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Molecular Mechanisms of Local Anaesthetic Action on Voltage-Gated Ion Channels

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"I'd like to know
what this whole show
is all about
before it's out"

-Gruck by Piet Hein

ABSTRACT

In this thesis I try to clarify some molecular mechanisms of local anaesthetic action on ion channels. The traditional view is that local anaesthetics eliminate action potentials by a direct block of Na channels. Other mechanisms, however, have been suggested. For instance bupivacaine has been proposed to affect Na channels in myelinated axons indirectly, by making the resting potential less negative. To test this hypothesis we analysed the effects of bupivacaine on voltage-clamped myelinated axons from *Xenopus laevis*. Contrary to the suggested hypothesis, the leak current and the resting potential were unaffected. Furthermore, the blocking effect on the K current was explained by two population-specific mechanisms.

In order to gain further insights in the molecular mechanisms of local anaesthetic action, we analyzed the effects of bupivacaine on a series of voltage-gated K (Kv) channels and various mutant channels, expressed in *Xenopus* oocytes. Two phenomenologically different blocking effects were seen; a time-dependent block of Kv1 and Kv3 channels, and a time independent block on Kv2.1. Swapping the S6 helix between Kv1.2 and Kv2.1 introduced Kv1.2 features into Kv2.1. The results suggest that bupivacaine blocks Kv channels by an open-state dependent mechanism, and that Kv2.1 deviates from the other channels in allowing a partial closure of the bupivacaine-bound channel. The results also suggest that the binding site in Kv2.1 is located in the internal vestibule and that residues in the descending P-loop and the upper part of S6 are critical for the binding.

The location of the binding sites was further investigated by automated docking and molecular dynamics methods, using homology models of Kv1.5. Two different models were constructed to describe an open channel. They were based on the crystallized bacterial channels KcsA and MthK. The first model corresponds to a PVP-type of bending hinge in the internal helices, while the second corresponds to a Gly-type of bending hinge. The automated docking and molecular dynamics calculations combined with free energy estimations predicted strongest binding to the PVP region. Surprisingly, no binding was predicted for the Gly-bend model. The results support our electrophysiological data showing that Kv1.5 is unable to close when bupivacaine is bound to the channel.

The standard view of local anaesthetic action assumes a preferential binding to channels in inactivated state. Recently, this view has been challenged. We investigated this issue by comparing the effects of bupivacaine on inactivating and non-inactivating Kv channels of similar subtypes, hypothesizing that the bupivacaine affinity is similar for the two types due to the structural similarities. The results can be explained by a simple kinetic scheme, deviating from the standard scheme in assuming local anaesthetic binding to channels exclusively in open state. By using data from Na channel experiments we could in simulations show that the basic scheme could be used to clarify controversial issues about local anaesthetic effects on Na channel inactivation.

LIST OF PUBLICATIONS

I. **Nilsson J**, Elinder F and Århem P (1998) Mechanisms of bupivacaine action on Na^+ and K^+ channels in myelinated axons of *Xenopus laevis*.
Eur. J. Pharmacol. 360, 21-29.

II. **Nilsson J**, Madeja M and Århem P (2003) Local anesthetic block of Kv channels: Role of the S6 helix and the S5-S6 linker for bupivacaine action.
Mol. Pharmacol. 63, 1417-1429.

III. Luzhkov VB, **Nilsson J**, Århem P and Åqvist J (2003) Computational modelling of the open-state Kv1.5 ion channel block by bupivacaine.
Biochim. Biophys. Acta 1652, 35-51.

IV. **Nilsson J**, Madeja M and Århem P. The role of inactivation for local anesthetic block: bupivacaine effects on naturally and mutationally inactivation removed Kv channels. (*Submitted*)

CONTENTS

1 INTRODUCTION

For thousands of years human beings have been aware of herbs, venoms, and food poisons that affect the nervous system. Agents causing pain, paralysis, cardiac arrest, convulsions, numbness, dizziness, and hallucinations have interested physicians of all cultures. We now know that all electrical activity of the nervous system and the brain, and thus all our behaviour, and our thinking and emotions, depends on the action of a certain class of membrane proteins, ion channels. It is, therefore, not unexpected that many of these agents act on ion channels.

Local anaesthetics are such agents. They have been clinically used for well over a century, used in controlling pain, cardiac arrhythmias and epileptic seizures, but the molecular mechanism by which they alter specific functions of the nervous system remained unclear for a long time. Investigations during the last few decades have presented evidence implicating the voltage-gated sodium channel protein in the nerve cell membrane as a target of local anaesthetic effects. Clinically important side effects may be produced by a block of voltage-gated K channels (Kv), also situated in the nerve cell membrane. For instance, bupivacaine has been reported to induce lethal arrhythmias by affecting cardiac Kv channels (Mather and Chang, 2001).

The blocking mechanism has been described as a complex state-dependent process in both Na (Hille, 1977; Hondeghem and Katzung, 1977; Butterworth and Strichartz, 1990; Hille, 2001) and K channels (Valenzuela et al., 1995), with binding preferentially in open or in inactivated channel states. The importance of S6 for the binding has been confirmed for both Na and K channels (Ragsdale et al., 1994; Franqueza et al., 1997). However, the molecular details of the blocking process are not well understood. This thesis aims to clarify some aspects of this issue. We have used the voltage-clamp technique on both the myelinated axons and on oocytes with expressed channels. In the oocyte experiments we focused on Kv channels. There are several advantages with using Kv channels. They have relatively slow and simple kinetics and do not show fast inactivation (making it possible to study the binding without complications caused by competition from the inactivation particle). They are structurally variable in the pore region, making our approach a natural site-directed mutagenesis.

To obtain further insights in the blocking mechanisms of buivacaine, we have made docking and dynamic calculations on the local anaesthetic action on homology model channels. The models are built from the crystallization data of the KcsA channel (Doyle et al., 1998) and the MthK channel (Jiang et al., 2002). These recent crystallizations have opened up totally new avenues to investigate binding processes in K channels, molecular dynamics simulations being one (Åqvist and Luzhkov, 2000).

As a background for the following discussion on the local anaesthetic blocking mechanisms, a brief account of the structure and function of ion channels will be given.

1.1 ION CHANNELS – THE TARGET MOLECULES

The modern race for a more detailed understanding of the electrical activity of nerve cells started with the pioneering work of Hodgkin and Huxley in 1952 (rewarded with a Nobel Prize in 1963). They showed that nerve impulses depend on specific ion currents (Na and K) through the nerve membrane. They also presented a quantitative model that has formed the basis for the present production of neural network simulations. However, the biophysical mechanisms for this model were far from understood. Pharmacological studies suggested that the fundamental units were specific proteins, and rough mechanistic models were explored in the 70's. An important step was taken when Neher and Sakmann (Nobel prize 1991) introduced the patch-clamp technique (Hamill et al., 1981) and it became evident that no other mechanism than diffusion through water-filled pores could explain the large currents through single channels. Some years later the first ion channels were cloned and sequenced (Noda et al., 1984; Tempel et al., 1987; Kamb et al., 1987) and the race for understanding nerve signaling went into a new phase, focusing on ion channel structure and dynamics.

1.1.1 Molecular organization

Ion channels come in different shapes and classes. The ion channels forming the functional background to propagated nerve impulses belong to the voltage-sensitive class. Three main families form the core of this class: K, Na and Ca channels. The structurally simplest are K channels and the evolutionary most recent ones are the Na channels. All channels show a common structure, based on a four-fold symmetry. K channels are tetramers, while Ca and Na channels are constructed as single four-domain proteins. Most likely Na channels evolved from K channels via Ca channels early in metazoan life (i.e. 800 million years ago), while the origin of K and Ca channels is much more early. K channels are found already among prokaryotes (perhaps more than 2400 million years ago).

The voltage-gated channels all comprise six helical trans-membrane segments (S1 to S6). For Na and Ca channels, a single large subunit suffices to make the functional channel, consisting of four domains forming the pore. In K channels however, the pore is built of four different subunits, four different proteins (Fig. 1). The fourth segment (S4) in all voltage-gated channel types shows a repetitive symmetrical arrangement of charged residues. Every third residue is arginine or lysine. This remarkable fact immediately made S4 the main candidate for being the voltage sensor of the channel (Noda et al., 1984). Already Hodgkin and Huxley predicted and quantitatively estimated charge movements in the membrane, gating the ion currents. Combined electrophysiological, mutational and chemical studies have confirmed the role of S4 as voltage sensor (Stühmer et al., 1989; Yang and Horn, 1995; Larsson et al., 1996).

K channels form the most diverse group of ion channels. Beside the voltage-gated channels this group also contains structurally simpler channels with only two transmembrane helices (2TM) instead of six (6TM), lacking the charged S4 helix. These channels are consequently voltage insensitive, but they show the same K^+ selectivity as the rest of the group. In 1998 MacKinnon (Doyle et al., 1998) succeeded in crystallizing such a voltage insensitive channel (KcsA) from the bacterium *Streptomyces lividans* and could reveal the structure of the central pore, common to all K channels (rewarded with the Nobel Prize of 2003). Suddenly, all the extensive theorizing about the selectivity mechanism was possible to check, and was found not to be far off the mark (Yellen, 1998). The central pore comprised, as hypothesized, a selectivity filter and a wide internal vestibule, delimited by a structure, easily conceived as a gate (Fig. 1). As will be seen, this internal vestibule is of fundamental importance for understanding the effects of local anaesthetics.

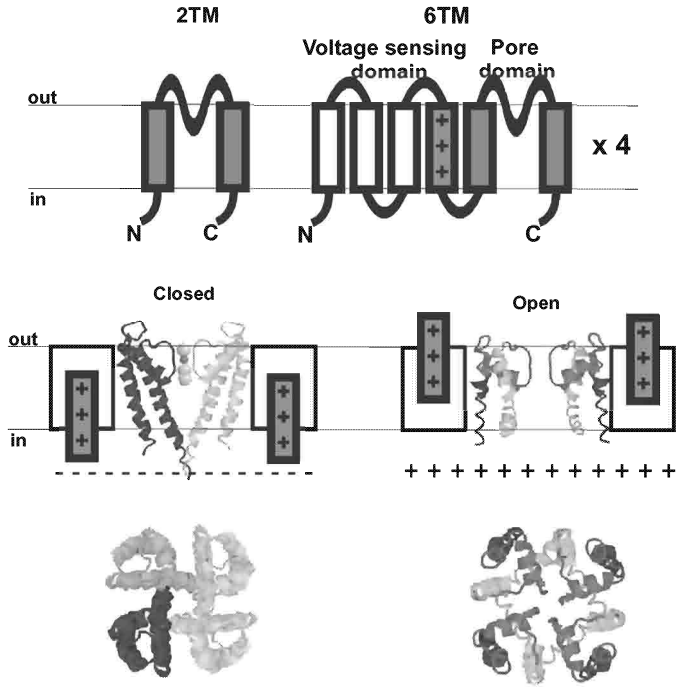

Figure 1. Molecular organization of K channels. The upper panel shows 2TM (2 Trans-Membrane segments) and 6TM subunits. Note the charged fourth segment (S4). The middle panel shows a 6TM channel in closed and open states. Note the changed positions of S4. The pore domain in the closed state channel is modeled after KcsA crystallization data and in the open state after MthK data. The lower panel shows the pore domains in closed and open states from above. (Figure from Elinder and Arhem, 2004).

Selectivity

The crystallization of KcsA thus explained the much of the problems with selectivity, and gave hints on how the gating functions. Since the detailed structure of K channels is a central theme in the studies of this thesis I will briefly discuss the main features revealed by the recent findings.

First to the selectivity filter. A typical action potential in a mammalian neuron requires millions of ions to flow in a millisecond. To accomplish this without using millions of proteins requires that each one have a very high throughput, while maintaining high selectivity, K channels are extremely selective in which ions they allow to pass, yet they allow transport rates close to the aqueous diffusion limit. The structural and architectural features of KcsA and K channels in general are thus perfectly adapted to fit their function. They solve the electrostatic problem of stabilizing ions, without making them too much more stable than they are in water, by using plenty of water and helix dipoles to counteract the unfavorable dielectric environment within the membrane. Furthermore, they solve the problem of stabilizing potassium in preference to sodium by precisely matching the configuration of oxygen atoms around a solvated potassium ion.

Voltage sensing

Even if the mechanism of voltage sensing could not be derived from the available KcsA data (KcsA lacked S4 segments), detailed suggestions were put forward on basis of mutational studies and theoretical modelling. Two main theories and two camps competed for acceptance. Both were based on the view that channels consist of densely packed transmembrane segments with the charged S4 in their midst. One camp assumed that S4 moved its charges from the intracellular solution to the extracellular solution by sliding spirally along the packed helices, helical screw model (Catterall, 1986; Guy and Setharamulu, 1986; Keynes and Elinder, 1999; Lecar et al., 2003). The other camp assumed S4 to rotate without sliding, thereby exposing the charges of S4 either to the extracellular or to the intracellular solution via deep crevices, helical twist model (Cha et al., 1999; Glauner et al., 1999). Up till last year the second view seemed to get the upper hand among the channelologists. It even made its way into the textbooks (Hille, 2001).

Last year, however, MacKinnon succeeded in crystallizing a voltage sensitive, six-transmembrane helix K channel and could reveal the location and the likely dynamics of S4 from the sturdy thermophilic archebacterium *Aeropyrum pernix* (Jiang et al., 2003a,b). Neither of the two leading theories was in line with the crystallization results. Unexpectedly, S4 occurred together with a portion of S3 in a hairpin loop, extending out from the perimeter of the channel protein. This *paddle* configuration immediately suggested a novel way of S4 motion at gating, deviating from that of the dominant views. The paddle could be envisioned to move up and down in the lipid bilayer at gating. This new view has not been generally accepted; rather the opposition seems to increase (Broomand et al., 2003; Ahern and Horn, 2003; Lainé et al., 2003; Neale et al., 2003). However, whatever the final outcome of the debate, this new alternative forces us to rethink some pharmacological mechanisms. At present, though, it is difficult to foresee any consequences for the local anaesthetic mechanisms discussed in the present investigation.

Gating: the activation process

Voltage-dependent ion channels respond to membrane depolarization with a rapid opening or "activation gating." This process involves a voltage sensor responsible for sensing the voltage across the membrane and a gate responsible for the actual opening and closing of the pore. Early pharmacological studies, using quaternary ammonium

compounds (for instance tetraethylammonium, TEA), suggested that the activation gate is located at the intracellular entrance to the pore (Armstrong, 1966, 1971).

The detailed process of gating is still controversial. The crystallization data from KcsA (Doyle et al., 1998) made it likely that the gate is the 'bundle-crossing' below the internal vestibule, formed by the ends of the four S6 segments. The S6 transmembrane region and its intracellular extension show high sequence conservation. Within the principal families of voltage-gated K channels (KcsA is a bacterial K channel), the conservation is very high, and at the bundle crossing there is a strongly conserved proline sequence (PXP or PXG). Studies of this region indicate that it is likely to bend S6 in both the closed and open channels (Holmgren et al., 1998; del Camino et al., 2000; del Camino and Yellen, 2001). A strong case for the view that the gating process in KcsA consists of rotational movements of the S6 segments, causing a widening of mouth at the bundle crossing, was found in EPR studies (Perozo et al., 1999).

However, the crystallization of MthK (Jiang et al., 2002) introduced new features to the picture, some of which standing in opposition to the prevailing view. A key property of this new view is a bending of the S6 helices at a glycine hinge and a simultaneous disassembly of the S6 helix bundle through rigid-body helical movements C-terminal to the gating hinge. The gating hinge is located near a pore helix, consistent with the finding of a cluster of gating sensitive amino acids in this helix. Our molecular dynamics simulations introduce a further alternative to the discussion.

Gating: the inactivation process

Another mechanism for gating the pore is the inactivation process. This process has been assumed to be of crucial importance for local anaesthetic action (the modulated receptor hypothesis, see below). There are different forms of inactivation processes, caused by different mechanisms. The classical K currents were not described as inactivating, but today we know of many inactivating K channels; perhaps the best-studied inactivating process is that of the Shaker channel. The fastest inactivation process has been named N-type (or ball-and-chain inactivation) and is either auto-inhibitory, caused by a segment of the N-terminus of the channel protein itself, binding to the internal pore mouth (Hoshi et al., 1990), or correspondingly caused by the N-terminus of an associated β-subunit (Rettig et al., 1994). The process closely resembles that of non-inactivating K channels after application of small organic blockers, such as quaternary ammonium compounds (Armstrong, 1966, 1971) or crown ethers (Århem et al., 1982; Kristbjarnarson and Århem, 1982). The N-type inactivation can be deleted by enzymatically or mutationally removing the critical N-terminus segment, and restored by addition of a soluble peptide of the same sequence to the intracellular bathing solution (Zagotta et al., 1990). In present investigation we will use the wild-type inactivating Shaker and Kv3.4 channels, and the wild-type non-inactivating channels Kv1.1, Kv1.2, Kv1.5, Kv2.1, Kv3.1 and Kv3.2. We will also use an inactivation-removed Shaker channel (IR-Shaker).

A slow inactivation, extensively studied in Shaker, has been named C-type. The name originates from the fact that the process depends on the C-terminus of the channel protein (Hoshi et al., 1991), the mechanism being a collapse of the selectivity filter (Lopez-Barneo et al., 1993; Liu et al., 1996; Larsson and Elinder, 2000). C-type

inactivation persists in Shaker channels when the inactivation ball is deleted (Hoshi et al., 1991), and it is quite sensitive to extracellular [K^+] and TEA (Choi et al., 1991). Preliminary experiments show that bupivacaine enhances the C-inactivation.

1.2 LOCAL ANAESTHETICS

Local anaesthetics are important clinical tools, used in controlling pain, cardiac arrhythmias and epileptic seizures. Although voltage-gated Na channels are the main targets for local anaesthetics, clinically important side effects may be produced by block of voltage-gated K channels. For instance, bupivacaine has been reported to induce lethal arrhythmias by affecting cardiac K channels (Albright, 1979; Mather and Chang, 2001).

The first clinically used local anaesthetic was cocaine. It was introduced by Carl Koller and Sigmund Freud. They noticed a numbing effect on the tongue after swallowing cocaine, and Koller, who was eager to find a drug to anaesthetize the cornea, knew that Freud had relieved pain with cocaine (Freud, 1884). In fact they could demonstrate on themselves, using pins to touch their cornea in front of a mirror, that within minutes of applying cocaine they could no longer feel touch or pain. In 1884 they reported that they had enucleated painlessly a dog's eye. Leonard Corning, a neurologist in New York City, had tried in 1885 to inject cocaine solution (2%) between the spinous processes in a young dog, which resulted in insensibility within 5 min (Corning, 1895); this procedure was subsequently tried on patients, with the drug presumably acting in the epidural space. Cocaine was widely used despite its disadvantages of high toxicity, short duration of anaesthesia, impossibility of sterilizing the solution and its cost (not to mention addiction). When in 1904 Alfred Einhorn, investigating degradation products of cocaine, synthesized procaine, he found that, ''the anaesthetic capability of cocaine is therefore a function of its acid group called by Paul Ehrlich the 'anesthesiophoric' group – the most potent being the benzoyl group'' (Einhorn, 1899). Today the majority of clinically used local anaesthetics consist of a benzene ring linked via an amide or ester to an amine group, and their names still end in 'caine'.

1.2.1 Mode of action – the modulated receptor hypothesis

The dominant view of local anaesthetic action on voltage-gated channels is today some version of the modulated receptor hypothesis, originally formulated in 1977 (Hille, 1977; Hondeghem and Katzung, 1977). In summary, it states that the local anaesthetics binds reversibly to a single channel site, which shows state-dependent affinity; for mostly local anaesthetics the affinity is lowest (but not zero) in closed and highest in inactivated state. A tight binding to inactivated channels is accompanied by an increased propensity to be inactivated. Mechanistically, the inactivation gate is more likely to be shut when the local anaesthetic is bound (Hille, 2001). This hypothesis is assumed to explain observed negative voltage shifts of the steady-state inactivation curve and a slow recovery times from inactivation. The central role of the inactivation for local anaesthetic block in the modulated receptor hypothesis, follows the tradition of a long line of research (see Weidmann, 1955; Khodorov et al., 1974, 1976; Courtney, 1975).

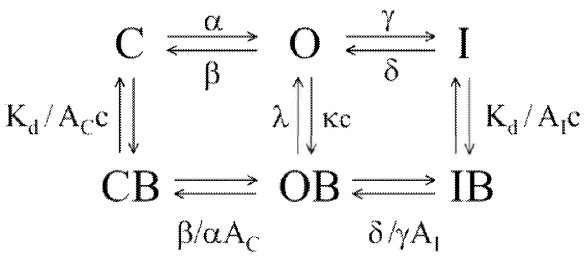

Figure 2. The standard version of the modulated receptor hypothesis. C, O and B denote lumped closed, open and inactivated states of the unbound channel; CB, OB and IB denote closed, open (but non-conducting) and inactivated states of the local anaesthetic bound channel; α, β, γ, δ, κ and λ denote rate constants and c local anaesthetic concentration. A_C and A_I are constants, reflecting the relative affinities in closed and inactivated states compared to that in open state. For the transitions to/from and between bound states only constants describing steady-state conditions are given (with the exception of the O – OB transition, where individual rate constants are shown). $K_d = \lambda/\kappa$.

A number of studies have extended and modified the original formulation. A high affinity between anaesthetic and channel in inactivated state has been reported in several studies, although not for all local anaesthetics (Hondeghem and Katzung, 1984). However, in a theoretical reinterpretation of the experimental data (the guarded receptor hypothesis), Starmer et al. (1984) questioned the high affinity of inactivated channels and suggested that local anaesthetics preferentially bind to open channels.

Attempts to resolve the conflicting viewpoints have been made by using irreversible inactivation modifiers like pronase, *N*-bromacetamide and chloramine-T (Cahalan, 1978; Yeh, 1978; Wang et al., 1987), and more recently, by using the substituted-cysteine accessibility method (Vedantham and Cannon, 1999). The results do not support a consensus view. Cahalan (1978) and Yeh (1978) concluded that the inactivation gate is important for anaesthetics inducing use-dependence and slow recovery (Cahalan, 1978), while Wang et al. (1987) reported that the blocking capacity and recovery were unchanged when inactivation was removed. Vedantham and Cannon (1999) found that the recovery rate from inactivation was unchanged by the anaesthetic, in spite of inducing a slow recovery from the block.

In summary, the role of the fast inactivation process in local anaesthetic block is far from resolved (see Scheuer, 1999). Furthermore, recent studies on the channel structure (Doyle et al., 1998; Zhou et al., 2001; Jiang et al., 2002) make a number of assumptions in the standard view questionable. How can the bupivacaine molecule enter and leave the inner vestibule when the channel is in inactivated state, i.e. with the inactivation particle blocking the entrance? Nevertheless, the standard view seems to prevail in the literature (see Hille, 2001).

1.2.2 Binding sites

The detailed location and nature of local anaesthetic binding sites are poorly understood. Several binding sites have been proposed in both Na (Khodorov et al., 1976) and K channels (Longobardo et al., 2000), but the most extensively discussed hypothesis assumes a single site, located in the internal pore mouth (Hille, 2001; Lipka et al., 1998), early postulated to be delimited by the S6 helix and the P-loop. The cloning of Na and K channels and the delineating of the primary structures have opened up new ways to detail the binding sites. The importance of S6 has also been confirmed for both Na and K channels in studies using this knowledge (Ragsdale et al., 1994; Franqueza et al., 1997). The crystallization of the KcsA and MthK channels (Doyle et al., 1998; Jiang et al., 2002) means that for K channels an even more detailed picture may suddenly be attainable, one way being through molecular dynamics simulations (Åqvist and Luzhkov, 2000; paper III).

2 AIMS

The general aim of this study is thus to clarify molecular mechanisms of local anaesthetic action on voltage-gated Na and K channels. The specific aims are:

- to examine whether bupivacaine blocking of Na channels in myelinated axons is direct or indirect via changing the resting potential (paper I),
- to examine the selectivity of bupivacine with respect to different Kv channels, expressed in Xenopus oocytes (paper II),
- to examine which amino acid residues are critical for bupivacaine binding to Kv channels (paper II),
- to examine the bupivacaine binding in homology models of Kv channels (paper III),
- to examine the role of fast inactivation for the bupivacaine block of Kv channels (paper IV).

3 MATERIALS AND METHODS

3.1 EXPERIMENTAL

3.1.1 Electrophysiology

The electrophysiological measurements were made with different versions of the voltage clamp technique, based on the classical work by Hodgkin and Huxley and by Cole (Cole, 1949; Hodgkin et al., 1949, 1952).

In the study of axonal channels (paper I) we used the version developed by Frankenhaeuser (Dodge and Frankenhaeuser, 1958). It consists of a two amplifier system controlling the voltage over the nodal membrane by injecting current from one cut end of the axon through the nodal membrane and keeping the axoplasmic current from the node to the other cut to essentially zero. The different parts of the axon are electrically isolated from each other in a recording chamber by petroleum jelly seals and the thus generated compartments filled with different extracellular solutions. The solution pools are connected to the amplifier clamp system by KCl bridges (120 mM) and by calomel electrodes. The amplifier system is constructed from operational amplifiers as described in Århem et al. (1973). The current is calculated from the recorded voltage at the output of the voltage-clamp amplifier, assuming an axoplasmic internodal resistance of 40 MΩ (see Dodge and Frankenhaeuser, 1958).

To improve the control of the membrane potential and to obtain good time resolution of the ion currents, the experiments were performed at relatively low temperature (8-10^{o} C). (At higher temperatures the inherent frequency response limitations of the system could cause oscillatory responses. Furthermore, the capacity current is relatively slower compared to the ion currents than at lower temperatures and consequently complicates the separation of currents).

In the present analysis single large myelinated fibers (axons) of different sizes were isolated from the sciatic nerve of decapitated toads (*Xenopus laevis)* as described by Frankenhaeuser (1957).

The measurements of the oocyte currents (paper II and IV) were made with a standard two-electrode clamp system (Stühmer, 1998); one electrode monitoring the voltage difference over the membrane and the other carrying injected current to compensate currents due to membrane permeability changes. The amplifier system used was a Dagan CA-1. The electrodes were pulled from borosilicate glass (Harvard apparatus Ltd GC150-10) and filled with 3M KCl. The resulting resistance was between 0.4 and 2.0 mohm. The experiments were performed at to locations; at the Department of Physiology, University of Münster, and at the Department of Neuroscience, Karolinska Institutet. The equipment used was similar at both locations. In the Münster lab a concentration-clamp system, developed by Madeja et al. (1991), was used.

Pulse generation and sampling in both types of voltage-clamp experiments were made using a TL-1 DMA interface (Labmaster, Axon Instruments, Foster City, CA) and the

pCLAMP software (Axon Instruments, Foster City, CA). The pulse rate was 0.5 Hz and the sampling interval 20-100 μs.

3.1.2 Solutions

In the myelinated axon experiments the test solution was applied to the pool containing the node of Ranvier, while the two end pools contained high **[K^+]** solutions, similar to the axoplasmic solution. The composition of the Ringer control solution was (in mM): NaCl 115.5, KCl 2.5, $CaCl_2$ 2.0, and Tris buffer (adjusted to pH 7.2) 5.0. The high **[K^+]** solution used in the end pools consisted of (in mM): KCl 120.0 and Tris buffer (pH 7.2) 5.0. The test solution consisted of bupivacaineHCl (Sigma Chemical Co, St Louis, USA) added to Ringer solution.

In the oocyte experiments two types of control solutions were used; normal Ringer type and high [K^+] solution, the latter being used for investigating tail currents. The composition of the control Ringer solution was (in mM): NaCl 115, KCl 2, $CaCl_2$ 1.8, HEPES 10; pH 7.2. The composition of the control high [K^+] solution was (in mM): NaCl 2.5, KCl 120, $CaCl_2$ 1.8, HEPES 10; pH 7.2. The test solutions consisted of bupivacaineHCl (Sigma Chemical Co, St. Louis), added to the control solutions.

The solution used for incubating the oocytes was a modified Barth medium (in mM): NaCl 88, KCl 1, $CaCl_2$ 1.5, $NaHCO_3$ 2.4, $MgSO_2$ 0.8, HEPES 5; pH 7.4, which was supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml).

3.2 THEORETICAL

3.2.1 Conductance and dose-response curves

The time course of the bupivacaine block was quantified by fitting a sum of two exponentials to the current recordings in the time window 2-500 ms with a non-linear correlation procedure (Chebyshev method; pCLAMP software, Axon Instruments, Foster City).

The steady-state K conductance g(V) was calculated as

$$g(V) = I(V)/(V-E_K) \qquad (1)$$

where I(V) is the steady-state current, V is the absolute membrane potential, and E_K is the equilibrium potential (assumed to be -80 mV).

The dose-response curves were fitted to the following equation,

$$y = 1/(1+ (c/K_d(V))^{nH}) \qquad (2)$$

where y represents either normalized steady-state current I(t), normalized steady-state conductance g(t) or normalized steady-state open probability Po(t) at potential V. In paper IV y also represents the initial peak current. c denotes bupivacaine concentration, n_H the Hill coefficient and $K_d(V)$ the dissociation constant of the reaction at potential V.

3.2.2 Kinetic model simulations

To quantitatively evaluate the models used in the present investigation, we fitted the numerical solution of a system of parallel differential equations to the experimental data. The probability P_i that the channel is in state i is described by the general equation

$$dP_i(t)/dt = \sum_{j=1}^{n} k_{ji} P_j(t) - \sum_{j=1}^{n} k_{ij} P_i(t) \qquad (3)$$

where n is the number of conformational states (n=6 in Fig. 2 and n=4 in Fig. 7) and k_{ij} is the transition rate from state i to state j. The initial condition was chosen such that the system is in state C ($P_1(0)=1$) at time zero. While the inactivation and deinactivation rate constants (γ and δ in Fig. 2 and 7), and the binding and unbinding rate constants (κ and λ in Fig. 2 and 7) were assumed to be voltage independent (for γ and δ, see Zagotta et al., 1990; Elinder et al., 1998), the activation and deactivation rate constants (α and β in Scheme 1 and 2) were assumed to be voltage dependent according to the following equations:

$$\alpha = k_{eq} \exp(z\, 0.5(V-V_0)F/(RT))$$
$$\beta = k_{eq} \exp(z\, 0.5(V_0-V)F/(RT)) \qquad (4)$$

where V, F, R and T have their usual thermodynamic meanings, $V=V_0$ when $\alpha=\beta$, k_{eq} is the rate constant when $V=V_0$ and z the valence of the transition. The voltage dependence of the blocking transition in paper II was assumed to be described by the factor $\exp(-\delta VF/(RT))$ multiplied with the unblocking rate constant λ. δ is the fraction of the field sensed by the charged bupivacaine molecule in its blocking action (the electrical distance). Eq. 3 was solved by an Euler integration method. The integration time interval used was 10 μs. For the cases (Kv1.1, 1.2 and 3.2) where the g(V) curves showed a clear saturation level within the investigated voltage range (< +60 mV) the electrical distance δ could be directly estimated from the g(V) curve by fitting the experimental data to the following equation:

$$g(V)/g_{ctrl}(V) = 1/(1 + c \exp(\delta VF/(RT)) / K_d(0)) \qquad (5)$$

where $g_{ctrl}(V)$ is the conductance in control solution and $K_d(0)$ is the dissociation constant at V = 0 mV.

3.2.3 Molecular dynamics simulations

To investigate the binding of bupivacaine to the Kv channels in more physical detail we used automated docking and molecular dynamic calculations. We used homology models of Kv1.5 rather than of Kv2.1, since data for *R(+)*- and *S(-)*- forms were available for Kv1.5 (Valenzuela et al., 1995), but not for Kv2.1. In our experimental

studies (papers I, II and IV) we used a racemic mixture of bupivacaine, partly due to the fact that this is the clinically used form. The template models were the KcsA and MthK crystal structures (Doyle et al., 1998; Jiang et al., 2002). The KcsA channel crystal is assumed to be in closed state, but the homology model was found to be open; mainly due to the introduced Kv channel conserved PVP segment. The MthK crystal is assumed to be open due to the Kv conserved glycine hinge.

The bupivacaine binding to the Kv1.5 homology models was investigated in basically three steps. The conformational properties and partial atomic charges of bupivacaine were determined from quantum mechanical calculations (HF/6-31G* with inclusion of solvent effects). The binding between bupivacaine and the two homology models of Kv1.5 was first analysed by automated docking calculations (AutoDock Program suite) and then by more detailed molecular dynamics calculations (program Q). Linear interaction energy (LIE) estimates were used to obtain the binding free energies.

4 RESULTS

4.1 BUPIVACAINE EFFECTS ON MYELINATED AXON CHANNELS – ROLE OF LEAK CHANNELS (PAPER I)

Most local anaesthetics, including the amide type anaesthetics, are described as state dependent Na channel pore blockers (Hille, 1977, 2001; Hondeghem and Katzung, 1977; for another opinion, see Butterworth and Strichartz, 1990). However, to explain its differential effect on axons with varying diameter, bupivacaine has been proposed to inhibit Na channels indirectly by depolarising the membrane (Bräu et al., 1995). The proposal was based on the finding that bupivacaine specifically blocks voltage independent flickering K channels, assumed to be essential for the resting potential (Koh et al., 1992). These channels are mainly found in thin myelinated axons, implying that these fibres are more sensitive to a bupivacaine block. However, these conclusions were based on patch-clamp experiments on axons that have been extensively treated by mechanical and enzymatic means, possibly affecting the channel properties. To test the hypothesis of indirect Na channel effects under more physiological conditions we analysed the effects of bupivacaine on voltage clamped intact nodes of Ranvier.

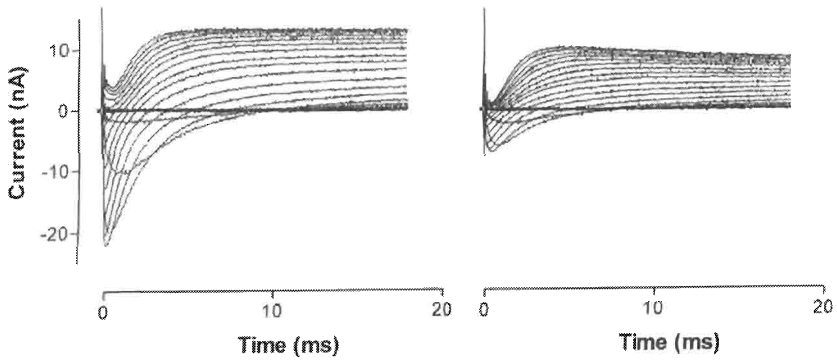

Figure 3. Current families in control (left) and 67 μM bupivacaine (right). Potential steps in 10 mV increments from a holding potential of -90 mV. Leak current subtracted.

No effect on the voltage-independent leak current or resting potential was found, not even at high concentrations (6 mM). In contrast, the voltage-dependent Na and K channels were affected at relatively low concentrations (see Fig. 3 at the concentration of 67 μM bupivacaine). Like other local anaesthetics (see Hille, 1977; Haydon et al., 1984), bupivacaine caused a voltage-independent decrease of Na and K steady-state activation curves (no shift), whereas it caused a clearly voltage-dependent decrease of the steady-state Na inactivation curve (a shift in negative direction). Also there was a reduction even at the low potential of –140 mV, (K_d = 44 μM). The results were

explained in terms of modulated receptor mechanisms (Hille, 1977; Katzung and Hondeghem, 1977).

A time dependent reduction of the K current is obvious in Fig. 3. At the potential of +50 mV, the induced inactivation was found to follow a monoexponential time course. The induced inactivation suggests an open-state dependent block; for simplicity described by a three-state kinetic scheme, including closed, open and blocked states (see Eq. 3, Methods). The experimentally estimated blocking rate constants were found to predict a K_d value of 617 μM. However, the experimentally estimated steady-state values indicated a higher bupivacaine affinity. The discrepancy between measured and predicted data suggests that the bupivacaine block also depends on, and is dominated by, a state-independent mechanism.

We thus concluded that the bupivacaine block of nodal K^+ channels can be explained in terms of two independent mechanisms, mediated by separate sites of action; one state-independent and one open-state dependent. We next investigated if the two effects were coupled to two sites on the very same channel, or if the sites were situated on different K- channel populations. Analysing the dose-response curves we tentatively concluded that the two tentative sites are located on channels belonging to two separate populations; one open-state dependent (K_d = 550 μM) and one state independent (K_d = 59 μM). The hypothesis that one of the populations comprised state-independent bupivacaine binding sites was strengthened by preliminary experiments on oocyte expressed Kv1.1 channels, showing no time dependent block. This, however, had to be reinterpreted, as discussed in paper II.

In conclusion, bupivacaine exerts its main anaesthetic action on myelinated nerve axons by a direct modification of Na channels and the modification of the K channels was explained by blocking two different channel types.

4.2 BUPIVACAINE EFFECTS ON VOLTAGE-GATED K CHANNELS – SENSITIVITY AND MOLECULAR MECHANISMS (PAPER II)

The traditional view of local anaesthetic binding is described by the modulated receptor hypothesis (Hille, 1977). Furthermore, the nature of the binding has been suggested to depend on aromatic amino acid residues (Ragsdale et al., 1994). These theories are built on experiments on Na channels. In paper II we have investigated this issue by using K channels as a model system. K channels have relatively slow and simple kinetics and do not show fast inactivation (making it possible to study the binding without complications caused by competition from the inactivation particle). They are structurally variable in the pore region, making our approach a natural site-directed mutagenesis.

We investigated the effects of bupivacaine on a series of wild-type Kv channels (Kv1.1, 1.2, 1.5, 2.1, 3.1 and Kv3.2) expressed in *Xenopus* oocytes. The results showed that bupivacaine at room temperature causes a time-dependent block of the studied Kv1 and Kv3 channels (K_d between 200 and 240 μM), and a time-independent block on Kv2.1 (K_d = 220 μM).

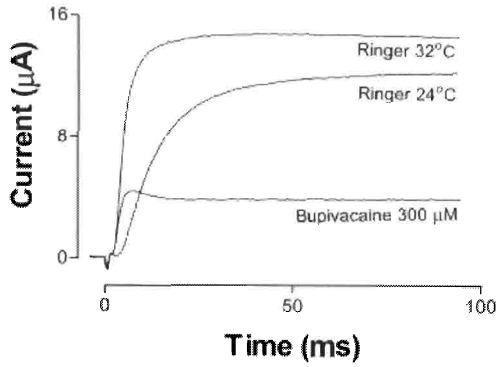

Figure 4. The effect of increased activation rate on bupivacaine block of Kv2.1. The figure shows the effect of bupivacaine at 32°C. Note the time dependence of the block, not observed at 24°C. For comparison control curves in Ringer solution at 24°C and 32°C are shown. Test step +60 mV and holding potential -80 mV.

The cause of for the non-peaked time course in Kv2.1 channel might be the slow activation time (compared to the other Kv channels), masking a time-dependent blocking time course. We tried to clarify this issue by increasing the activation rate. There are two easy ways to experimentally accomplish this: to increase the temperature (compared to the previously used room temperature) and to increase the potential (compared to the previously used highest potential +60 mV). We used both ways, and both showed that the block was indeed time dependent. Figure 4 illustrates the effect at 32°C. However, further electrophysiological analysis showed that the bupivacaine block of Kv2.1 deviated from that of the other investigated channels. Kv2.1 was able to trap the bupivacaine molecule in the internal vestibule at repolarisation. This was not the case for the non-Kv2.1 channels.

We therefore investigated the effect of replacing Kv2.1 residues with corresponding Kv1.2 residues (Fig. 5). Experiments showed that N-terminal end substitutions in S6 introduced Kv1.2 features with respect to the bupivacaine block. Critical residues were positions 395 and 398. The triple substitution of residues 372, 373 and 374 in the S5-S6 linker decreased the bupivacaine affinity five-fold (K_d increased from 220 μM to 1170 μM).

The results suggest that bupivacaine blocks Kv channels by an open-state dependent mechanism. The data are consistent with the existence of a binding site, located in the internal vestibule. Numerical simulations suggest that the effect on Kv2.1 deviates from that on the other channels in allowing a partial closure of the channel in bound state. The mutational experiments suggested that residues in the pore helix and the upper part of S6 are critical for the binding, most likely by an allosteric mechanism. A simple mechanistic scenario could explain the observations. A crucial feature of this scenario is the small distances between the critical residues and the conserved glycine residue in the S6 helix recently shown to act as a gating hinge (Jiang et al., 2002; Yifrach and

MacKinnon, 2002). Thermodynamic considerations suggest that the interaction between bupivacaine and the channels is hydrophobic.

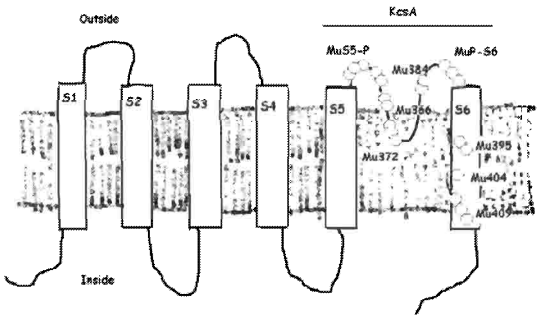

Figure 5. Transmembrane topology of a Kv subunit with location of the substitutions used in paper II. The region corresponding to the KcsA channel (the pore region) is marked.

4.3 BUPIVACAINE BINDING TO KV1.5 – MOLECULAR DYNAMICS SIMULATIONS (PAPER III)

To clarify the nature and localization the binding sites of bupivacaine Kv channels, we in paper III studied the binding of bupivacaine to Kv1.5 homology channels, by automated docking and molecular dynamics methods. Homology models were built, using the 3D structures of the KcsA and MthK channels as templates. The packing of transmembrane α-helices in the KcsA structure corresponds to a closed channel state, while the MthK channel corresponds to an open channel state. Opening of the KcsA channel may be reached by a conformational transition yielding a bent structure of the internal S6 helices. We thus used two Kv open-state models: One involving a PVP-type of bending hinge in the internal helices, while the second model corresponded to a Gly-type of bending hinge as found in the MthK channel (Fig. 6).

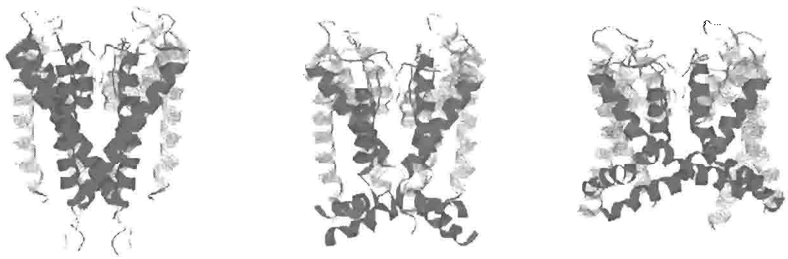

Figure 6. The 3D structures of the KcsA channel, and the PVP-bend and Gly-bend homology models of Kv1.5 channel. The internal M2 and S6 α-helices are displayed as solid ribbons.

The bupivacaine binding to these models was probed, assuming binding from the intracellular side. Conformational properties and partial atomic charges of bupivacaine were determined from quantum mechanical calculations. The automated docking and MD calculations for the PVP-bend model predicted that bupivacaine could bind either in the central cavity or in the PVP region of the channel pore. Linear interaction energy (LIE) estimates of the binding free energies for bupivacaine predicted strongest binding to the PVP region. Surprisingly, no binding was predicted for the Gly-bend model. These results are in line with the electrophysiological data in paper II where the Kv1.5 channel was shown unable to close when bupivacaine is bound.

4.4 BUPIVACAINE EFFECTS ON INACTIVATING K CHANNELS – ROLE OF THE INACTIVATION GATE (PAPER IV)

Paper IV investigates the molecular mechanisms of local anaesthetic action on the inactivation of voltage-gated channels. The dominant view today, the modulated receptor hypothesis, states that the local anaesthetic binds reversibly to a single channel site which shows state dependent affinity, with the highest affinity for inactivated channels and with bound channels more easily inactivated (see Introduction; Fig. 2). In molecular terms, this view suggests that the local anaesthetic directly affects the inactivation gate. Some years ago this standard view was challenged by (Vedantham and Cannon, 1999) using a substituted-cysteine accessibility method to show that the recovery rate from inactivation was unchanged by the anaesthetic, in spite of an induced slow recovery from the block.

We reanalyzed the issue by partly using a novel approach. We investigated the effects of bupivacaine on a series of (artificial and wild type) inactivating and non-inactivating voltage-gated K channels. The use of Kv channels gave us many advantages; among others we have better structural knowledge about K channels than about other channels (Doyle et al., 1998; Jiang et al., 2002), as well as to detailed knowledge of the kinetics of a bupivacaine block in a series of non-inactivating Kv channels (paper II). An underlying hypothesis in this approach was that the bupivacaine affinity is similar for the inactivating and non-inactivating subtypes due to the close similarity between primary structures. We thus analyzed effects on the inactivating wild type and a non-inactivating mutated Shaker (Shaker vs Shaker-IR) and inactivating and non-inactivating wild type Kv3 channels (Kv3.4 vs. 3.2). Both wild type Shaker and Kv3.4 display a fast inactivation of N-type (Zagotta et al., 1990; Stephens et al., 1996).

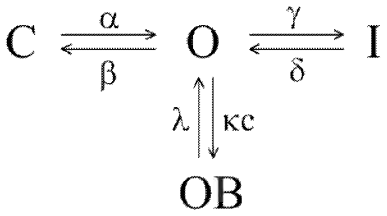

Figure 7. Alternative scheme. Same notations as in the standard scheme in Fig. 2.

Non-inactivating channels were more effectively blocked than inactivating ones (IC_{50} for Shaker-IR and Kv3.4 being 210 and 80 μM, and for Shaker and Kv3.4 1600 and 1200 μM). The time course of the inactivation was modified, a fast and a slow component being induced. The steady-state inactivation curve was shifted to the left (at 1000 μM -5 mV for Shaker and -8 mV for Kv3.4). The results can be explained by a simple kinetic scheme (see Fig. 7), deviating from the standard scheme (see Fig. 2), in assuming local anaesthetic binding to channels exclusively in open state, thus without direct effect on the inactivation gate. The close similarity between the anaesthetic effects on Kv channels and on Na channels, suggests that the present scheme can also be used to clarify controversial issues about local anaesthetic effects on Na channel inactivation.

5 DISCUSSION

5.1 DIRECT EFFECTS ON NA AND K CHANNELS IN MYELINATED AXONS – NO ROLE FOR LEAK CHANNELS (PAPER I)

The main conclusion from the work on the anaesthetic action of bupivacaine in myelinated axons is that the effect primarily is due to inhibition of the Na channels and not to a depolarisation, due to effects on the leak current as suggested by Bräu et al. (1995). The inhibition of Na channels was found being caused by a combination of a reduced maximum open probability and a shift of the Na steady-state inactivation curve. This was tentatively explained by a single binding-site model. The effect on K channels was more complex, comprising effects on two channel populations. The mechanistic implications of these hypotheses will be explored below. First, however, possible reasons for the lack of leak current effect will be outlined and discussed.

5.1.1 Reasons for experimental discrepancies on leak current

The reason for the discrepancy between the present negative results on the resting potential effects and the positive results reported by Bräu et al. (1995) is not clear. One possible explanation is that the reported flickering channels in the present study were inaccessible for bupivacaine in the external solution due to their location in the paranodal region. This implies, however, that a possible flickering channel effect is clinically irrelevant. Another possible reason for the discrepancy is that the present results are obscured by an extra axonal current under the myelin sheath. This explanation can, however, be excluded. An extra axonal current was first inferred by Dodge and Frankenhaeuser (1958), and has recently attracted renewed interest (see Ritchie, 1995). The existence of such a current would mean that the presently measured leak current comprises both a nodal (and paranodal) transmembrane current and a longitudinal extraaxonal current. Consequently bupivacaine effects on the nodal component will be partially masked by the unaffected extraaxonal component. However, as shown by Dodge and Frankenhaeuser (1958), the extra-axonal current is negligible in axons cut at full internodal length. Another possible explanation can be the size of the axons. Bräu et al. (1995) studied axons of a diameter of about 5 μm (personal communication), while the diameters in our study, are in the range of 10 to 20 μm.

5.1.2 Molecular implications for axonal Na channels

The found inhibition of the Na channels was characterized by a decrease of maximum open probability and a shift of the steady-state inactivation curve. This dual effect was discussed in paper I to be explained by the modulated receptor hypothesis (Hille, 1977). The essence of the model is that bupivacaine binds to a single site in closed or open configuration, shifting the equilibrium state of the channel towards the inactivated state. This deviates from the tentative conclusions presented in paper IV. In the paper I study, however, we did not take data from experiments on non-inactivating channels into account, making the weight of the conclusions less than that of the paper IV conclusions. The paper I model was constructed to comply with the view that the intracellular linker between the third and fourth domain of the channel forms an

inactivation "lid" that binds to a hydrophobic pocket in the internal mouth of the pore (see Patton et al., 1992, and Zamponi and French, 1994). Bupivacaine can be assumed to bind to the hydrophobic pocket, thereby increasing the probability of the linker to bind to its site (Kuroda et al., 1996).

5.1.3 Implications for axonal K channels – selective effects on two populations

The effect on the K channels could be explained by a model suggesting two independent mechanisms mediated via two separate binding sites: one low-affinity open-state dependent site and one high-affinity state independent site. The results further suggested that the two tentative binding sites are located on separate channel populations, the population with the high-affinity site comprising about 75 % of the total population. Different studies have reported different Kv populations in the node of Ranvier (Dubois, 1981) found two that show fast activation kinetics (f_1 and f_2) and one that shows slow (s). In paper II we report different bupivacaine effects on different Kv channels expressed in oocytes. We also show that an open state dependent block can give the same time course as a state independent block, given that the blocking transition is much faster than the activation transition. The bupivacaine effects on the nodal K currents were therefore explained by two open-state dependent effects on two different Kv channel populations. This is compatible with recent findings of several Kv channel populations (Kv1.1, Kv1.2 and Kv3.1) in the nodal membrane of myelinated axons (Rasband and Shrager, 2000; see Hille, 2001; Devaux et al., 2003). Furthermore it is compatible with the findings of paper II that the bupivacaine block of Kv1 channels shows less time dependence than Kv3 channels. This also explains why we in paper I interpreted the preliminary bupivacaine results on Kv1.1 expressed in oocytes as a state independent.

5.2 DIFFERENT BLOCKING MECHANISMS FOR KV2.1 AND KV1.2 (PAPER II)

In order to gain insights in the molecular mechanisms of anaesthesia we analyzed the effects of bupivacaine on a series of voltage-gated K channels (Kv1.1, 1.2, 1.5, 2.1, 3.1 and 3.2) and various mutant channels derived from Kv2.1.

Numerical simulations of the different types of electrophysiological recordings showed that the block could be described by a simple unitary state diagram (Fig. 9) in which the deviation of Kv2.1 is seen in the relatively high probability to pass into a bound closed state at repolarization. This means that in Kv2.1 the bupivacaine molecule is partly trapped in the internal vestibule of the channel at repolarization, while in the other channels bupivacaine prevents the channel to close.

A deviating blocking effect described on Kv2.1 compared to Kv1 and Kv3 channels is in line with results from other studies, suggesting Kv2.1 to be selectively sensitive to certain pharmacological compounds, such as 4-aminopyridine and propafenon (Kirsch et al., 1993; Madeja et al., 2003).

5.2.1 A digression: the importance of the blocking mechanism for the cellular firing pattern

So far we have discussed differences in mechanisms of block on a channel level. How do these differences show up on a cellular level? Do differences at a channel level become irrelevant at a cellular level? To investigate this we constructed a hippocampal model neuron, based on a voltage clamp analysis of a subset of hippocampal interneurons (Johansson and Århem, 1992), and applied two model anaesthetics, selectively blocking Kv channels. One model anaesthetic was assumed to block state-independently and the other open-state dependently. Figure 8 shows the result of simulating the effects of the two model anaesthetics on the oscillatory dynamics of the hippocampal model neuron. The upper panel shows the control case at a constant stimulus current, the middle panel the case of the state-dependent block and the lower panel the case of the state-independent block. In the both the block situations a steady-state block of 50% was assumed. As seen, the two ways of blocking the channels show drastically different results. The state-independent block increases and the state-dependent block decreases the frequency of the oscillations. The reason for the differential result is that the state-independent block shortens the recovery time and poises the membrane at a level close to threshold while the state-dependent block induces a longer recovery time due to a "foot-in-the-door" effect. The two types of mechanism thus theoretically disrupt oscillatory activity in different ways. The mechanism of block can be of fundamental importance at higher complexity levels, and therefore of clinical relevance.

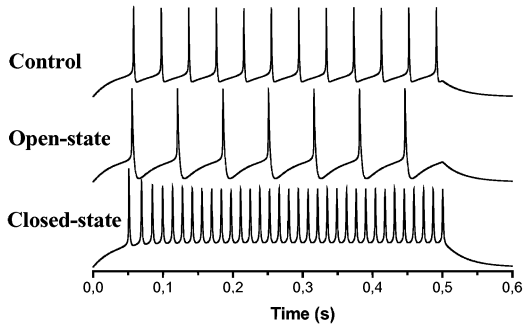

Figure 8. The effect of different blocking mechanisms on the repetitive firing of a hippocampal model neuron. The upper panel shows the firing at constant current injection. The middle panel shows the effect of selectively blocking K channels by an open-state dependent mechanism. The lower panel shows the effect of a state-independent block of K channels.

5.2.2 Critical residues for binding in Kv2.1

Introducing Kv1.2 residues in positions 395 (G395S) and 398 (C398A) of Kv2.1 induced Kv1.2 features, i.e. decreased probability of closing, while substitutions of other S6 residues did not modify the bupivacaine block. Introducing Kv1.2 residues into positions 372 (T372V), 373 (I373V) and 374 (T374S) of Kv2.1 decreased the

bupivacaine affinity. Summarizing the mutational studies, the results can be schematically illustrated as below.

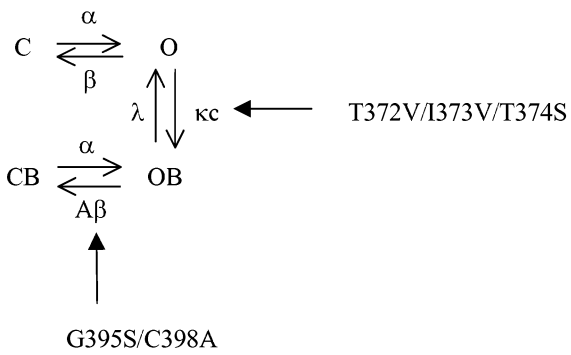

Figure 9. Schematic diagram of the mutational effects on the bupivacaine block of Kv2.1. Parameter A reflects the probability of the channel to trap the bupivacaine molecule.

The triple mutation T372V/I373V/T374S leads to modified binding of bupivacaine (described by a decreased affinity), and the double mutation G395S/C398A to modified closing of the channel in bound state.

Homology simulations, in which Kv2.1 and chimeras were constructed on basis of the KcsA channel (see Methods), suggest that the distances between the double and triple mutations above range from 3.2 to 7.5 Å. The shortest distance is between the side chains of T374 and C398. We hypothesised that the apparently different effects of these two groups of mutations with reference to the blocking behavior are caused by a unitary mechanism.

Binding of local anaesthetics to an open-state dependent site in an internal vestibule was early postulated (Hille, 1977), as was the binding of other channel blocking compounds (Armstrong, 1971; Kristbjarnarson and Århem, 1982). Molecular studies have later confirmed these early suggestions (Yellen et al., 1991; Choi et al., 1993; Kirsch et al., 1993; Ragsdale et al., 1994; but for another view see del Camino et al., 2000), most recently by using crystallographic methods (Zhou et al., 2001; Jiang et al., 2002).

The fact that point mutations in the N-terminal end of S6 modify the blocking kinetics so drastically (from trapping to non-trapping) is at first thought remarkable, in view of the distance between the substituted residues (395 and 398) and the assumed gating region, the S6 bundle crossing. However, some recent findings may make our working hypothesis less far-fetched. One is that a more closely located glycine (G401) may be the critical hinge for the gating mechanism (Jiang et al., 2002; Yifrach and MacKinnon, 2002). Another is that the I470C mutation in Shaker (corresponding to I405 in Kv2.1) induces trapping of TEA and C_{10} (Holmgren et al., 1997). Also, V373 in Kv2.1 have been shown to be of importance for the TEA binding, possibly without being part of the binding site (Kirsch et al., 1992).

5.2.3 Physical nature of the binding site in Kv2.1 (paper II)

The exact location and the physical nature of the site(s) and bond(s) between channel and bupivacaine are unknown. The residues of the S6 segment reported to be critical for local anaesthetic binding in Na channels are aromatic (Ragsdale et al., 1994). Likewise, the residues critical for antiarrhythmic binding in HERG channels have been reported to be aromatic (Mitcheson et al., 2000). Both studies suggest that local anaesthetics and antiarrhythmic drugs form ion-dipole and dipole-dipole π bonds with the channel. However, no aromatic residues are present in the S6 segment of the channels studied. Thermodynamic considerations of our data on Kv2.1 suggest that they are hydrophobic. The temperature experiments allowed us to calculate the binding energy, supporting the hypothesis of hydrophobic bonds between bupivacaine and Kv2.1 (Cantor and Schimmel, 1980).

Mutations in the PVP-region of the S6 segment have been reported to be important for the bupivacaine affinity in Kv1.5 (Franqueza et al., 1997). This is in line with our results in paper II showing that the Kv1.5 and the other non-Kv2.1 channels are unable to close the gate when the molecule is bound. The importance of the PVP-region for bupivacaine block of Kv1.5, was confirmed in the molecular dynamic calculations in paper III. Our finding in paper II that blocking Kv2.1 is insensitive to mutations in the PVP-region suggests another binding site in Kv2.1 than in Kv1.5.

Most substitutions in the present investigation concern residues not directly constituting a part of the internal vestibule wall. Thus we suggested that bupivacaine mainly forms hydrophobic bonds with internal vestibule residues, not modified in the present investigation.

5.2.4 Pharmacological implications

An open-state dependent blocking mechanism is assumed to be the working principle for widely used antiarrhythmic and antiepileptic drugs. But what about the special feature of trapping a blocker molecule? What clinical consequences can this finding have? It can be shown that the trapping process can have important consequences for the use-dependent behavior of therapeutic channel blockers (Holmgren et al., 1997). Also the finding that a single double mutation in Kv2.1 can change this channel from one that does trap bupivacaine to one that does not trap bupivacaine is in itself quite remarkable, and should be given a thought. It suggests that the difference between channels that do or do not trap blockers does not depend on major architectural features. Instead, it suggests that this property only requires minor channel remodelling. Furthermore, this example of a minor amino acid residue switch, causing a blocking mechanism change, suggest that there could be significant species differences involved. It may be important to resolve the properties of the precise human molecular target, rather than using an animal homologue, when testing channel active compounds for clinical use.

5.3 BUPIVACAINE BINDING TO KV1.5 – ROLE OF THE PVP REGION (PAPER III)

In this work two open-state homology models of the Kv1.5 channel was built to explore their bupivacaine binding properties. The first one, based on the architecture of KcsA, has a partially open pore entry due to the internal α-helical bends at the conserved PVP sequences (PVP-bend model), and the second one has an MthK-like structure with the bends at G504 and a fully open internal cavity (gly-bend model). The presented channel structures necessarily involve uncertainties inherent for most homology models. However, these two models suggest two different ways of making a Kv pore open, i.e. using either a Gly-type bending hinge or a PVP-type bending hinge, and in this way they provide possibilities to trace the corresponding differences in the binding pattern of blocker molecules.

5.3.1 Bupivacaine properties and binding

As other potassium channel blockers the structural properties of bupivacaine is given by a combination of hydrophobic peripheral groups and a quaternary nitrogen centre (Snyders and Yeola, 1995). The additional presence of the polar amide group ensures a potentially rich hydrogen bond pattern to the channel. According to the quantum mechanical calculations, bupivacaine is a fairly rigid molecule with restrained internal rotations of the phenyl and piperidine rings, which improves the calculation conditions. Furthermore, only one low-energy conformation was found, making the molecule easier to use in docking and dynamic calculations. The docking calculations predict two major binding regions for the *R(+)*-bupivacaine in the internal pores of both homology models. The first binding region is in the upper part of the internal cavity for both models. The second binding region however, is formed by the internal S6 α-helices for the PVP-bend model and for the Gly-bend model it is located between the S6 and the S5 α-helices. In each of the two binding regions, several types of ligand-channel complexes were found.

5.3.2 Possible binding sites in the two open-state channel models

In the PVP-bend model, when comparing all binding possibilities the MD/LIE results show that binding of the *R(+)*-bupivacaine to the PVP region is more favorable than binding to the upper part of the cavity. The PVP site does not seem to include any side chains that can make specifically strong interactions with bupivacaine. However, the H-bonds disruptions by proline residues in the α-helical backbone at the PVP bend make several backbone carbonyl groups to participate in forming H-bonds with the ligand. The binding position is in line with mutational studies of bupivacaine on Kv1.5 (Franqueza et al., 1997; paper II).

Even if the energetically most favourable binding mode was in the PVP part of the cavity another alternative was in the upper part of the channel, closely resembling the predicted position for internal blocking by TEA (Luzhkov and Åqvist, 2001) and the experimentally determined binding position for the tetrabutylammonium analogue in KcsA (Zhou et al., 2001). In this part of the channel, the most important factor for ligand stabilisation is the electrostatic interaction of the ligand charge with the short pore helices, which is similar to the effect of K^+ stabilization in the same position.

5.3.3 PVP-bend or Gly-bend model?

There has been considerable progress in recent years towards understanding what kind of structural changes occur when potassium channels open. The first reported structure of a potassium channel (KcsA; Doyle et al., 1998) described the closed state of the channel. Soon after that, a site-directed spin labelling and EPR study (Perozo et al., 1999) showed that opening of the KcsA channel is accompanied by rotational and translational rigid-body movements of the internal M2 helices as well as by less dramatic changes in the positions of the outer M1 helices. The crystal structure of the calcium-gated MthK channel provided another picture for the open potassium channel (Jiang et al., 2003a,b), where the gating is reached due to the outward rotation of the outer helices and bending of the internal helices by ~30^{o}. The open structure of MthK is much broader and gives better access for blocker molecules than the earlier EPR-derived open structure of KcsA. Recently the X-ray structure of the voltage-gated potassium bacterial channel KvAP in a complex with Fab fragments was published by MacKinnon and coworkers (Jiang et al., 2003a). In similarity to MthK and KcsA it does not contain the PVP sequence characteristic for mammalian Kv channels. The observed conformation of KvAP, which presumably corresponds to the open channel state, is similar to that of MthK but not as wide at the intracellular opening (Jiang et al., 2002). However, this structure shows that there may indeed be some structural variation among (open-state) K channels in that region. The mammalian Kv channels contain glycine at the same position in the S6 segment as MthK, KvAP, and KcsA. In addition, the S6 segment of the Kv channels includes a highly conserved PXP sequence. The conserved character of the PXP sequence indicates its important functional role, which in fact can be interpreted in terms of forming the bending hinge in the α-helical structure.

The results of the paper III study as well as other published data make at least a qualitative discussion possible about the structure of open Kv channels. First, the existing electrophysiological data in paper II provides evidence that bupivacaine binds to Kv1.5 in open state and prevents the subsequent closure of the channel. The molecular dynamic calculations for the PVP-bend model predict that the PVP region is the best binding site for bupivacaine in Kv1.5. Inspection of the corresponding ligand-channel complexes suggests that the open-state channel cannot transform to the KcsA-like closed-state structure while having the bupivacaine bound at the PVP hinge. This also agrees with the electrophysiological data on Kv1.5 in paper II. On the contrary, binding of bupivacaine to the PVP region of the Gly-bend (MthK-like) model does not seem to provide any obvious blocking effect since this site is too remote from the ion permeation path. Second, a bent PVP structure has been suggested by electrophysiological experiments (del Camino et al., 2000), as well as in MD simulations of the isolated Shaker S6 α-helix (Tielman et al., 2001). Importantly, in the calculations of paper III, the PVP-bend appeared much more flexible than the Gly-bend in these simulations. Still both the glycine and the PXP regions are both conserved in most of the eukaryote voltage-gated K channels. Thus, we should also consider the possibility that the actual gating mechanism in the Kv channels might involve both bending sites.

5.4 NO DIRECT EFFECT OF BUPIVACINE ON THE INACTIVATION GATE (PAPER IV)

The main conclusion of the paper IV investigation is that the inactivation process does not play the central role for local anaesthetic block of Kv channels, assigned to it by the received view, the standard version of the modulated receptor hypothesis (see Introduction). The conclusion is based on results from a novel experimental approach: to compare local anaesthetic effects on non-inactivating and inactivating channels of closely related structural subtypes (both artificial and natural), hypothesizing that the "proper" affinity is equal in non-inactivating and inactivating channels of the same subtype.

We found that the effect of bupivacaine on Kv channels could be explained by a simple model, assuming binding to the channel exclusively in open state, i.e. without any binding to the channel in closed or inactivated state. This model (Alternative scheme of Results; Fig. 7) was found to quantitatively explain the essential features of the present results; especially features not explained by the standard model (Standard scheme in Introduction; Fig. 2), the apparent lower affinity of the inactivating channels compared to the non-inactivating, and the lack of phasic block. Furthermore, estimations of the "proper" bupivacaine affinities to the inactivating channels by global fitting of the alternative scheme to the experimental data confirmed that they were, as initially hypothesized, close to the more reliable estimations of non-inactivating channel affinities. This conclusion is in line with the conclusions from a previous study on bupivacaine effects on Kv channels (paper II), showing that the affinities vary little over a wide range of channel subtypes (see Table 1 and 2 in paper IV). The presented alternative model also seems us to be better in line with the present understanding of the K channel structure and the binding of the inactivation particle and local anaesthetics than the standard model; with the PVP region being the gate region (Doyle et al., 1998; Yellen, 1998) as well as binding site for the inactivation particle (Zhou et al., 2001) and bupivacaine (paper III), thus excluding bupivacaine bound closed and inactivated states.

5.4.1 Implications for Na channel inactivation

The standard scheme discussed above was originally developed to explain effects on voltage-gated Na channels. The bupivacaine action on the inactivating Kv channels described above shows qualitative similarities to that on Na channels but also differences (e.g. lack of use dependence in Kv channels). In the next step of our analysis we therefore investigated whether the alternative scheme also quantitatively explains local anaesthetic effects on Na channels. We even asked whether it could be used to clarify some of the controversies surrounding local anaesthetic action on the inactivation of Na channels. For this reason we analyzed the experimental results reported for local anaesthetic action on Na channels in which the inactivation had been pharmacologically removed (Cahalan, 1978; Yeh, 1978; Wang et al., 1987) or modified (Vedantham and Cannon, 1999).

Thus, we first compared the predictions of the standard and the alternative schemes with respect to tonic and phasic block. Some qualitative predictions are summarized in Table 3 of paper IV, together with estimations from reported experimental results for Na channels with and without the fast inactivation removed. Comparing the experimental results with the qualitative predictions (considering the variability due to different preparations and experimental methods), it is clear that the alternative scheme provides a better explanation of local anaesthetic block of Na channels than the standard scheme.

Next, we compared the predictions of the standard and the alternative schemes with respect to the rate of recovery from inactivation and from local anaesthetic block. For this we used simulations partly based on the experimental findings of a substituted-cysteine accessibility study by Vedantham and Cannon (1999). This study suggests that lidocaine does not affect the inactivation recovery rate in spite of slowing down the rate of recovery from block. The simulations based on the alternative scheme predicted such a lack of effect on the inactivation recovery, in contradiction to the predictions of the standard model.

Finally, we compared the predictions of the two schemes with respect to shifting the inactivation curve. The dissociation constant (K_d) of the blocking reaction uniquely determines the shift of inactivation curve in the case of the alternative scheme, but not in the case of the standard scheme, which involves several free parameters. Assuming that we obtained a reasonable estimation from studying a non-inactivating channel we could predict the shift for the corresponding inactivating channel. With this strategy we thus estimated K_d for different local anaesthetics from reported experimental studies of artificially modified, non-inactivating Na channels (Cahalan, 1978; Wang et al., 1987), and calculated the shifts. The values are listed in Table 4 of paper IV together with experimentally measured shifts. From the comparison we conclude that the alternative scheme well predicts the experimental results.

In summary, we conclude that a kinetic scheme of the alternative type better explains the experimental results on local anaesthetic effects on inactivation modified Na channels than the standard scheme. This means that the inactivation does not play the role for local anaesthetic block of voltage-gated channels traditionally assumed; neither for K channels nor for Na channels. This conclusion seems us to be in line with recent insights in the detailed structure of channels (Doyle et al., 1998; Yellen, 1998) and with insights in the inactivation mechanism (Zhou et al., 2002) and the binding of local anaesthetics (paper II; paper III). This does not seem to be altered by the recent reports, supporting the standard version of the modulated receptor hypothesis. They do not test the alternatives discussed here (Balser et al., 1996; O´Leary et al., 1994) and they are based on less direct measurements (paper I).

6 CONCLUSIONS

- Bupivacaine blocks activity in myelinated axons by a direct effect on Na channels – not indirectly by affecting the leak current.

- Bupivacaine blocks Kv channels (Kv1.1, 1.2, 1.5, 2.1, 3.1 and 3.2) in open state.

- Bupivacaine blocks Kv2.1 and Kv1.2 differently – Kv2.1 can partly close and trap the bupivacine molecule at repolarization, while the Kv1.2 (and Kv1.1, 1.5, 2.1, 3.1 and 3.2) cannot.

- Residues G395 and C398 on the S6-segment are critical for trapping bupivacaine in Kv2.1.

- Bupivacaine binds to the PVP-region of the internal vestibule in Kv1.5.

- Bupivacaine does not directly affect the inactivation gate of inactivating Kv channels.

The effect of local anaesthetics on the Na channel inactivation is traditionally assumed to be the basis for their clinically important anti-epileptic and anti-arrhythmic action. The blocking processes presented here mean a slightly different way of understanding the molecular mechanisms of local anaesthetic action than that of the traditional modulated receptor hypothesis. This in turn may suggest new approaches in drug-design, and forms a background for the ongoing attempts to understand the physical nature of blocking voltage-gated channels in molecular dynamics simulations.

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