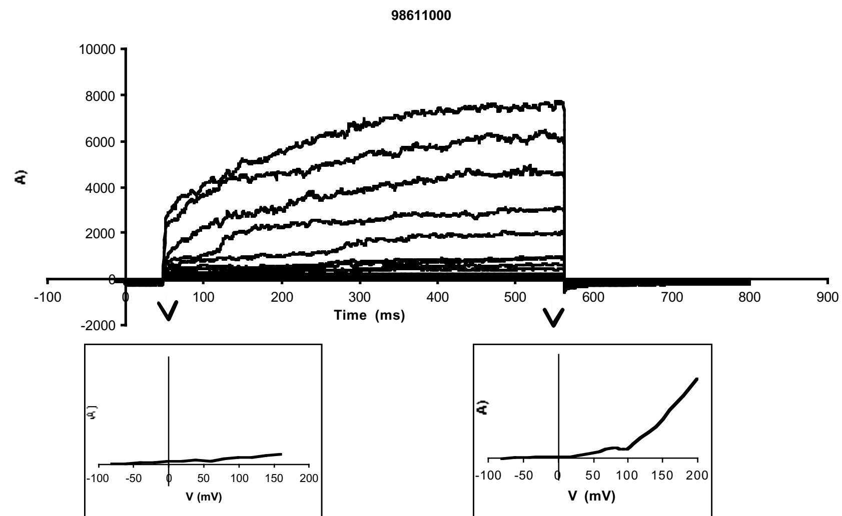


Figure 6A

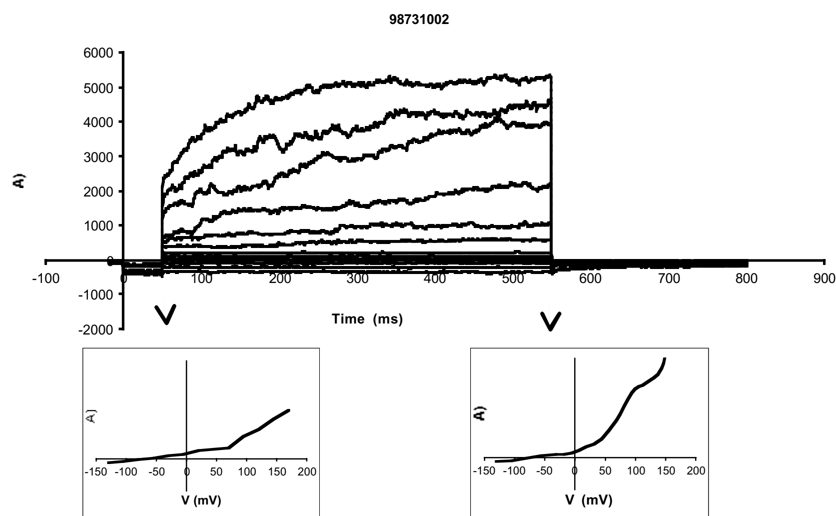


Figure 6B

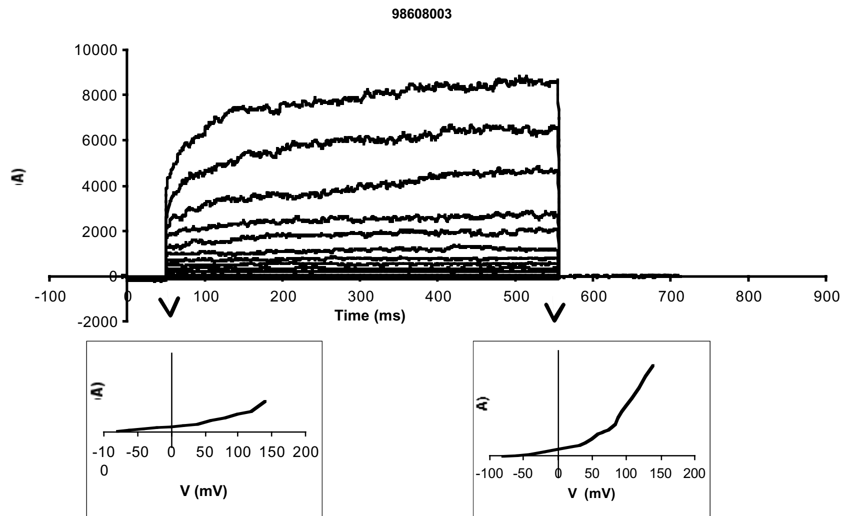


Figure 6C

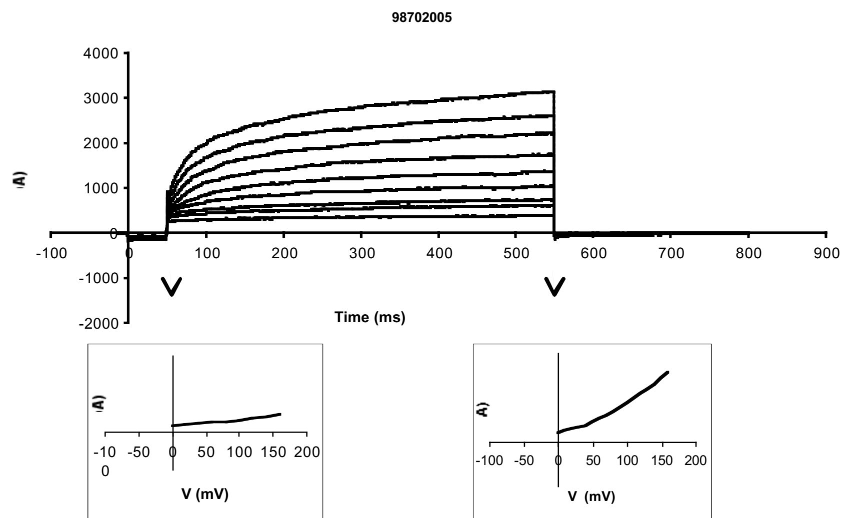


Figure 6D

Figure 6. Examples of perforated patch recordings. All recordings were filtered at 5 kHz, sampling rate 20 kHz. I-V curves are scaled according to the I-t curves. Stimulation was different for each cell. Before the variable voltage stimulation(V_{stim}), a hyperpolarizing voltage step (V_{hyp}) was applied, in order to close LVA channels. 4A: V_h= -80, V_{hyp}= -130, V_{stim}=0 + 10 steps of 20 mV. 4B V_h= -70, V_{hyp}= -90, V_{stim}= -80 +10 steps of 20 mV. 4C V_h= -70, V_{hyp}= -100, V_{stim}= -130+ 14 steps of 25 mV. 4D V_h= -70, V_{hyp}= -90, V_{stim}= -80+12 steps of 20 mV.

experiments ($0<\tau<1000$ ms for a first order fit), and are not voltage dependent as expected. So, despite strong evidence that we are indeed recording voltage

dependent ion channels, characterization of the kinetics is difficult. With these recordings, we can't get certainty about the channel types that are present in these cells. Adding specific channel blockers would be helpful in this respect, but as mentioned before, even the slightest mechanical stress will disrupt the gigaseal. Changing media during the experiments is therefore not feasible.

2.6. Control experiments

Although we had previous experience with whole cell patch clamp recording method (Karst *et al*., 1997), the lack of results prompted us to check the setup with a cell type known to be 'patch clamp-able'. Therefore we did several control experiments along the way to check our capabilities. A few batches of cultured mouse N1E-115 neuroblastoma cells were kindly provided by C. Meulenberg, RITOX, Utrecht. Figure 7 gives an example of a recording we made from one of these cells. Similarly we have recorded from dissociated guineapig trachea hair cells, kindly provided by R. ten Broeke, Department of Pharmacology and Pathophysiology, Utrecht University. An I-V curve from a recording of those cells is illustrated in figure 8. The success rate of these control recordings was high. The figures 7 and 8 show the sum of the voltage dependent membrane currents of the cells, because we didn't manipulate the cells pharmacologically, nor with special voltage protocols. Further interpretation of the results of these control experiments is beyond the scope of this chapter.

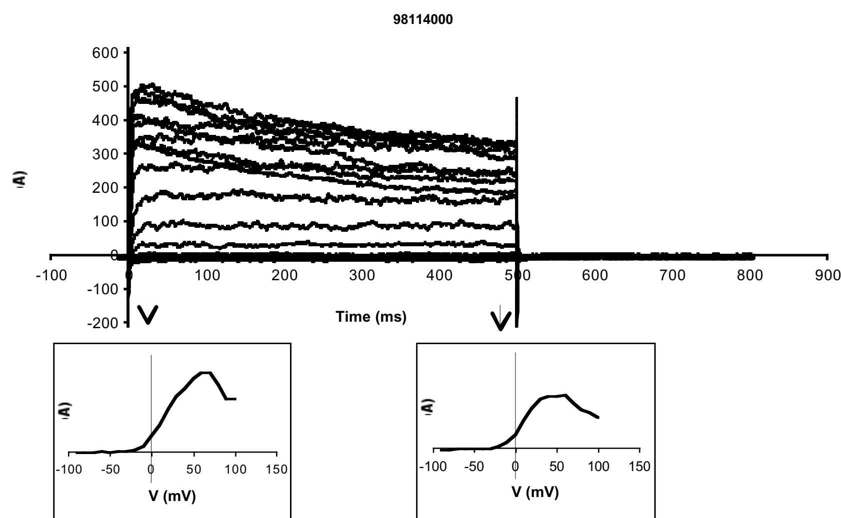


Figure 7. Whole cell recording of a mouse N1E-115 neurobastoma cell. The recordings were filtered at 5 kHz, sampling rate 20 kHz. Y-axis were scaled identical. Intracellular medium in mM: 150 KCl, 10 NaCl, 10 Hepes, 1 $MgCl_2$, pH 7.2. Extracellular medium in mM: 125 NaCl, 5.5 KCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, 20 Hepes, 25 glucose, 36.5 sucrose, pH 7.3. I-V curves are measured 20 ms after the onset of the stimulus and 1 ms before the end of the stimulus.

3. General discussion

From the data presented in this chapter, it is hard to draw unambiguous conclusions. However there are a few observations that bring us closer to resolving the mechanisms of transduction in electroreceptor cells. From the single channel recording we know that functional ion channels are present in the electroreceptor cells. This might seem a conclusion that hardly needs mentioning, but still it had never been shown directly in this cell type. The conclusion that the single channel we recorded is a chloride channel is, we realize, somewhat speculative. Still this option covers the data best, as chloride channels are abundant in epithelial cells.

The perforated patch currents we showed were inward currents. So the outward single channel current mentioned above is not of the predominant type. The kinetics of these perforated patch currents can not be determined explicitly. The differences in membrane

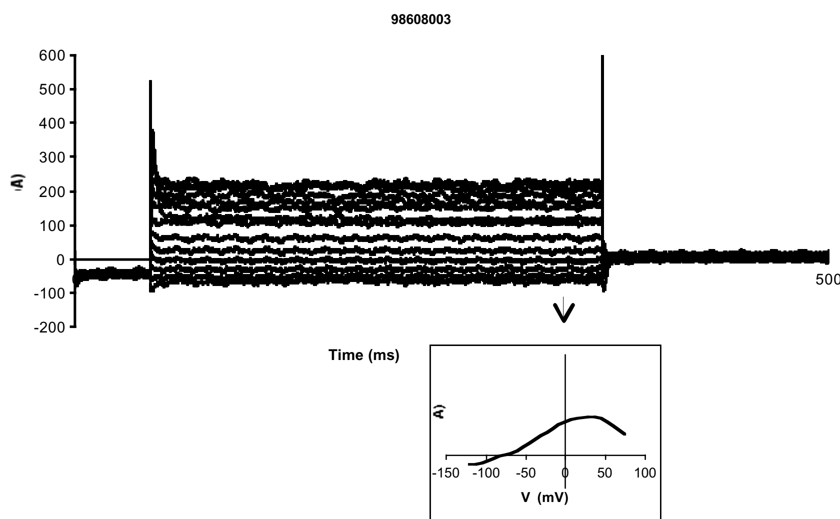


Figure 8. Whole cell recording of a guinea-pig trachea cilliairy epithelial cell. The recordings were filtered at 5 kHz, sampling rate 20 kHz. Intracellular medium in mM: 110 KCl, 2 NaCl, 5 EDTA, 10 Hepes, 1 $MgCl_2$, pH 7.3. Extracellular medium: Krebs ringer. I-V curve is the result of all voltage dependent currents. The currents presented are small, as can be expected in a non-excitable cell.

conductance between the recorded cells is too large, and most likely the membrane potential varies among the cells. Since the cells do not generate action potentials, we do not expect a large population of voltage gated Na^+ channels in the cell membrane. Therefore we think that the non linear component of these currents are due to the presynaptic voltage sensitive Ca^{2+} channels.

The currents we found with the perforated patch experiments were rather large. Part of the magnitude of the currents is due to a linear or leakage component. We can estimate the value of this ohmic component from the I-V curves by extrapolation of the most hyperpolarized part of the I-V curves. The values obtained are in the range of 60 to 440 MΩ. It has already been proposed that the apical membrane of the receptor cells contain leakage channels through which the external stimulus

enters the cells (Bennett and Clusin, 1979; Bretschneider *et al*., 1991; Teunis *et al*., 1991). The large linear current we find may well be passing through those leakage channels. If so, the values we found gave an estimation of the apical membrane resistance. Because the hatching treatment deteriorates the cells we expect that ion channels will have disappeared from the cell membrane, due to normal turn over and perhaps some lysosomal activity. Therefore the lowest membrane resistance we found most likely reflects the actual apical membrane resistance best. As mentioned above, the lowest value of the ohmic component of the perforated patch currents was 60 $M\Omega$. This is in the right order of magnitude of earlier estimates (Bretschneider *et al*., 1991; Heijmen and Peters, 1995).

4. Concluding remarks

Our efforts to record ion currents directly from electroreceptor cells were not rewarded with the results we had hoped for. Nevertheless, they loosely confirm our current ideas about electroreceptor functioning. The future of the patch clamp work on electroreceptor cells may lie in a different preparation. Ampullae of Lorenzini for example do not have the stern osmotic differences between the apical and the basolateral sides. These cells may be easier to dissociate and once dissociated, easier to recognize. Also an *in situ* preparation of a different fish species may work well.
For direct measurements of electroreceptor cell functioning we have chosen to pursue fluorescence imaging. The results of this are presented in chapter 2.

Chapter two

Simultaneous measurements of calcium mobilization and afferent nerve activity in electroreceptor organs of anesthetized *Kryptopterus bicirrhis*

Summary

The transduction pathway of ampullary electroreceptor organs involves ionic currents. It has been shown that calcium, as well as both sodium and potassium play important parts in this process. In this study we examine the stimulus-evoked changes in Fura-2 ratio in electroreceptor cells. Furthermore, we recorded stimulus-evoked Fura-2 ratio changes while Na^+ and K^+ channels were blocked by amiloride and TEA. Simultaneously, extracellular recordings of the afferent spike activity were made .

The results show the presence of stimulus-evoked fluctuations in Fura-2 ratio. These fluctuations can be abolished by application of Cd^{2+}, TEA, and amiloride. Also, the stimulus-evoked activity of the afferent nerve was decreased by application of these drugs.

We conclude that the transduction current is carried by Na^+, K^+, and probably Ca^{2+}. This fits an existing model on transduction in electroreceptors.

1. Introduction
The ampullary electroreceptor organs of the transparent catfish *Kryptopterus bicirrhis*, are sensory organs dedicated to sense electrical fields. The organs are sensitive to electrical stimuli as weak as a few microvolts and in the frequency range of approximately DC to 50 Hz. An electroreceptor ampullary organ consists of a cavity in the skin where about 20 receptor cells reside. The apical membranes of the cells face the fresh water around the fish, whereas the basolateral membranes face tissue fluid. Each receptor cell is connected to the afferent nerve fiber (going from the receptor toward the brain) with about 5 chemical synapses. The current hypothesis on stimulus transduction was formulated by Bennett and Clusin in 1979 (Bennett and Clusin, 1979) and refined later by Bennett and Obara (Bennett and Obara, 1986). It involves a low series-resistance apical membrane. Thus, the electrical stimulus current is supposed to pass the apical membrane practically unattenuated. Almost the entire potential difference will drop over the basolateral membrane. The basolateral membrane is believed to be regenerative and is thought to contain glutamatergic synapses. In glutamatergic synapses, a depolarization of the receptor cells will lead to a presynaptic calcium influx, which in turn induces neurotransmiter release. The nature of the synapses of electroreceptor cells of freshwater fish has been studied by Andrianov and colleagues (Andrianov *et al*., 1995; Andrianov *et al*., 1992b). In effect, the electrical stimulus directly stimulates the synapse.
The low electrical resistance of the apical membrane is most likely due to the presence of ion channels. Application of channel blockers influences the sensitivity of catfish electroreceptor organs to electrical stimuli (Andrianov *et al*., 1992b; Peters *et al*., 1989; Zwart *et al*., 1988). In *Plotosus* ampullary organs and in *Skate* ampullea of Lorenzini, AC as well as DC currents were recorded in the lumen of ampullae of Lorenzini. The recorded currents originate in the electroreceptor cells, and can be blocked by cation channel blockers (Clusin and Bennett, 1979; Lu and Fishman, 1995a; Lu and Fishman, 1995b; Sugawara and Obara, 1984a). In this paper we present more direct data about the role of Ca^{2+}, Na^{+} and K^{+} in electrosensory transduction, obtained using Fura-2 ratio imaging.
Fura-2 is a ratiometric fluorescent calcium indicator that enables the detection of changes in free cytosolic calcium concentration. Since the discovery of Fura-2 it has been used in many experiments, on many different cell types. Often, either cultured or dissociated cells are used because a fairly transparent preparation is necessary for appropriate illumination with and emission of fluorescence. We are able to perform *in vivo* experiments because we use a transparent test animal; the glass catfish *Kryptopterus bicirrhis*. The anesthetized fish can be placed in a dish on a microscope stage, and studied for several hours. Organs such as blood, muscle, and electroreceptor organs can be observed very well this way (Bretschneider and Brus, 1999). The use of this transparent fish has great advantages because the organs we measure do not have to suffer destructive excision.
Combining intracellular free calcium measurements with afferent spike recordings enabled us to study the behavior of electroreceptor cells in response to an electrical stimulus. We hypothesize that a lumen-positive electrical stimulus, which excites the afferent nerve fiber, will evoke an increase in free cytosolic calcium. Furthermore we

think that other cation currents are important in the signal transduction. We test this by applying ion channel blockers.
We found that electrical stimulation with a lumen-inward current of the receptor cells leads to mobilization of Ca^{2+} in these cells. Also such a stimulation leads to an increase in spike frequency. Both the spike frequency and changes in intracellular free calcium ($[Ca^{2+}]i$), could be measured. By applying blockers for other cation channels, we determined their role in electroreception.

2. Materials and methods
The animals were kept in copper-free tap water at 26 °C. The fish were anaesthetized by submersion in water containing Saffan (Alphaxalone (0.9%), Alphadolone (0.3%) (Schweiz. Serum & Impfinstitut, Bern), 200 µl/100 ml for 20 minutes. Subsequently they were incubated for an hour in the membrane permeable Fura2-AM (4 µM) in tap water containing Saffan. Fura-2-AM solution was prepared from stock (2 mM in DMSO). By this treatment, the electroreceptor cells were loaded whereas the surrounding tissue showed no significant Fura-2 loading. After incubation the fish were rinsed and placed in a transparent dish on the microscope stage. During the experiment the fish were kept in 40 ml tap water containing Saffan. After the experiment the fish were sacrificed by means of a lethal dose of Saffan (400 µl Saffan/100 ml tap water).
Single unit activity was recorded extracellularly as described previously (Peters *et al*., 1988). A tungsten microelectrode was placed in the lumen of the ampulla for single unit recording. A silver wire was placed in the surrounding tap water to serve as stimulus electrode, to apply the low frequency stimulus. The reference electrode a silver strip of approximately 5 cm long and 1 cm wide, was also placed in the tap water. The stimulus and reference electrodes were placed on opposite sides of the fish. The electroreceptor organs were stimulated with sinusoidal currents of 0.025-0.01 Hz. This frequency was chosen because it excites the cells long enough to let a detectable amount of calcium into the cells. Sensitivity of the organs to this frequency range is -20 dB compared to the optimum of the frequency spectrum, which is at about 10 Hz. Therefore we chose a relatively high stimulus strength of 0.42 - 4.2 $\mu A/cm^2$ (Bretschneider *et al*., 1985). Stimuli were generated by a Wavetek model 132 function generator. Interspike intervals were recorded, and averaged over one second time intervals. These averages were inverted to read the instantaneous spike rate.
Fluorescence intensity was recorded using a PTI (Photon Technology International, South Brunswick, USA) ratio recording setup combined with an Olympus BX50WI fluorescence microscope. The microscope was equipped with water immersion objectives. Excitation wavelengths were 340 nm (calcium bound) and 380 nm (calcium free). A xenon lamp provided light which was filtered to obtain the two excitation wavelengths. These two wavelengths illuminated the preparation alternately by means of a rotating mirror. The light was conducted from the lamp to the microscope by an optic fiber. Emitted light was filtered at 510 nm using a dichroic cube and recorded by a photon multiplier. Sampling rate of the recordings was 2 Hz. For the following reason we did not attempt to calibrate the ratio values for true calcium concentrations. Application of high concentrations of ionomycine and EGTA, needed for calibration, results in whitening of the preparation. Stressing

the animals with such chemicals mediates aggregation of proteins and results in decreased transparency. Not only application of chemicals has this effect, but for example sickness also causes the same decrease in transparency. This alters the reflective properties of the fish and would make such a control incomparable with the recordings. However, using exactly the same recording setup, two of us (M.S. and R.S.) performed experiments with cultured heart muscle cells. The calibrations used for that case give a rough estimate for the measurements on the electroreceptors. To estimate the $[Ca^{2+}]i$, we used a standard equation for intracellular calcium concentrations introduced by Grynkiewicz (Grynkiewicz *et al*., 1985):

$$[Ca^{2+}]i = Kd \cdot Q \frac{(R-R_{min})}{(R_{max}-R)}$$

R is the fluorescence ratio, R_{min} and R_{max} are the minimum and maximum ratio values. K_d is the dissociation constant (140 nM). Q is the ratio of the emission intensity at 380 nm in the absence of Ca^{2+} and the emission intensity at 380 nm in the presence of Ca^{2+} .

Cadmium chloride (0.5 mM), tetra-ethylammonium chloride (TEA) (10 mM) and amiloride chloride (1 µM) were used to block calcium, potassium and epithelial sodium channels, respectively. The blockers were added manually from a 1000x aqueous stock, and mixed by triturating. All ion channel blockers showed immediate an effect. Incubation time was equal to the recording time: 30 minutes for all 3 agents. Washout was accomplished by replacing the external medium. This has been shown to be an adequate way of manipulating the electroreceptors pharmacologically (Peters *et al*., 1989; Roth, 1973).

3. Results

When no electrical stimulus was applied, the Fura-2 ratio was stable and always between 0.3 and 0.4. According to our calculations this corresponds to $[Ca^{2+}]i$ of 5-20 nM. An electrical stimulus-evoked changes in the Fura-2 ratio (from here on called the Fura-2-response). As figure 9 shows the Fura-2 ratio changes accordingly when an alternating electrical stimulus is applied.

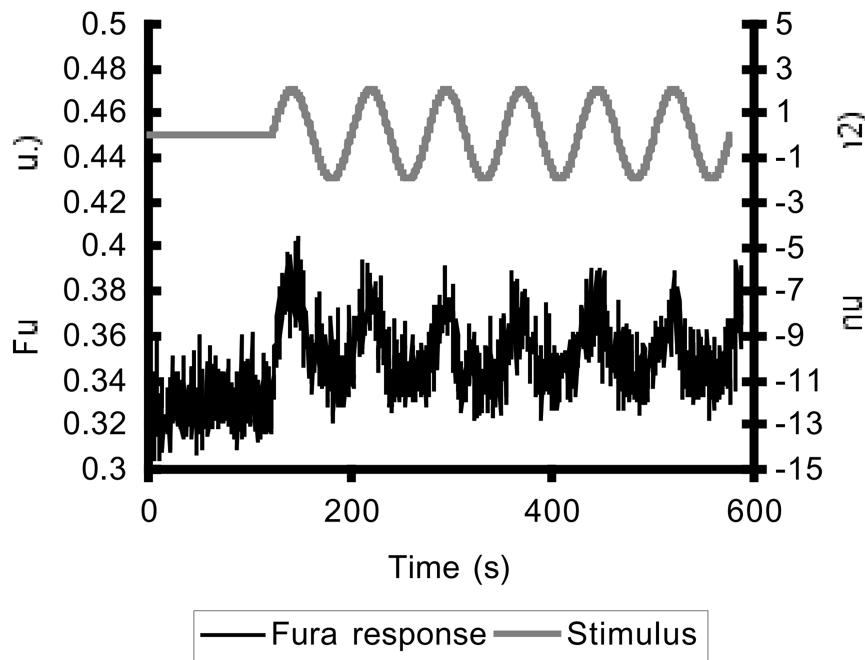


Figure 9. Raw data of the changes in the Fura-2 ratio due to an electrical stimulus. The upper trace represents the electrical stimulus. The scale is displayed on the secondary y-axis. The first 125 s no electrical stimulus is applied. The lower trace is the recorded Fura-2-response to this stimulus.

Figure 10 is a typical example of both the Fura-2-response and the afferent nerve activity in response to a stimulus. The spike rate can vary from 0- 200 Hz. The response is extremely non-linear and rises steeply due to the positive (lumen inward) stimulus. A maximum is reached, even before the stimulus is at its peak. The spike rate then decreases, and reaches a plateau phase. When the stimulus becomes negative, the spike frequency drops, and is minimal before the stimulus is. Then it increases slowly, and when the stimulus gets positive again, the steep rising slope reappears. This pattern is a typical response to these low frequency stimuli (Bretschneider *et al.*, 1985). It remains similar even after a great number of repetitions.

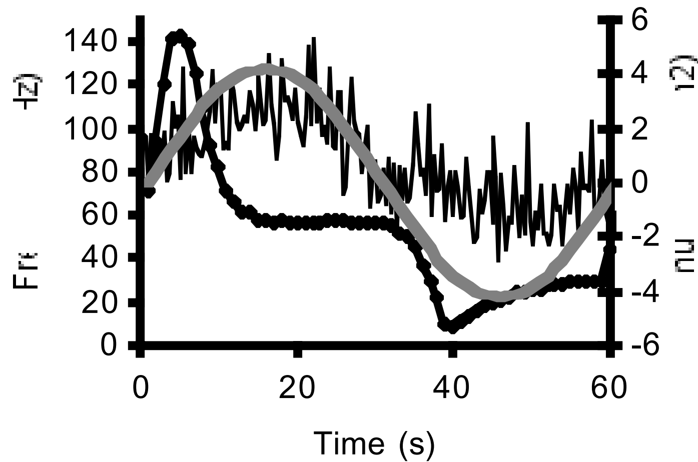


Figure 10. A typical example of the fura-2-response and the response of the afferent nerve to an electrical stimulus of 0.01 Hz. Each point in the nerve response trace represents the instantaneous spike frequency of the afferent nerve. The spike frequency scaling is displayed on the primary y-axis , the stimulus strength on the secondary y-axis. Stimuli are depicted as gray lines.

To further investigate the Fura-2-response, 0.5 mM $CdCl_2$ was added to the cells. Cd^{2+} blocks the Ca-channels irreversibly. Results are depicted in figure 11. Each trace in figure 11 shows one period of the average Fura-2-response of the electroreceptors. Due to the Cd^{2+}
treatment the Fura-2-response disappears. Separate control experiments showed that the stimulus-evoked response of the organs did not change in the course of time. This indicates that the preparation did not deteriorate during those experiments (figure 11).
In figure 12 the change in stimulus-induced modulation of the spike train due to Cd^{2+} treatment is depicted. As expected, application of cadmium decreases the spike rate modulation by almost 50%.
Earlier work suggests (Lu and Fishman, 1995a; Lu and Fishman, 1995b; Peters *et al.*, 1989; Sugawara and Obara, 1984a) that sodium and potassium ions play a part in the signal transduction of the electroreceptor. To investigate this we used TEA to block potassium channels and amiloride to block sodium channels. The results are displayed in figure 13. TEA as well as amiloride decrease the Fura-2-response to an electrical stimulus reversibly.

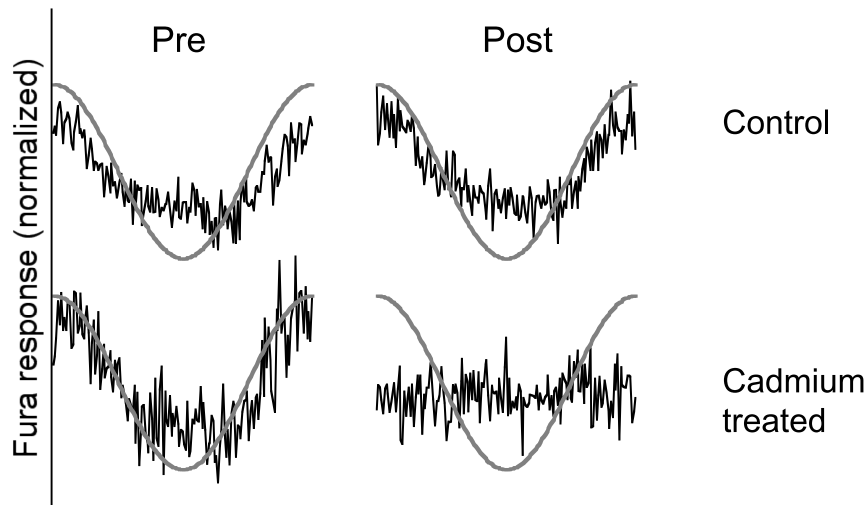


Figure 11. The average change in Fura-2-response for one 0.01 Hz stimulus cycle, as a result of cadmium administration. Time and amplitude scaling is identical for all traces. For clarity traces are shifted along the axis. To eliminate individual differences, data are normalized by setting the baseline value to one. For averaging of a trace, data of 2 fish were used. For each fish and each condition, at least 15 stimulus repetitions were used. Stimuli are depicted as gray lines.

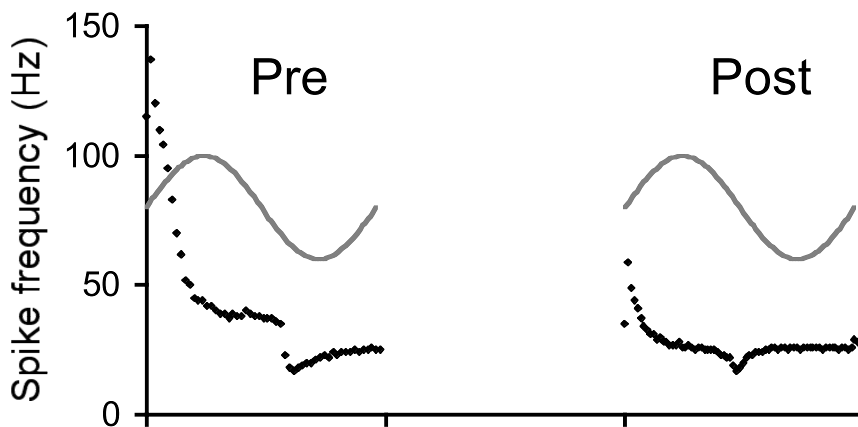


Figure 12. Example of the effect of Cd^{2+} administration on the afferent nerve activity. Cd^{2+} administration decreases the electrical stimulus-evoked modulation of the spike frequency. Traces are recorded before (Pre), and after 30 min administration of Cd^{2+} (Post). The 0.01 Hz stimuli are depicted as gray lines.

4. Discussion

In this paper, we showed that a positive (lumen inward) electrical stimulus induces Ca^{2+} mobilization in electroreceptor cells. This Ca^{2+} mobilization is associated with an increase in spike rate of the afferent nerve fiber. The calcium current can be inhibited by blocking Na^{+} as well as K^{+} channels. Although the involvement of Na^{+}

and K^+ channels has been suggested many times, unto now there was only indirect evidence of ion currents in electroreception. Both intracellular and patch clamp recording in this cell type are very difficult.
Pharmacological studies performed with electroreceptors sometimes involve an Ussing-chamber-type setup. With this experimental design, the voltage and currents recorded in the ampullary lumen reflect depolarization of the electroreceptor cells. The spike activity of the afferent nerve cannot be simultaneously recorded using this method. Alternatively, extracellular recordings from the afferent nerve can be made. However, this method does not allow recording from receptor cells. The method we use here allows both direct recording from receptor cells as well as recording from the afferent nerve.

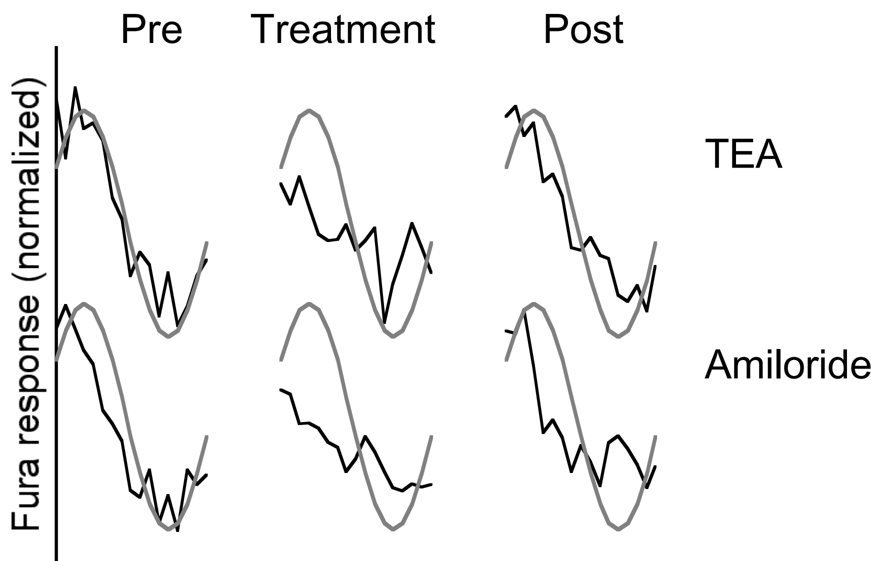


Figure 13. Effects of amiloride and TEA administration. Average change in Fura-2 response for one cycle. Time and amplitude scaling is identical for all traces, stimulus frequency is 0.025 Hz. For clarity traces are shifted along the axis. To eliminate individual differences data are normalized by setting the baseline value to one. Traces are recorded before (pre), during (post) drug administration, and after wash out (wash). For averaging of a trace, data of 2 fish were used. For each fish and each condition, at least 15 stimulus repetitions were used. Stimuli are depicted as gray lines.

The reason why the loading procedure appears to selectively label ampullary cells, is the relative accessibility of these cells. The entire skin of the fish consists of an extremely tight epithelium, dedicated to maintain the separation between the internal and external milieu. Because of this, Fura-2 is able to specifically enter the receptor cells. Loading of electroreceptor cells by a fluorescent tracer has been described previously by Jorgensen (Jorgensen, 1992).
The raw data (figure 9) already show the stimulus-dependent variation in the Fura-2-response, and thus in the calcium concentration. The transformation of Fura-2 ratio to intracellular free calcium concentrations is precarious. Precise values can not be obtained but we made a fair estimate of the intracellular calcium concentrations,

which is 5-20 nM. In other types of hair cells baseline intracellular calcium-concentrations of between 30 and 88 nM were found (Devau, 2000; Fujiyama *et al*., 1998; Zviman *et al*., 1996). The values we found are of the same order of magnitude. We did not express our data in terms of calcium concentrations because of the uncertainty of the calibration factors.
We think that the stimulus-evoked calcium fluxes took place at the synapse as well as at the apical membrane. Considering the physiological properties of synaptic voltage-dependent calcium channels (for a recent review see; (Tareilus and Breer, 1995), it is possible that the increase of the intracellular calcium level was due to the influx at the synapses on electroreceptor cells. Previously it has been shown that Cd^{2+} diffuses into the electroreceptor cells (Zwart *et al*., 1988). Therefore Cd^{2+} may well act intracellularly at the synapse. On the other hand, replacing the tap water by water with either high or low calcium content has a strong immediate effect on the sensitivity of the organ (Peters and Westerink, 1999; Peters *et al*., 1989; Roth, 1971). This suggests that there are Ca^{2+} channels in the apical membrane that are involved in stimulus conduction.
The afferent spike activity exhibits a non-linear response. For low frequency stimuli, such non-linearity has been described previously (Bretschneider *et al*., 1985). They suggested that the plateau-phase in the stimulus can be due to depletion of neurotransmitter. This is supported by our Fura-2 measurements, because the plateau-phase did not occur in the Fura-2-response. This means that its origin must be in a later stage of the signal transduction, like neurotransmitter release. Furthermore, the plateau-phase is not due to permanent damage because the pattern does not change after successive stimuli.
Cadmium, in the concentration used, did not completely block the spike frequency modulation, but the Fura-2-response became undetectable. Part of the difference in these results lies in the difference in signal-to-noise ratio of the two types of recording. Also, because of synaptic amplification, a decrease in Ca^{2+} influx is more obvious in the recordings than the resulting decrease in spike frequency modulation.
We found that amiloride, an epithelial sodium-channel blocking agent as well as TEA, a potassium-channel blocking agent suppressed the Fura-2-response reversibly. Both agents are known to block passive (not voltage dependent) ion channels (Iliev and Marino, 1993; Kleyman and Cragoe, 1990). This suggests that these ion currents are involved in the stimulus transduction pathway. An additional effect of amiloride is blocking of the Na/Ca exchanger. This only happens at high concentrations (> 1 mM). The concentration we used (1 μM) however was far too small to induce such an effect (Kleyman and Cragoe, 1990).
The present data support the existing hypothesis on the transduction mechanism formulated by Bennett and coworkers (Bennett and Clusin, 1979; Bennett and Obara, 1986). By applying channels blockers we make evident that Na^{+}, K^{+} and most likely Ca^{2+} are responsible for the transduction current through the apical membrane. This transduction current depolarizes the cell membrane. In turn the depolarization leads to activation of presynaptic calcium channels, and thus to activation of the synapse.

Acknowledgement
The authors would like to thank dr. Lindemann (Saarland University, Germany) for his help and advice.

Chapter three

Spontaneous nerve activity and sensitivity in catfish ampullary electroreceptor organs after tetanus toxin application

Summary
Functioning of electroreceptor organs of *Ictalurus sp.* was investigated by inhibiting synaptic transmission by administration of tetanus toxin *in vitro*. A piece of *Ictalurus* skin of about 20 mm diameter was mounted in an Ussing type chamber. TeTx was applied basolaterally for 150 min in 66.7 pM and 400 pM concentrations, while the single unit nerve activity was recorded extracellularly. Spontaneous spike activity and sensitivity of the electroreceptor organs were measured.
The results show that TeTx reduces sensitivity to less than 20% of its original value, whereas the spontaneous activity is unaffected by the treatment. This indicates that the afferent nerve is capable of generating impulses independent of receptor-cell neurotransmitter release. In the discussion we suggest two alternative mechanisms for the emergence of the spontaneous spike activity.

1. Introduction

The ampullary electroreceptor organs of *Ictalurus Sp.* form a sensory system specialized to detect low-frequency electrical stimuli in the environment (Bretschneider and Peters, 1992; Dijkgraaf, 1968). The organs consist of a cavity in the skin in which about 20 electroreceptor cells of epithelial origin are situated. These electroreceptor cells are all innervated by one single afferent nerve fiber. This afferent nerve fiber is spontaneously active, which means that it generates spike activity even if the receptor organ is not stimulated electrically. The generally accepted model of the functioning of the electroreceptor organ assumes that a positive electrical stimulus depolarizes the receptor cells. The change in membrane potential modulates voltage-sensitive Ca^{2+} channels in the presynaptic membrane of the receptor cells. The resulting rise in Ca^{2+} concentration results in neurotransmitter release at the synapse. The neurotransmitter in this synapse is identified as glutamate (Andrianov *et al.*, 1992a; Andrianov *et al.*, 1992b; Andrianov *et al.*, 1994b; Bennett and Clusin, 1979; Heijmen *et al.*, 1994). The release of glutamate evokes excitatory post synaptic potentials in the afferent nerve. Since the afferent nerve fiber is spontaneously active, the ongoing spike train is modulated by the stimulus, rather than initiated by it. The advantage of such a system is that the output of the organ can be modulated in two directions: up modulation by a positive electrical stimulus, and down modulation by a negative electrical stimulus.

It has been proposed that the depolarization necessary to maintain the spontaneous activity of the afferent nerve originates in the electroreceptor cells. The cells would be continuously depolarized in order to establish a continuous neurotransmitter release (Bennett and Clusin, 1979). Such a depolarization of the cells could be the result of a so called 'bias current' (Bennett and Obara, 1986; Sugawara and Obara, 1989), which has indeed been shown to exist in similar sensory systems. Also Andrianov showed that the spontaneous activity can be completely abolished by applying 30 mM Mg^{2+}. Since Mg^{2+} blocks synaptic transmission, he argued that the spontaneous spike activity is the result of a presynaptic process (Andrianov *et al.*, 1992a). These experiments support the hypothesis that the depolarization necessary to maintain the spontaneous activity originates in the receptor cells.

However, there are also indications that the spontaneous activity and the spike train modulation of the electroreceptor organs have different causes. In 1980 Bretschneider et al. described that prolonged inhibition of the ampullary organs has a long-lasting effect on the sensitivity (Bretschneider *et al.*, 1980). The spontaneous activity however was restored much faster. These results were acquired by means of a sinusoidal stimulus superimposed upon an inhibiting D.C. component. Initially, the recurring spontaneous spike activity was regular and independent of the sinusoidal component, suggesting independence of neurotransmitter release. Additionally, denervation results in complete disappearance of the spontaneous activity, while maintaining the sensitivity of the electroreceptor organs (Teunis *et al.*, 1989). This shows that spontaneous activity is not a prerequisite for sensitivity of the organs, and thus that the two parameters have different origins.

In order to resolve the discrepancy between single-cause and dual-cause theories, and to determine whether the spontaneous nerve activity originates in the nerve itself, or is a result of continuous neurotransmitter release of the receptor cell, the synaptic transmission needs to be inhibited. This can be achieved by applying the

neurotoxin tetanus toxin (TeTx) (Bruns *et al*., 1997; Herreros *et al*., 1995; Van Vliet *et al*., 1989). TeTx blocks synaptic transmission of glutamate by specific enzymatic cleaving of the presynaptic docking protein synaptobrevin (Bruns *et al*., 1997; Herreros *et al*., 1995; Sudhof, 1995). Application of TeTx will have one of two possible results. If TeTx impairs the spontaneous activity as well as the sensitivity, the spontaneous activity must be a result of neurotransmitter release by the electroreceptor cells. On the other hand, if TeTx affects the sensitivity but not the spontaneous activity, the spontaneous activity must be a nerve property.
In this paper we present evidence that the spontaneous activity is not challenged by synaptic blockage. Therefore it must be a property of the nerve rather than a result of continuous neurotransmitter release of the receptor cells.

2. Experimental procedures
The parameters measured are sensitivity of the organ to electrical stimuli, and the spontaneous activity of the afferent nerve. One has to keep in mind that both parameters are determined using the afferent nerve spike activity. The word sensitivity relates to the stimulus-evoked modulation of the spike frequency, whereas the word spontaneous activity relates to the nerve activity when the organ is not stimulated.

2.1 Skin preparation
Ictalurus sp. (n=8) weighing 100-240 g were kept in 250 l glass containers in Utrecht copper-free tap water at 16 °C, for at least one month. These animals were anaesthetized with 24 mg/kg body weight Saffan (Glaxovet, Harefield, UK) by means of i.m. injection. After excision of a piece of skin the fish were given an additional lethal dose of 96 mg/kg body weight. Care was taken to prevent the animals from regaining consciousness.
The methods used for preparation in this study, have been described previously (Andrianov *et al*., 1992b). In summary, a round piece of skin was selected from the dorsal head region, and placed in an Ussing type chamber. It was about 20 mm diameter, and contained 3-10 electroreceptor organs. The serosal compartment was perfused (1 ml/min) with saline (containing in mM; 167 NaCl, 5 KCl, 3 $CaCl_2$, 1.5 $MgCl_2$, 10 hepes, pH 7.2) and 10% FCS (Gibco/RBL, Rockville, USA). TeTx was applied at the serosal side at a concentration of 66.7 pM, or 400 pM. The mucosal side was kept in fresh water that was refreshed regularly.

2.2. Electrophysiology
Single unit activity was recorded extracellularly at the mucosal side, with a tungsten microelectrode. A silver reference electrode was placed in the tap water compartment, and a silver stimulus electrode was placed at the serosal side. The electroreceptor organs were stimulated with sinusoidal currents with frequencies of subsequently 1, 4, 8, 10, 12, 16 and 20 Hz in order to avoid influence of changes in frequency characteristics on the results. Each experiment consisted of a 15-30 min control period, followed by a maximum of 120 min of TeTx administration. Control experiments consisted of up to 150 min recording periods. Sensitivity and spontaneous activity were recorded every 10 (control and low TeTx) or 15 minutes (high TeTx). Stimulus strength was 0.17-1.7 $\mu A/cm^2$. The stimulus was kept within

the linear range of the I/O-curve of the receptor organ. Amplified spike trains were processed by a computer into peristimulus time histograms (PSTH). Sine curves were fitted through these PSTHs by means of the method of least squares in order to estimate the amplitude and the phase shift. At weak response amplitudes the sensitivity and phase shift do no loner represent a meaningful relation between the stimulus strength and the response amplitude. Therefore sensitivity at a certain frequency (S_f) was quantified by processing the amplitude (A) of the fit, and the phase shift (φ) and the stimulus strength (I_{stim}). The responses were vectorially averaged according to the following calculation:

$$\mathbf{S_f} = \overline{A} / I_{stim}$$

$$\overline{A} = \sqrt{(\Sigma x^2)} + (\Sigma y^2)$$

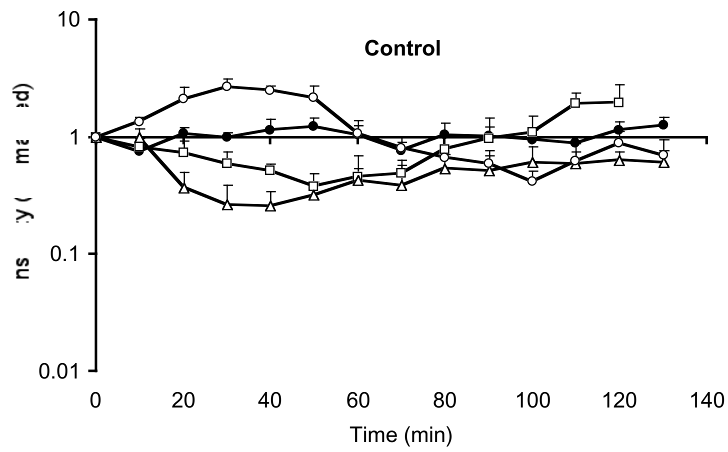
Where:

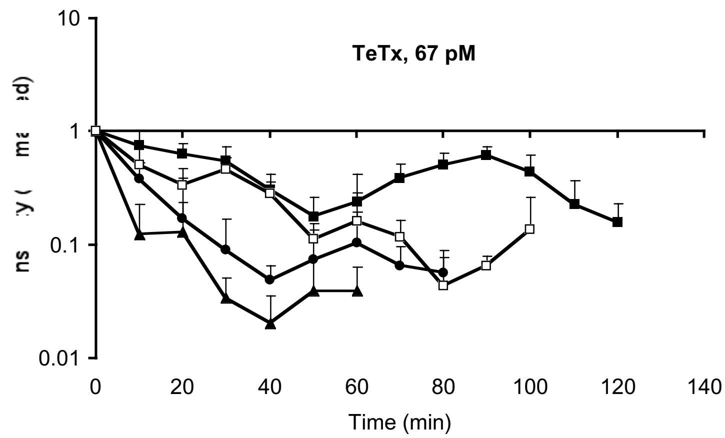
$x = A*\cos(\varphi)$ and $y = A*\sin(\varphi)$

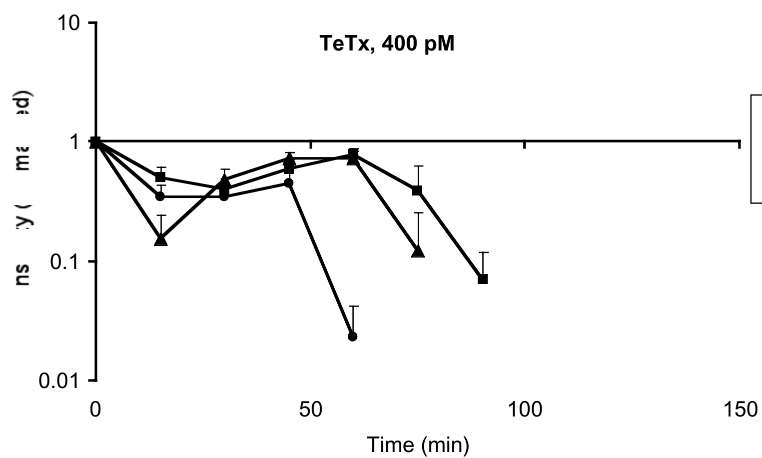
Spontaneous activity was quantified by the mean of the corresponding interspike interval histogram (Peters *et al*., 1988).
All data were normalized by setting the values for sensitivity and spontaneous spike activity of the initial control period for each experiment to 1. Since TeTx is an irreversible neurotoxin (Bruns *et al*., 1997; Herreros *et al*., 1995; Schmitt *et al*., 1981; Sudhof, 1995), we could not include a control period after the administration period.

Next page:

Figure14.Effects of TeTx on the sensitivity of the electroreceptor organs.
Scaling of the sensitivity (y-axis) is logarithmic. In all figures dots represent the average value of the normalized sensitivity for all 7 recorded frequencies. Error bars are standard deviations. Figure 14A illustrates 4 control experiments, where no TeTx is added. The sensitivity remains stable in the course of time. In figures 14B and 14C the results of TeTx application are shown for two concentrations of TeTx. All experiments (n=7) ultimately show a decrease of the sensitivity to below 20% of the original value.


Control
10
1
0.1
0.01
0
20
40
60
80
100
120
140
Time (min)
c 1
c 2
c 3
c 4


TeTx, 67 pM
10
1
0.1
0.01
0
20
40
60
80
100
120
140
Time (min)
exp 1
exp 2
exp 3
exp 4


TeTx, 400 pM
10
1
0.1
0.01
0
50
100
150
Time (min)
exp 5
exp 6
exp 7

3. Results

3.1. Sensitivity

Figure 14 A shows the change in sensitivity in the course of time for each of the 4 control experiments. There are some individual fluctuations in the control experiments. However, on average, the control experiments retain their value. Non-normalized data have been statistically tested for equal variances, using single factor ANOVA. No significant difference was found ($P>0.01$). This indicates that preparation of the skin flap does not affect the electroreceptor organs functioning for the duration of our experiments.
The effect of TeTx on the sensitivity of the electroreceptor organs is illustrated as a function of time in figure 14B and 14C. Figure 14B illustrates the change in sensitivity of electroreceptor organs in 4 separate experiments when 67 pM TeTx is added to the skin preparation. In all 4 experiments, the sensitivity decreases to less than 20% of the original value. In some cases there is even a 98% reduction (experiments 1 and 7).
There are individual differences between the experiments. The time it takes for the TeTx to take effect differs per experiment, and within the experiments there are some fluctuations. Single factor ANOVA of the non-normalized data showed that the effect is significant ($P<<0.05$).
In order to see if we could speed up the effects of TeTx application, we performed an additional experiment during which the effective dose of TeTx was increased by factor 6.
Figure 14 C illustrates the change in sensitivity in the electroreceptor organs in 3 separate experiments when 400 pM is added. Again, the sensitivity of all electroreceptor organs was decreased to less than 20 % of the original value. The time it takes to reach this minimum has not decreased compared to the low concentration. Also, the degree of inhibition scatters somewhat. Although the higher concentration doesn't seem to make the effect stronger, the decrease in sensitivity due to the treatment is evident. Again, single factor ANOVA of the unnormalized data showed that the effect is significant ($P<<0.05$).

3.2. Spontaneous activity

The other parameter we use in this study is the spontaneous activity of the afferent nerve fiber. The effect of TeTx treatment on the spontaneous activity in the course of time, is illustrated in figure 15. It shows that the spontaneous activity does not change significantly over time in the control experiments. Also, neither the low (66.7 pM) nor the high (400pM) concentration TeTx affects the spontaneous activity of the afferent nerve. The data in the figure are normalized. Non-normalized values were between 20 and 50 spike/s.
In summary, the sensitivity of electroreceptor organs is decreased by TeTx application, whereas the spontaneous activity remains unaffected.

4. Discussion

4.1. *Incomplete block*

Application of TeTx reduces the sensitivity of the electroreceptor organs (figure 14), even at concentrations as low as 66.7 pM. Therefore we can conclude that the applied TeTx indeed inhibits the neurotransmitter release in the electroreceptor

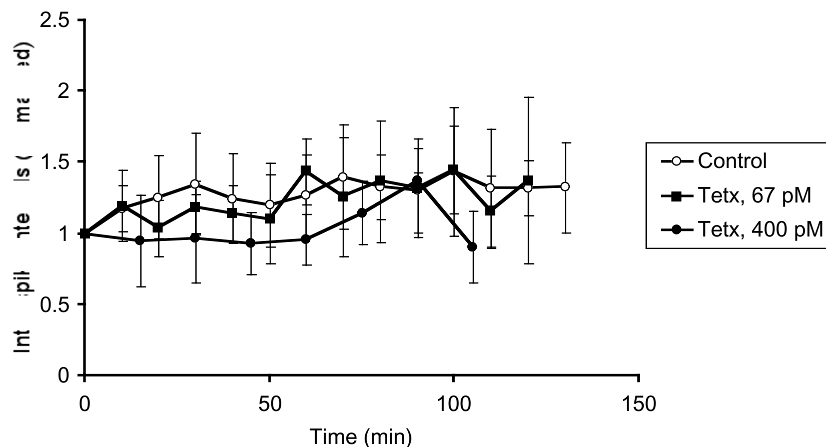


Figure 15. Effects of TeTx on the spontaneous activity of the electroreceptor organ. Dots represent the average value for all experiments (n=4 for control, n=4 for 67 pM TeTx, n=3 for 400 pM TeTx). Error bars represent standard deviations. TeTx does not have an effect on the spontaneous spike activity.

organ. The sensitivity was reduced to 2-20% of its original value. This means that the synaptic transmission was not completely blocked by the TeTx. Most likely, even the 400 pM concentration of TeTx wasn't quite high enough to be a 100% effective. Also, some of the variation between the results may be due to individual variation in the skin patch preparation. Occasionally, fragments of muscle or adipose tissue stuck to the skin patch, making it more difficult for the TeTx to reach the electroreceptor cells.

4.2. Latency

The relatively long latency of the effects can be ascribed to the fact that TeTx is an enzyme. Enzymatic reactions are usually slower than for example ion channel blockage. In cultured cells and synaptosomes, TeTx incubations over night or for 24 hours are not unusual (Herreros *et al.*, 1995; Van Vliet *et al.*, 1989). Also, the experiments were performed at room temperature, where enzymatic activity is relatively low.

4.3. Specificity

TeTx is a specific protease that cleaves synaptobrevin at the peptide bond between Gln76 and Phe77 (Schiavo *et al.*, 1992). Therefore it is unlikely that TeTx affects anything but synaptic transmission.

In conclusion, the decrease in sensitivity, shown in figure 14B and 14C, indicates that TeTx has the intended effect, especially since there is no such decrease in the control experiments (figure 14A). Figure 15 demonstrates that the inhibition of neurotransmitter release leaves the spontaneous spike activity unchanged. Apparently, the spontaneous activity is not the result of continuous neurotransmitter release.

4.4. Alternative hypotheses
How then, is the spontaneous activity generated? If we assume normal Hodgkin and Huxley kinetics supplemented by a potassium A channel (K_A), low frequency repetitive firing can be induced in the neuron (Connor and Stevens, 1971; Hille, 1991).
The firing rates of such a system are between, say, 1 and 100 Hz. As mentioned in the results section, and confirmed by others (Obara and Sugawara, 1982; Peters and Bretschneider, 1981; Schaefer *et al*., 1990), the spontaneous activity of our system is well within this range. An assumption we make with this model is that spikes are generated in the afferent nerve fiber, at the resting membrane potential. This can be due to for example a small shift in resting membrane potential, which can be established by variation in the ion-ATP-ase content. The (K_A) has been found in many types of neurons in a great variety of animals, and it is reasonable to assume such a channel in our system.

4.5. Autorelease
Another mechanism known to generate spontaneous activity is autorelease of glutamate, which binds to NMDA receptors. For example; motor rhythms can be induced by NMDA application, as reviewed by Daw, Stein and Fox (Daw *et al*., 1993). Also, NMDA receptors play a part in the modulation of the electric organ discharge frequency in electric fish (Daw *et al*., 1993; Dye *et al*., 1989; Kawasaki and Heiligenberg, 1990). More recently it has been shown in thalamocortical organotypical cultures that the spontaneous activity occurring in these systems is NMDA-receptor mediated and that it can be diminished by application of extracellular magnesium (Coulter and Lee, 1993; Hentschke and Antkowiak, 1999). However, the spontaneous discharges found in these studies are in the frequency range of 0.1-0.5 Hz, which is much slower than the firing rate found in our system.
The hypothesis for autorelease of glutamate is supported by the finding that strong immunoreactivity to glutamate antibodies was found in the cytoplasm of the afferent nerve (Heijmen *et al*., 1994). This hypothesis can explain the findings of Andrianov et al. mentioned in the introduction. They found that application of magnesium to the electroreceptors blocks the sensitivity as well as the spontaneous activity. It is likely that the magnesium did not only affect synaptic glutamate receptors, as they concluded, but also NMDA receptors that are involved in the generation of spontaneous activity.

5. Conclusion
In this study we dissociated the spontaneous activity of the afferent nerve of the electroreceptor organ from the activity of the epithelial electroreceptor cells. We found that the spike activity continues, even if the synaptic transmission is blocked. Therefore we conclude that the spontaneous activity of the afferent nerve fiber of ampullary electroreceptor organs, is not generated by continuous neurotransmitter release.
The two alternative mechanisms by which the spontaneous activity can be generated, as suggested above are, as far as we can see equally likely. Future research will have to discriminate between these options.

Chapter four

The dendritic tree of an ampullary electroreceptor organ; morphology and functional modeling

Summary
1. The afferent nerve fiber of an electroreceptor organ was stained immunocytochemically. This staining shows that the afferent nerve branches heavily, so there are more nerve terminals than there are electroreceptor cells in the ampulla.
2. A three dimensional image was made using a confocal scanning laser microscope. The morphology obtained from this picture is numerically reconstructed with a semi-automatic image analysis system.
3. The morphology of the afferent nerve fiber was used to formulate a model, involving passive membrane properties and Hogkin and Huxley type kinetics. Simulations then show that some of the behavior of the electroreceptor organ can be reproduced with this relatively simple model, indicating that the shape of the tree is important for the receptor organs' functioning.

1. *Introduction*

Ampullary electroreceptor organs of catfish are dedicated to sense electric fields that occur in the habitat of the fish. A receptor organ consists of an ampulla in which approximately 20 electroreceptor cells can be found. All receptor cells of the organ project to one afferent nerve that is part of either the posterior or the anterior lateral line nerve (McCormick, 1982). The afferent fiber exhibits spontaneous activity, which is modulated by synaptic activity of the electroreceptor cells.

In previous research, the functioning of the organ as a whole was investigated. While applying an extracellular stimulus, single unit recordings of the afferent nerve were obtained. This way of studying sensory organs has a noteworthy advantage, because it allows recordings from the intact, living animal. The results obtained this way resemble the true, *in vivo*, input-output characteristics. A disadvantage is, that the organ is considered as a "black box". The successive steps in the transduction chain, such as the contribution of the receptor cells, the dendritic tree, and the spike generating zone cannot be distinguished with this method. Pharmacological studies cast some light on these questions (Peters *et al*., 1997b; Peters and Westerink, 1999; Peters *et al*., 1989), but a complete model of all transduction steps in the process of electroreception has not yet been described. Here we attempt to take a step toward such a model. Previous chapters of this thesis have dealt with experiments that were designed to investigate transduction in the electroreceptor cells. In this chapter we focus our modeling attempts on the functioning of the afferent nerve.

The properties of the afferent nerve are an important step in the functioning of the electroreceptor organ. As described in the previous chapter, the afferent nerve autonomously produces the spontaneous activity which seems essential for the functioning of the electroreceptor organ. Also, it has been shown that convergence leads to increased sensitivity (Peters and Ieperen, 1989; Peters and Mast, 1983). The mechanism of this sensitivity increase was not explained, but it was suggested that the unmyelinated fibers provide a structure where generator potentials are summed.

For these reasons it is assumed that the afferent nerve is responsible for some of the features of the receptor organs. Therefore we decided to study the afferent nerve more closely.

We did this by analyzing the geometry of the dendritic tree. The unmyelinated branches of the afferent nerve fiber was stained immunocytochemically using a neurotubulin marker. Subsequently, the geometrical parameters of the fiber were determined and analyzed with the use of a semi-automatic image analysis system. The geometrical parameters are entered into the neuron simulation program NEURON (by Michael Hines and John W. Moore, Department of Neurobiology, Duke University). Implemented in the model are passive membrane properties and Hodgkin and Huxley (HH) kinetics (Hodgkin and Huxley, 1952). This model will be shown to simulate the behavioral properties of the electroreceptor organs fairly well and enables us to explain some properties of the system.

2. Methods

2.1 Staining procedure

A tropical fresh water catfish *Kryptopterus bicirrhis* was kept in Utrecht copper free tap water at 25°C. It was sacrificed with an overdose Saffan (Glaxo, Harefield, UK).

The anal fin was removed and fixated overnight in 4% paraformaldehyde. After rinsing with phosphate-buffered saline with 0.5% Bovine serum albumin (PBS/BSA) (Sigma, St Louis, USA), the fin was incubated overnight in goat anti-mouse Monoclonal Anti-Acetylated Tubulin T-6793 (Sigma, St Louis, USA). The fin was rinsed with PBS/BSA and incubated over night with anti-goat antibody labeled with Cy3 (Sigma, St Louis, USA). Finally, the fin was rinsed with PBS/BSA again, dehydrated in an alcohol series and embedded in Depex (British Drug Houses, Poole, England).

2.2. Confocal microscopy

Data was gathered using a confocal laser scanning microscope (CLSM) (Carl Zeiss Vision, Germany). Of each organ, a stack of 32 scans of 512*512*31 voxels was made. Spatial resolution of the scans was 0.195 µm/voxel for x and y directions, for z direction 0.78 (± 0.173) µm/voxel. In this way, the data of each ampulla is represented by a stack of 32 images, each image being about 100*100 μm^2, separated by 0.78 µm.

2.3. Image processing

The stacks were analyzed semi-automatically with an IBAS imaging system (Carl Zeiss Vision, Germany). Of each segment, the start and end-points were marked. The distance between those markings was calculated using the Pythagorean theorem, which resulted the length of the segments.
The stack was transformed into a single layer projection image. In this projection image the length and area of each projected segment were determined. This area, divided by the length, provides an approximation of the average thickness of each segment.

2.4. The mathematical model

The simulation program NEURON was obtained from http://www.neuron.yale.edu.
The geometry of the dendritic tree as obtained above was entered into the model. Trifurcations were avoided by adding a small (0.1 µm) extra segment with the thickness of the parent branch. The initial segment was given a length of 100 µm to simulate an open end. A spike generating mechanism was inserted in this segment, involving a leak current I_{leak} and 2 voltage dependent currents: a sodium current I_{Na}, a potassium current I_K, using conventional HH kinetics, with an integration time step of 250 µs. The currents can be described with $I = g_{max}a^x b(V-E)$, where g_{max} is the local conductance density, a is an activation variable with order x kinetics, b is an optional inactivation variable, V is the local membrane potential, and E is the reversal potential for the ionchannel. Full descriptions of the kinetics used can be obtained from the literature (Hodgkin and Huxley, 1990; McCormick and Huguenard, 1992). Values used for g_{max} and E are given in table 1. All segments had the following passive membrane properties: axial resistance (R_a) 200 Ωcm, specific membrane resistance (R_m) 10 $k\Omega/cm^2$, specific membrane capacitance (C_m) 1 µF/cm.

	I_{Na}	I_K	I_{leak}	I_{pas}
g_{max} (mho/cm^2)	0.36	0.108	0.0003	0.0001
E (mV)	50	-77	-54.3	-70

Table 1. Values for g_{max} and E used in the quantitative description of membrane currents.

By choosing these parameters, we kept the number of model components as small as possible. Postsynaptic input was simulated with a 10 Hz sinusoidal current clamp, with varying amplitude. Interspike-intervals were measured. The reciprocal of the inter-spike-intervals is defined as the instantaneous spike frequency. The sensitivity of the organ was defined as the difference between the maximum and the minimum instantaneous spike frequency divided by the stimulus amplitude. Spontaneous activity was simulated by inserting a 3 nA D.C. current clamp in the first segment. This elicited a spike train.

3. Results

3.1. Morphology

Figure 16 shows a projection image of an afferent nerve fiber. This particular electroreceptor organ contained 16 receptor cells. The afferent dendrite of the electroreceptor organ branches strongly. All branches originated from one afferent myelinated fiber only, called the single parent afferent. This single parent afferent is indicated with a freckled structure in figure 16. The myelin sheath presumably prevented the antibodies to access the neural membrane, therefore it didn’t show clearly on the fluorescent staining. However, with normal light microscopy, it could be seen that the myelinated branches connected to form the single parent afferent (Bretschneider, 1991). We assume that the single parent afferent is the spike generating zone. In order to simplify the terminology, we refer to the single parent afferent as the soma in the model nerve fiber. However, one has to keep in mind that the soma of a real fish' electroreceptor afferent nerve cell is situated in the brain stem.

The length of the segments varies between 7 and 82 µm. The branches vary in thickness from 1.75-13.36 µm, they taper and are thinnest at the terminals. The afferent has 45 terminals, connecting to 16 cells. In other words, each receptor cell forms more than one synapse with the afferent nerve. This implies that the convergence is considerably stronger than we initially thought: not 16 cells to1 nerve fibre per organ, but 45 synapses to 1 nerve.

3.2. Spike activity

An example of the soma membrane potential as determined in the model, is given in figure 17. The frequency of the spontaneous activity is 74.5 Hz. The average number of spikes per time interval does not change; the response is only modulated.

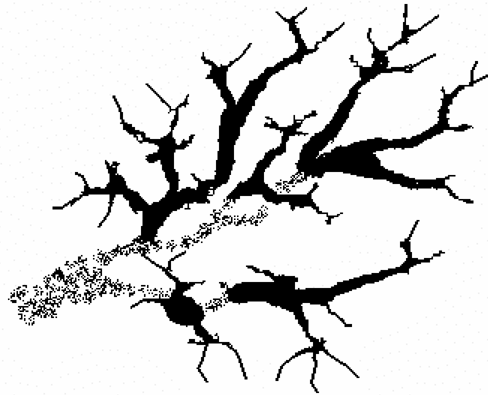


Figure 16. Projection image of the afferent nerve fiber. In black are the branches as measured by the IBAS system. The single parent afferent is indicated with the freckled structure.

3.3. Stimulus-response curves

The stimulus response curves have sigmoid shapes for electroreceptor organs (Bretschneider *et al.*, 1980). In order to see if our model fits the same function, we tested several stimulus amplitudes. Every ending of the afferent nerve has a sinusoidal current clamp inserted with the same amplitude, the value we set for each of the 45 current clamps refers to the stimulus amplitude .

An example of the spike train resulting from strong stimulation (0.1 nA) is given in figure 18. In this figure the stimulus is added as a gray trace in arbitrary units. With the positive phase of the stimulus the spike frequency increases, but the spike amplitude decreases. Then, at the negative phase of the stimulus the nerve is silent until the stimulus increases again, and crosses the spike initiation threshold. At this stimulus strength, the model nerve is outside its linear range.

The decrease in amplitude of the spikes can be explained with the inactivation curve of the I_{Na}. Figure 19 is similar to figure 18. The spike frequency is depicted in gray and the black trace represents the action of the HH h-gate. A low value of h means a high percentage of inactivated channels. At the peak of the stimulus, the spike frequency is high and the inactivation of the I_{Na} is not removed sufficiently to produce a spike of the same amplitude as the previous one. During the negative pulse of the stimulus, the inactivation is removed to a large extent, so at the next action potential a lot of channels participate resulting in a extra large spike.

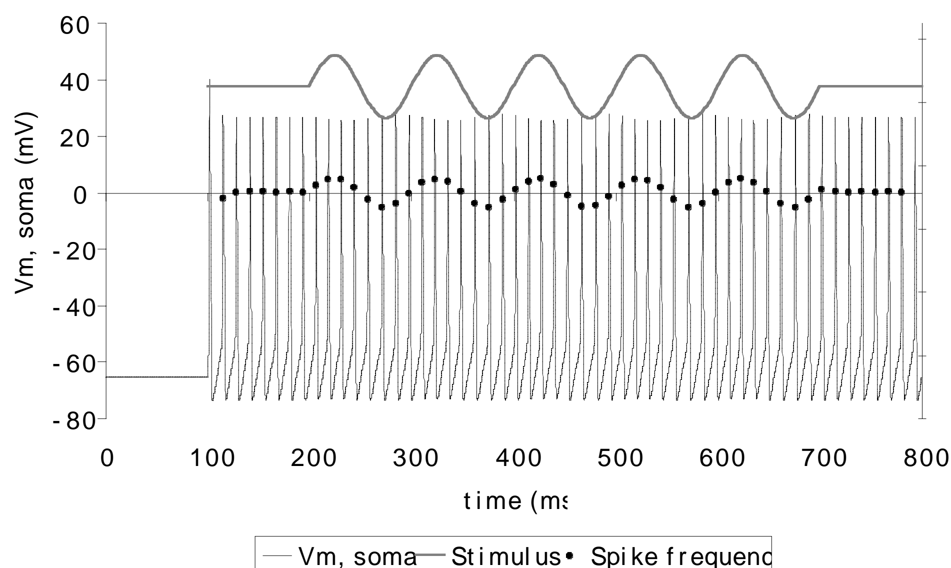


Figure 17. Soma membrane potential with and without a stimulus. The stimulus waveform is depicted in gray, the soma membrane potential in black. From 0 to 100 ms, the model is allowed to reach the steady state values. The 3 nA current clamp is switched on 100 ms after the beginning of the trial inducing spontaneous activity. At 300 ms the sinusoidal stimuli are switched on at the ends of the dendritic tree. These stimuli result in a linear response of the spike frequency. At 700 ms the sinusoidal stimuli are switched off, and the system resumes the spontaneous activity. The black dots represent the instantaneous spike frequency. Each dot represents a sample (spike), the shift of the dots along the Y-axis represents the inverse of the interval times between the subsequent spikes.

The input-output curve constructed from the responses to different stimulus strengths is shown in figure 20**.** Positive stimuli, and weak negative stimuli show a linear relation in the range that was measured. Stimuli more negative than -0.001 nA result in non-linearity. At stimulus strengths between -0.01 and 0.01 nA the I/O-curve behaves linearly. The slope of this linear part of the curve represents the sensitivity and has a value of 472 Hz/nA. The intercept with the Y-axis represents the spontaneous activity and amounts to 76.3 Hz.
At stimulus strengths exceeding 0.01 nA, the model nerve fiber shows non-linear behaviour, which is also asymmetrical around the Y-axis. *3.4. Geometrical variations*
Unto now, we simulated a synapse (current clamp) on all endings of the dendrite. However, we do not know whether all endings bear a functional synapse, and if so, if they are all functional. Furthermore it may be that not all cells are functional. Since the electroreceptor cells form the outer cell layer of the fish they are susceptible to damage. The turnover may therefore be high, as already suggested by Jørgensen (Jorgensen, 1992).

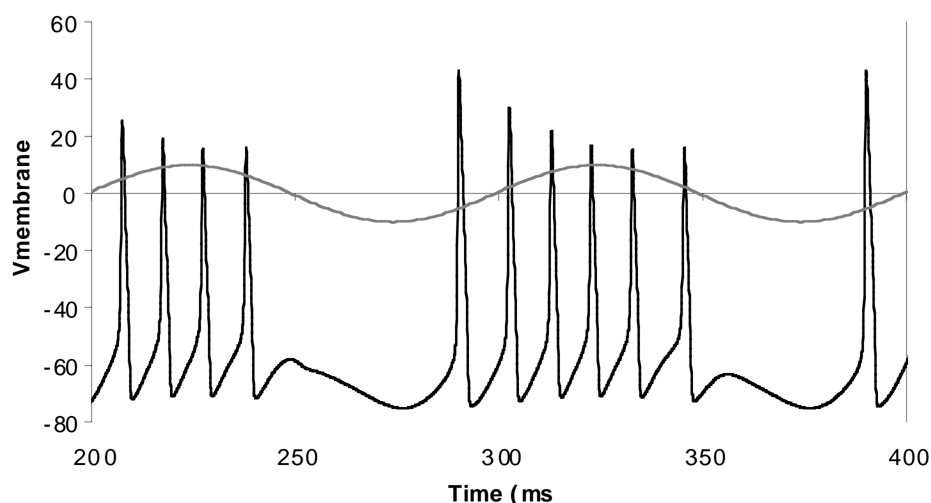


Figure 18. Soma membrane-potential changes due to strong stimuli. In gray, the stimulus is depicted, in black the membrane potential. The negative pulse of the stimulus is below the action potential initiation zone. Therefore the response is out of the linear range.

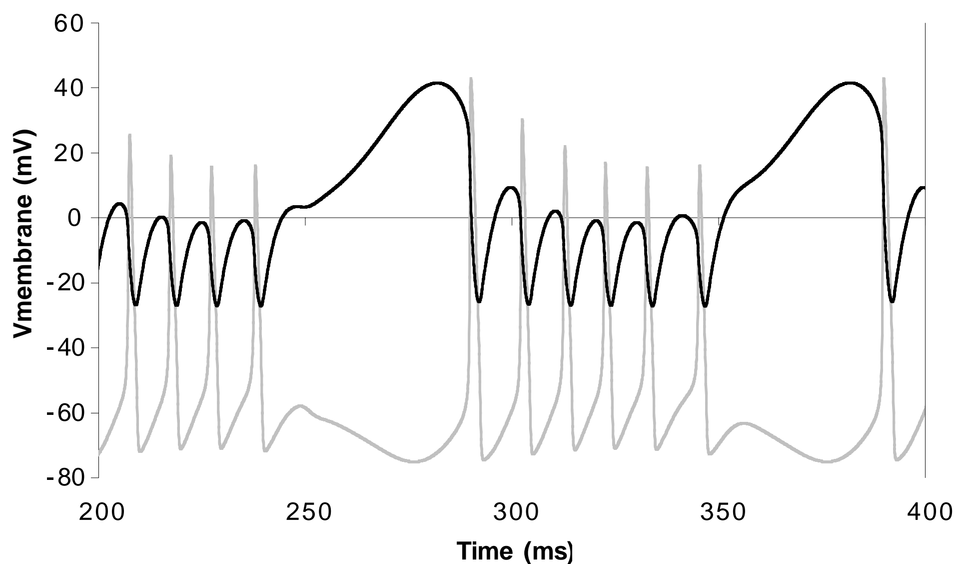


Figure 19. Gating properties of I_{Na} in the spike generator. Spike frequency is depicted in gray, the I_{Na} h-gating in black. A low value of h means high inactivation. The strong stimulus prevents removal of the inactivation. This leads to a decrease in spike amplitude.

In order to test the effects of a reduced number of inputs on the sensitivity, we simulated 3 experiments with different geometrical parameters. The first experiment (A) is similar to the results we already showed, it represents the entire dendritic tree, with at every terminal a sinusoidal I-Clamp to simulate a synapse. The second experiment (B) represents the entire dendritic tree with a sinusoidal I-Clamp at 5 terminals only. In the third experiment (C) only part of the model dendritic tree was simulated, namely the part that leads to the same 5 endings as in experiment B. Again I-Clamps were inserted, at these 5 terminals to simulate synapses. The results of the simulations are depicted in figure 21. In experiments A, B and C only the linear part of the stimulus-response characteristic is considered. In the graph, the crossing of the curves with the Y-axis represents the spontaneous activity of the system.

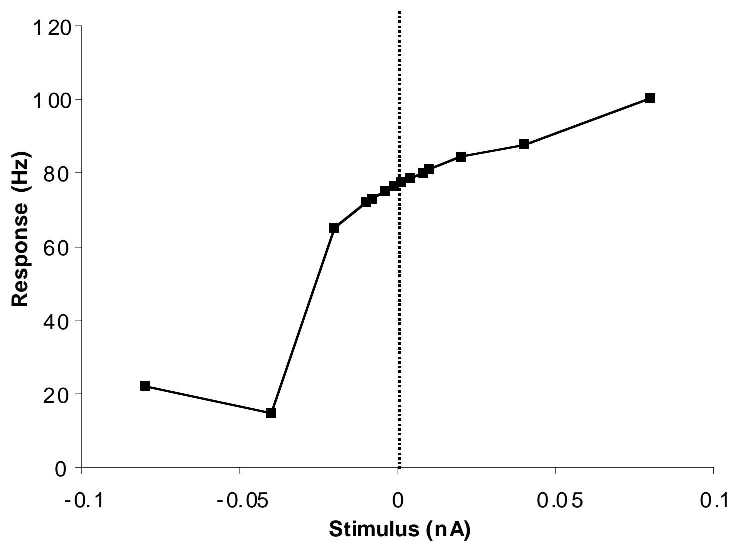


Figure 20. Stimulus response curve of the model afferent nerve.

The slopes of the curves represent the sensitivity. The values for sensitivity and spontaneous activity of the 3 experiments are given in table 2. The entire tree with all synapses, as simulated in experiment A is the most sensitive (472 Hz/nA). The spontaneous activity of experiment B is similar, but the sensitivity is much smaller: 49.3 Hz/nA. In experiment C the sensitivity is approximately equal to that in experiment B (44.6 Hz/nA), but the spontaneous activity is raised to 83 Hz. This increase in spontaneous activity is a result of the smaller area of membrane. The passive membrane of the dendritic tree contains ion channels with a reversal potential of –70 mV (table 1). A large passive area will contain a lot of those channels, which will hyperpolarize the cell membrane potential towards –70 mV. Thus, a smaller passive membrane area will have a membrane potential closer to that of the regenerative membrane which is –65 mV. Therefore a fiber with a relatively small passive dendritic tree will have a high spontaneous activity.

Experiment	Shape	Input	Sensitivity (Hz/nA)	Spontaneous activity (Hz)
A	Entire tree	45 synapses	472	75
B	Entire tree	5 synapses	49	75
C	One branch	5 synapses	45	83

Table 2. Values for the sensitivity and the spontaneous activity of the experiments with geometrical variations.

4. Discussion

The branching of the dendritic tree proved quite extensive. The structure suggests that all electroreceptor cells receive mult Its does contribute to the functional properties of the electroreceptor organ. This is illustrated by the fact that the passive membrane area influences the spontaneous spike rate (figure 21). Furthermore, the number of functional synapses influences not only the sensitivity of the system, but also the range of the linear behavior.

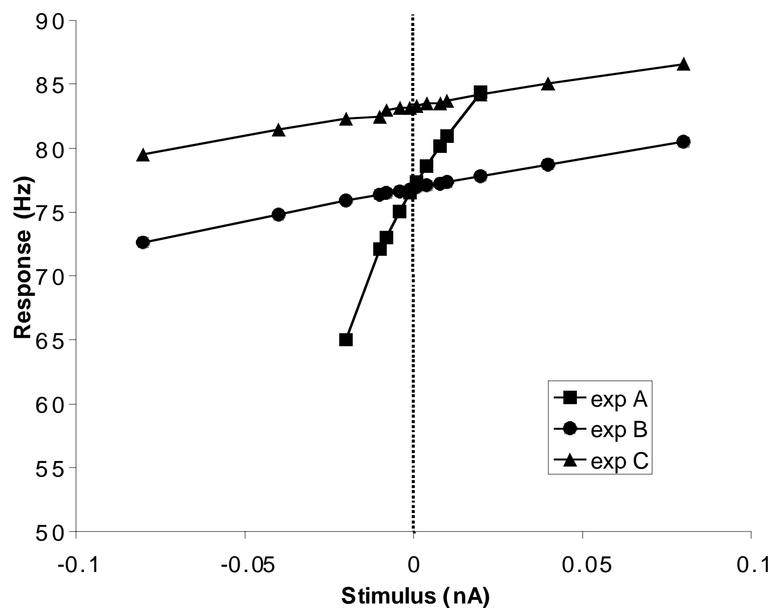


Figure 21. Stimulus response curve of the model afferent nerve, for different geometrical variations. Experiment A engages the entire afferent nerve fiber, with at every terminal a current clamp to simulate a synapse. Experiment B involves the entire dendritic tree with a sinusoidal current clamp at 5 terminals only. In experiment C only part of the model dendritic tree was simulated, with 5 terminals containing a current clamp.

4.1. The model organ versus the electroreceptor organ

Phenomenologically, the model simulates real recordings quite well as far as the following aspects are concerned. The average frequency over longer time periods is,

within limits, independent of the stimulus strength. Furthermore, reduction of spike amplitude occurs at strong stimuli. In the past it was hypothesized that this could be due to saturation of the sodium and potassium currents. With our model we showed that inactivation behavior of I_{Na} is sufficient to explain this behavior of the afferent fiber. In experiments where the ampullary lumen was electrically isolated, the sensitivity of electroreceptor organs is estimated to be between 250 and 11,300 Hz/nA, (Bretschneider *et al*., 1991). The maximum sensitivity we found with the model was 472 Hz/nA, which is within the empirical range. The spontaneous activity of the model nerve is 76.3 Hz, which approximates the spontaneous activity of the real organ (Bretschneider *et al*., 1985).

We can argue that only a subset of the synapses is active, either because they are simply not all functional or due to the turnover of the receptor cells (Jorgensen, 1992). This results in a decrease of sensitivity (figure 21). But if only a subset of the synapses is functional why then is the branching of the dendritic tree so extensive? It has been shown experimentally that convergence leads to increased sensitivity (Peters *et al*., 1997a; Peters and Ieperen, 1989; Peters and Mast, 1983). From figure 21, experiment C it can also be inferred that a large area of passive membrane does not reduce the sensitivity of the organ. Therefore it may pay off to just make as many potential places for a synapse as possible. Every extra synapse adds to the sensitivity, whereas surplus passive membrane area doesn't seem to be a substantial load on the system.

In vivo, the stimulus amplitude-response curves of the electroreceptor organ have a symmetrical, sigmoidal shape with a minimum spike frequency of 0 Hz, and a maximium of 200 Hz. In the extracellular experiments, a signal averager was used. With a signal averager, intervals with no spikes lead to a stimulus amplitude of 0. In the model nerve fibre we do not find a spike frequency of 0 Hz because of a difference in methodology. In our simulation we simply measured the interval between two spikes. This never leads to a spontaneous activity of zero. Still, a minimum value is reached at a stimulus amplitude of -0.08 nA. In order to match the symmetrical, sigmoidal stimulus response curve, we expect a saturation of the maximum response at a stimulus amplitude of 0.08nA. However, in the model cell, the spike frequency does not saturate at these stimulus strengths. This makes it likely that in the real electroreceptor organ, the maximum spike frequency is limited by neurotransmitter release of the electroreceptor cells, rather than the spike generating mechanism. Neurotransmitter release is not implemented in our model, and that is why we do not see a flattening of the curve at strong stimuli (figure 20).

4.2. Concluding remarks

In this chapter we presented the shape of the dendritic tree of an electroreceptor organ afferent nerve fiber. With a relatively simple mathematical model, we simulated some of the properties of the receptor organ. Indeed, the shape of the dendritic tree seems to have functional implications for the sensitivity. The mathematical model may help to explain the mechanism of convergence. Also, it will be interesting to expand the model with more realistic synapses, and maybe even receptor cell models. The spike generator should be refined in order to match the kinetics to the tropical temperatures, of the normal habitat of our experimental habitat of our experimental animal.

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General discussion

Signal transduction in the receptor cells of the ampullary electroreceptor organ is different compared to other sensory systems. The stimulus and the response are of the same modality: electricity. The result of this is that the various steps in the signal transduction make use of similar components: ion channels. Previous investigations to the transduction mechanism involved pharmacological manipulations of the electroreceptor organ (Bennett and Clusin, 1979; Lu and Fishman, 1995a; Lu and Fishman, 1995b; Peters *et al.*, 1989; Roth, 1982; Sugawara, 1989; Sugawara and Obara, 1984b; Zhadan and Zhadan, 1975). The effects on the system were apparent, but which part of the system was affected by the chemicals was hard to determine. The main effort of this thesis is to separate the functioning of the electroreceptor cells from that of the afferent nerve fiber. This is not trivial, since the functional components of the output signal, spontaneous activity and sensitivity, are believed to have separate origins (Bretschneider and Peters, 1992). Firm evidence for this hypothesis is presented in chapter 3, where the neurotransmission of the electroreceptor cells to the afferent nerve fiber has essentially disappeared. This leads to the suppression of the sensitivity of the organ, whereas the spontaneous activity remained unaffected. The conclusion of this result is that reading the output of the afferent nerve fiber does not necessarily yield the output of the electroreceptor cells. Therefore, the transduction mechanism of electroreceptor cells cannot be investigated by just reading the input-output characteristics of the entire organ, they have to be investigated separately of the rest of the system. The conclusion that the sensitivity and the spontaneous activity can be manipulated independently was already drawn by Roth. He found that application of Co^{2+} and La^{2+} decreased the sensitivity but not the spontaneous activity of a *Kryptopterus* electroreceptor organ (Roth, 1982). However, in the same study he found that changing the content of the water with other ions, such as Ca^{2+} or K^{+} did affect the sensitivity as well as the spontaneous activity. Similar effects are found by others (Bauswein, 1977; Zhadan and Zhadan, 1975) Furthermore, apically administered ion channel blockers also reduce both sensitivity as the spontaneous activity (Peters *et al.*, 1989). The latter experiments show that the two functional properties of the electroreceptor are not completely independent of each other. It may be that there is a causal relation between the two. More likely, the generating mechanisms of the spontaneous activity and the sensitivity are both influenced by the application of the ions and the ion channel blockers.

The patch clamp experiments described in the first chapter were meant to do this, and would have been the best way, if it weren't for the experimental difficulties. One thing that the patch-clamp experiments showed is a large passive component in the membrane currents. The passive component probably originates in the apical membrane of the electroreceptor cells, where it guarantees a low electrical resistance. The relevance of a low electrical resistance in the apical membrane is that the stimulus is able to enter the cells unattenuated. The calcium measurements in chapter 2 gave us the opportunity to study what is going on in normal functioning electroreceptor cells. Indeed a depolarizing stimulus induces a calcium current in the electroreceptor cells; evidence for calcium channels in the membrane of the electroreceptor cells. This calcium current reflects the stimulus waveform (figure 9),

showing that the calcium current is voltage dependent. The stimulus-evoked calcium channel could be repressed by application of sodium and potassium channel blocking agents. Blocking sodium and potassium channels, leads to the attenuation of the stimulus at the apical membrane, and consequently to a smaller response. We conclude that sodium and potassium channels, perhaps in cooperation with other channel types, conduct the ion currents that depolarize the electroreceptor cells.

Now we can adapt the original model of Bennett with the findings in chapters 1 through 3. These adaptations are illustrated in figure 22. Sodium and potassium channels are added to the apical membrane, and the spontaneous spike activity is an intrinsic property of the fiber, rather than the result of a continuous depolarization of the receptor cells.

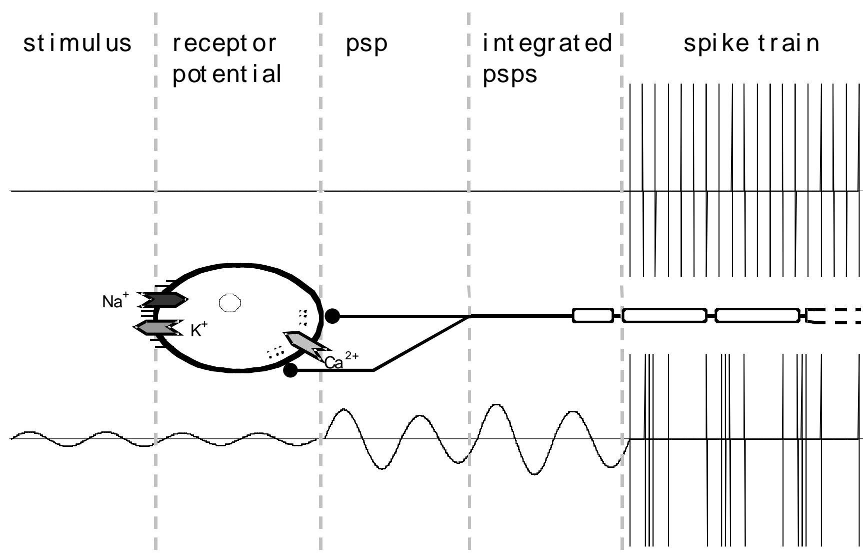

Figure 22. Schematic model of the electroreceptor organ functioning. The scheme is adapted from the Bennett model (figure 2) according to our findings. Compared to the scheme shown in the introduction there are a few adaptations. Sodium and potassium channels are added in the apical membrane. In the upper trace no stimulus is present, and the receptor cells are in resting condition. The spontaneous activity is autonomously generated in the spike generating zone.
The lower trace represent the stimulus induced response. Since the resting condition is defined as zero (see text), the post synaptic potentials vary around zero.

In figure 22 the nerve potential as it is when no electrical stimulus is applied, is defined as the resting potential. One has to keep in mind that the spiketrain is up and down modulated by an electrical stimulus. This implies that somehow, there must be a continuous activity in both the electroreceptor and the afferent nerve. In the present model, we define the resting condition as the condition when the organ is not stimulated. This is customary in other types of neural systems, for example the retina. The photoreceptor cells in the vertebrate retina are continuously depolarized when not stimulated: in the dark. This is referred to as the resting membrane

potential. Only when the photoreceptor cells in the retina are stimulated with photons, the depolarization is relieved.
As for the mechanism of up and down regulation of the spike activity a few remarks can be made. It is highly unlikely that the system engages a push-pull mechanism with two neurotransmitter types: one for depolarization (glutamate) and one for hyperpolarization (GABA). The main reason for this is that Saffan, the anaesthetucum we use in our experiments inhibits the GABA-ergic neurotransmission. If GABA is involved in the sensitivity of the electroreceptor organ, it will be impaired by Saffan administration, which is not the case. In earlier days, the fish were often sedated with the anesthetic MS222, or immobilized with the muscle relaxant Flexedil. No structural differences in either sensitivity or spontaneous activity can be found with any of the 3 drugs.
Continuous neurotransmitter release could be a mechanism involved in the up and especially the down regulation of the spike train by an electrical stimulus. In chapter 3 it is shown that the spontaneous activity is not dependent on neurotransmitter release. However, the sensitivity is. There may be a continuous release of neurotransmitter going on merely to ensure the two way modulation of the electroreceptor organs output.
Alternatively, the answer to this problem may be solved by adaptation of the receptor cells. In chapter two, figure 9 It can be seen that the Ca^{2+} influx follows the sine-shape of the stimulus. When the stimulus is switched on, the average $[Ca^{2+}]_i$ is elevated, and the $[Ca^{2+}]_i$ does not get lower than the resting level. This way, the receptor cells seem to adapt to the stimulus.

In chapter four we continue with our attempts to separate the components of the system. We showed that the afferent nerve fiber shows extensive branching. With a mathematical model we showed that the branching pattern has functional implication for the electroreceptor organ. The model of the afferent nerve fiber only allowed us to elucidate some aspects of the systems behavior. For example, the sigmoidal shape of the input-output characteristic as recorded *in vivo*, does not show in the model, indicating that it must be the result of a process earlier in the system such as neurotransmitter release.

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Samenvatting in het Nederlands

Dit proefschrift handelt over signaaltransductie en -transmissie in ampullaire electroreceptor organen van meervallen. Electroreceptororganen spelen een sleutelrol in het vermogen van meervallen om electrische velden waar te nemen: electroreceptie.
Alle in het water levende organismen genereren een electrisch veld, dit ontstaat door bv. Ademhaling met de kiewen, stofwisseling, wondjes aan de huid etc. Omdat het electrische geleidingsvermogen van water veel beter is dan dat van lucht zijn electrische velden voor waterdieren bruikbaar als zintuigelijke stimulus. Electroreceptoren komen daarom vrijwel uitsluitend voor bij waterdieren. In het modderige water waar meervallen leven is het zicht vaak slecht .Dit maakt electroreceptie een belangrijke eigenschap om te overleven. De informatie die in het electrische veld van een organisme besloten ligt, is bijvoorbeeld de grootte, en de plaats van het waargenomen object. Is het een dier of de plant, een prooi of een predator? Beweegt het of niet? Soortgenoten worden aan hun electrische veld herkend, en misschien is zelfs te bepalen of er sprake is van een manlijk of een vrouwelijk individu. Voor het onderzoek dat in dit proefschrift wordt beschreven zijn twee soorten vissen gebruikt; de Amerikaanse meerval *Ictalurus sp*. en de Aziatische meerval *Kryptopterus bicirrhis* ofwel glasmeerval. Van beide soorten staat een afbeelding op de voorkant van deze dissertatie. Meervallen zijn specifiek gevoelig voor wisselspanningen in de grootteorde van een microvolt per centimeter (μV/cm) tot enkele millivolts per cm (mV/cm). Ter verduidelijking: 1 μV/cm = 1,5 volt per 15 kilometer. Dit betekent dat een electrische veld zo groot als dat van van een penlight batterij, beginnend bij Urk tot in Lelystad door de meerval kan worden waargenomen!
Om electrische velden te kunnen waarnemen hebben meervallen de beschikking over gespecialiseerde orgaantjes: de ampullaire electroreceptor organen. Ampullaire electroreceptor organen zijn kleine holtes in de vissehuid waarin electroreceptor cellen liggen. Figuur 1 (p 6), is een afbeelding van een electronenmicroscopische opname van een dwarsdoorsnede van zo'n receptororgaan bij een glasmeerval. In deze figuur is te zien dat de kleine ronde electroreceptorcellen (RC) geclusterd op de bodem van de ampul liggen. Via de ampullaire opening (O) staan de receptor cellen direct in verbinding met het buitenmilieu dit is het zoete water van het meer of de rivier waarin de vis zwemt. Figuur 3 (p 14) is een bovenaanzicht van zo'n orgaan te zien.
De receptorcellen zijn verbonden met een zenuwvezel middels een zogenaamde chemische synaps. Een chemische synaps is een structuur die voor communicatie tussen cellen dient. Als cel A een signaal door wil geven aan cel B, scheidt cel A bij de synaps een bepaalde stof af; een neurotransmitter. Deze neurotransmitter wordt door cel B herkend, en die kan er op reageren. De hoeveelheid afgegeven neurotransmitter is een maat voor de sterkte van het signaal.
Met bestaande gegevens kan er een hypothese geformuleerd worden over hoe het electroreceptororgaan werkt, een proces dat in principe uit twee fasen bestaat: stimulustransductie en stimulustransmissie. Het electrische veld van bijvoorbeeld een prooidiertje uit de omgeving wordt opgevangen door de electroreceptorcellen en brengt daarin een electrischepotentiaal verandering teweeg. Deze potentiaalverandering wordt doorgegeven naar de synaps waar het zorgt voor de opening van ionkanalen. Ionkanalen zijn poortjes in de membraan van de cel die al dan niet doorlatend kunnen zijn voor geladen moleculen (ionen), zoals calcium (Ca^{2+}), natrium (Na^{+}), kalium (K^{+}) en chloride (Cl^{-}). Het openen en sluiten van de kanalen wordt geregeld door de electrische spanning

over de celmembraan. Door de potentiaalverandering bij de synaps gaan er calcium-kanalen open waardoor er Ca^{2+} de cel in stroomt. Het Ca^{2+} in de cel zet op zijn beurt het mechanisme aan dat voor de neurotransmitter-afscheiding zorgt. De afgescheidde neurotransmitter induceert in de zenuwvezel ook een electrische potentiaalverandering, die voor verdere verwerking naar de hersenen wordt doorgegeven. Het opvangen van de stimulus en het doorgeven ervan naar de synaps heet transductie. Het overdragen van de stimulus naar de zenuwvezel heet transmissie.

Hoofdstuk 1
Signaaltransductie in de electroreceptoren gaat middels ionkanalen. De externe stimulus gaat door de apicale membraan (dit is het stuk van de cel membraan dat in contact is met het externe milieu) en geleiding van electriciteit gaat in de biologie altijd middels ionen. De vraag is echter welke ionen door de apicale membraan kunnen. Om dit uit te zoeken hebben we geprobeerd gebruik te maken van een electrofysiologische techniek die sinds de jaren tachtig van de vorige eeuw gangbaar is: de patch clamp. Bij deze methode wordt een glazen electrode tegen de membraan van de cel gezet, waardoor er een electrisch dichte verbinding ontstaat. De electrode is in wezen een hol glasbuisje dat een zoutoplossing bevat. Met wat geluk komt de electrode precies op de plek waar een ionkanaal in de membraan zit. Het openen en sluiten van een ionkanaal is een zeer snel alles-of-niets proces dat in een electrofysiologische meting goed zichtbaar is. In de electroreceptor cellen hebben we een keer zo'n meting kunnen doen. Deze is weergegeven in figuur 5 (p 19).
Bij de patch clamp metingen gebruikten we *Kryptopterus bicirrhis* als proefdier. Deze 7 cm lange vis heeft een hele speciale eigenschap: hij is voor het grootste deel transparant en wordt daarom ook wel glasmeerval genoemd. Een verdoofde vis kan onder de microscoop geplaatst worden, waardoor de electroreceptor organen duidelijk zichtbaar zijn. De receptorcellen kunnen via de ampullaire opening met een patchelectrode aangeraakt worden.
Helaas was deze methode niet erg succesvol. Ook variaties van de patch clamp techniek, waarbij alle ionkanalen in de hele cel tegelijk gemeten kunnen worden, leverden niet het gewenste resultaat. De experimentele moeilijkheden die we ondervonden zijn grotendeels te herleiden op de slechte conditie van het preparaat. Omdat de electroreceptor cellen de buitenkant van de vis vormen, worden ze door van alles bedreigd. Daarom zijn ze extra goed beschermd op een aantal manieren zoals bv een dikke laag met harde suikermoleculen die aan de apicale membraan kleeft en in de ampullaire holte bevindt zich een slijmachtige substantie. Dit maakt het formeren van de noodzakelijke electrisch dichte verbinding zeer lastig. Bovendien hebben de celletjes microvilli, een soort haren op de apicale membraan, welke ook in de weg zitten bij het formeren van de verbinding.
In andere preparaten is het niet ongebruikelijk losse cellen te patch clampen die uit het weefsel gehaald zijn. Dit vergemakkelijkt de methode aanzienlijk. Bij electroreceptoren is dit wat moeilijker omdat de apicale membraan gewend is aan zoetwater, terwijl de basolaterale membraan (het deel van de membraan dat aan de binnenkant van de vis ligt) omgeven is door weefselvloeistof. Als je de cellen uit de vis haalt (dissocieert) kun je ze bijna niet goed houden: in gewoon water gaan ze kapot omdat de basolaterale membraan daar niet tegen kan, en in een weefselvloeistof gaan de cellen kapot omdat de apicale membraan daar niet tegen kan. Daarom bleek het werken met geïsoleerde

cellen ook niet succesvol. In hoofdstuk 2 hebben we hetzelfde probleem anders aangepakt.

Hoofdstuk 2

In dit hoofdstuk wordt opnieuw gebruik gemaakt van de doorzichtige *Kryptopterus bicirrhis*. De electroreceptor cellen worden "geladen" met een fluorescente stof : FURA-2. Het bijzondere van deze stof is, dat de fluorescente eigenschappen anders zijn als het aan Ca^{2+} gebonden is, dan wanneer het ongebonden is. Op deze manier is het mogelijk met behulp van licht de hoeveelheid Ca^{2+} in de cel te meten. Omdat we te maken hebben met een doorzichtige vis kunnen we deze experimenten in een intacte vis uitvoeren, een groot voordeel zoals we in hoofdstuk 1 hebben ontdekt.
Met deze methode vonden we inderdaad dat de hoeveelheid Ca^{2+} in de cel varieert ten gevolge van een electrische stimulus (dit is geïllustreerd in figuur 9, p 31). Een Ca^{2+} instroom speelt dus een belangrijke rol in electroreceptie. Het grootste deel van de Ca^{2+} stroomt waarschijnlijk bij de synaps de cel in, zoals de hypothese voorspelt.
Verder kon de stimulus-geïnduceerde Ca^{2+}-stroom gemanipuleerd worden door andere typen ionkanalen (n.l. Na^{+}- en K^{+}-kanalen) te blokkeren met specifieke chemicaliën. Hiermee is bewezen dat de signaaltransductiestroom via Na^{+}- en K^{+}-kanalen gaat. Door deze transductiestroom ondergaat de basolaterale membraan bij de synaps een potentiaal verandering, waardoor het openen en sluiten van de synaptische Ca^{2+}-kanalen wordt geregeld.

Hoofdstuk 3

Eén van de bijzonderheden van het electroreceptororgaan is dat het zowel positive als negatieve stromen kan meten. Dit kan doordat het orgaan 'spontaan actief' is. Spontane activiteit is het verschijnsel dat de zenuwvezel van het orgaantje in rust toch zenuwpotentialen genereert. Zenuwpotentialen zijn korte electrische pulsen die met een electrode in het ampullaire lumen gemeten kunnen worden. Door een positive stimulus aan te bieden gaat de activiteit van de zenuw omhoog (meer zenuwpotentialen per seconde), en door een negative stimulus gaat de activiteit van de zenuw omlaag (minder zenuwpotentialen per seconde).
De centrale vraag in dit hoofdstuk is hoe de spontane activiteit tot stand komt. Aan één kant zijn er aanwijzingen dat de electroreceptorcellen continu neurotransmitter afgeven, waarop de zenuw continu zou reageren. Aan de andere kant zijn er aanwijzingen dat de spontane activiteit in de zenuw zelf gegenereerd wordt. Om uitsluitsel te geven over deze twee mogelijkheden, is in de experimenten van hoofdstuk 3 de synaps tussen de electroreceptorcellen en de zenuwvezel uitgeschakeld met het zenuwgif tetanus toxine. Het gif werd toegediend aan de binnenkant van een plakje vissehuid zodat het direct bij de synaps kon komen. Voor de experimenten werd de amerikaanse meerval *Ictalurus sp* gebruikt.
Het resultaat van het toedienen van het gif was dat de gevoeligheid van de electroreceptoren zeer sterk afnam: de zenuw kreeg geen informatie meer van de receptorcellen over het aangeboden electrische veld. De spontane activiteit van de zenuw ging echter onverminderd door.
Aan de hand van dit experiment kan er geconcludeerd worden dat de spontane activiteit autonoom door de zenuw gegenereerd wordt.

Hoofdstuk 4
In dit hoofdstuk is er een kleuring gemaakt van de zenuwvezel van een electroreceptororgaan van de glasmeerval. De zenuwvezel blijkt sterk te vertakken, zodat hij met elke electroreceptorcel meerdere synapsen heeft. De vorm van een zenuwvezel is belangrijk voor de manier waarop hij functioneert. We hebben, met behulp van een kleurings methode, de vorm van de zenuwtakjes nauwkeurig geanalyseerd en gekwantificeerd. Die gegevens zijn in een wiskundig model verwerkt. Het gedrag van het wiskundige model is vervolgens vergeleken met metingen die we in het echte electroreceptororgaan gedaan hebben. Voor een aantal aspecten bleken het model en de werkelijkheid goed overeen te komen. Een aantal grootheden die een belangrijke rol spelen in het model is echter moeilijk te bepalen. Dit is wellicht een taak voor de volgende AIO bij de neuroethologie groep.

Dankwoord

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Curriculum Vitae

De schrijfster van dit proefschrift werd geboren in Heemskerk op 28 december1970.
Op het Berlingh college te Beverwijk haalde zij een MAVO-D (1987), HAVO (1989) en een VWO (1991) diploma. In de zomer van 1991 ging zij medische biologie studeren aan de Universiteit van Amsterdam. Tijdens deze studie deed zij drie stages. De eerste was aan het Nederlands Instituut voor Hersenonderzoek, bij de groep Neuronen en Netwerken onder begeleiding van Drs. R.R.H Nuijtinck. Het onderwerp van deze stage was het karakteriseren van een primaire hippocampuskweek. Daarna ging zij terug naar de biologie faculteit in Amsterdam voor de tweede stage. Deze voerde zij uit onder leiding van Dr. T.A. Werkman. Het betrof een onderzoek naar de ontwikkeling van calcium-stromen in normale ratten en ratten zonder bijnier. De derde stage vond plaats aan het Karolinska Instituut in Stockholm. Hier werkte de schrijfster mee aan een Positron Emitting Tomography-onderzoek naar functionele aspecten van de motorcortex. Deze stage werd zij begeleid door Prof. Dr. H. Uylings (N.I.H., Nederland) en Prof. Dr. P. Roland (K.I., Stockholm). In augustus 1996 studeerde zij af, en werkte een half jaar als vrijwilliger op het Academisch Medisch Centrum in Amsterdam bij de vakgroep Experimentele Neurobiologie onder leiding van Dr. F. Baas. Het betrof hier een moleculair biologische studie naar transgene neuroblastoma cellen.
Op een april 1997 volgde een aanstelling van vier jaar als assistent in opleiding bij de vakgroep Neuroethologie van de Universiteit Utrecht. Het betrof een onderzoek naar signaaltransductie en -transmissie in ampullaire electroreceptor organen van de meerval. Het onderzoek werd begeleid door promotor Prof. Dr. Ir. W.A. van de Grind en Co-promotoren Dr. F. Bretschneider en Dr. R.C. Peters. Per 1 Juli 2001 is zij aangesteld als Post-doc bij het Interfacultair Oogheelkundig Instituut.

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