'Butamben, a specific local anesthetic and aspecific ion channel modulator'

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CHAPTER 1

GENERAL INTRODUCTION

Pain is a functional property of the human body. It is a warning for danger and damage. Yet, if pain is accompanied by the inability to take away its cause, it loses its usefulness and can become a chronic nuisance. It directly affects the 'quality of life'. Besides the personal tragedy for the people involved, there are also economic effects due to the fact that the pain prevents people from functioning normally in our society. Therefore, pain is of a growing concern in the medical field nowadays.

The methods that are currently being used in the treatment of intractable pain all have limited success or severe side effects. This is most obvious in the case of nerve lesioning (Candido and Stevens, 2003) and the use of systemic morphine (Donnelly et al., 2002). So, alternatives are more than welcome and many of them are being studied at this moment. The ideal alternative would have a reversible action, which would last as long as necessary and would only block the malicious pain without affecting any other system of the body. The present thesis describes a study of the mechanism of pain suppression by a local anesthetic, butamben, applied as a suspension on the hard membrane (dura mater) enveloping the spinal cord (Shulman, 1987). This method has an ultra-long duration (a few months), has a reversible action and selectively suppresses pain without affecting motor function (Korsten et al., 1991). Furthermore, it does not involve expensive or difficult to handle chemicals. These ideal features make the method really interesting, however, the exact mechanism of action remains unclear to date. Therefore, the role of this drug is investigated in this thesis by studying its effects on ion channels in pain signal producing neurons, which is a group of sensory neurons dedicated to the detection of possible tissue damage.

Pain physiology

Pain receptors, or nociceptors, are merely free nerve endings appearing in many tissues of the body. They are sensitive to various stimuli, causing a local depolarization of the membrane. The cell bodies (somata) of these primary afferent pain neurons are located in the dorsal root ganglia, like for all somatic sensation. The dorsal root ganglion (DRG) neuron extends a single process, which then bifurcates into a branch to the periphery at one side and a branch that turns to the central nervous system at the other side (Figure 1).

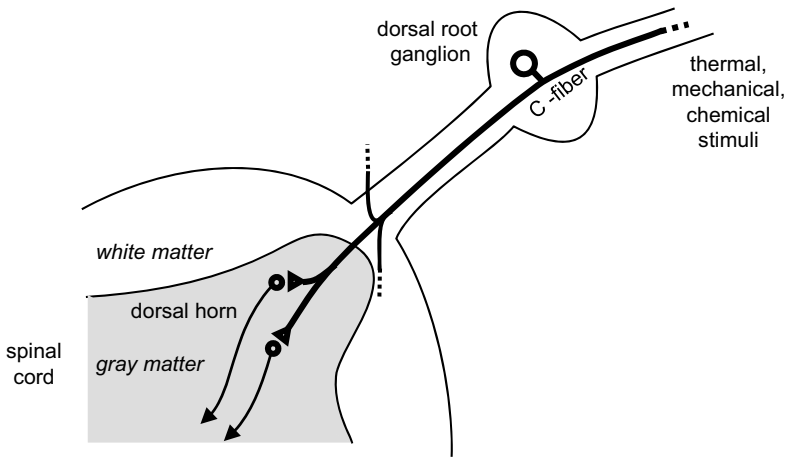

Figure 1: Schematic diagram of the location of the dorsal root ganglion (DRG) with its sensory neurons, including pain sensitive neurons. A pain fiber transmits its pain signaling action potentials from the periphery to the external layers of the dorsal horn of the spinal cord.

Pain is transmitted through two different kinds of nerve fibers (Ganong, 2003). Fast myelinated fibers, Aδ- and to a smaller extent Aβ-fibers, conducting the pain signals in the form of action potentials with a conduction velocity, largely depending on species, of 5-60m/s (Djouhri and Lawson, 2004). These nerves are aroused by either heat (>45˚C) or mechanical stimuli. These events are accompanied by a sharp or pricking pain sensation. Secondly, there are the slow unmyelinated C-fibers, characterized by their slower conduction velocity of 0.5-2 m/s. These fibers are useless for stimuli that require fast action in order to prevent tissue damage. The nerve endings of C-fibers can be found in the skin as well as deep tissues and are not only activated by thermal and mechanical but also by chemical stimuli. The latter are often associated with cell damage, such as tissue acidity (protons), which stimulates the vanilloid pain receptor VR-1 (Ganong, 2003). The pain sensation resulting from aroused C-fibers can be described as long lasting and burning. The C-fibers are at the origin of chronic pain.

The DRG neurons project their sensory information on the dorsal horn of the spinal cord (Ganong, 2003). Just before entering the dorsal horn the nerves

bifurcate and ascend as well as descend in order to enter nerve tracts that lead to the dorsal horns of neighbouring spinal cord segments. Most nerves end in the outer or marginal layers I-III of the dorsal horn on relay- or interneurons. Substance P has been identified as one of the neurotransmitters released by the dorsal root ganglion neurons. From the dorsal horn the pain information is sent to the thalamus and subsequently to other higher brain areas. Finally, the perception of pain is generated in the cortex.

The signal transmission for the whole tract from the pain receptors to the cortex is mediated by dynamic ion fluxes across the membranes of the neurons involved. These ionic fluxes are mediated and regulated by the ion channels in these neuronal membranes and are the subject of this thesis.

ION CHANNELS

Ion pumps and channels in the lipid membranes of cells are used by the cell to regulate the ionic composition of the intracellular (and extracellular) fluid. This regulation is a dynamic process and is essential for a wide variety of processes that can be divided into cell maintenance or development at one side and signal transduction and information processing at the other side. To this end, ion channels are tunnel-shaped macromolecular proteins that vary in their selective permeability for ions and their gating properties (Hille, 2001). The permeability of the ion channels can be highly specific for one type of ion, like for instance calcium, or it can be permeable to merely every ion, like gap junctional channels. An open channel passively conducts the ions along the electrochemical gradient, as opposed to ion pumps, which bring ions across the membrane against the electrochemical gradient at the cost of energy in the form of ATP. Gating of the channel can be regulated by numerous stimuli, some of which are membrane potential, temperature, ion concentrations and the presence of ligands. The actual opening of the channel occurs by a gate and is called activation, whereas closing of that so-called activation gate is called *de*activation. It is also possible however, that within a channel a different gate is closing causing a block of the

ionic flow. This is called *in*activation. Re-opening of that inactivation gate is called recovery from inactivation or de-inactivation. The two or more gates of

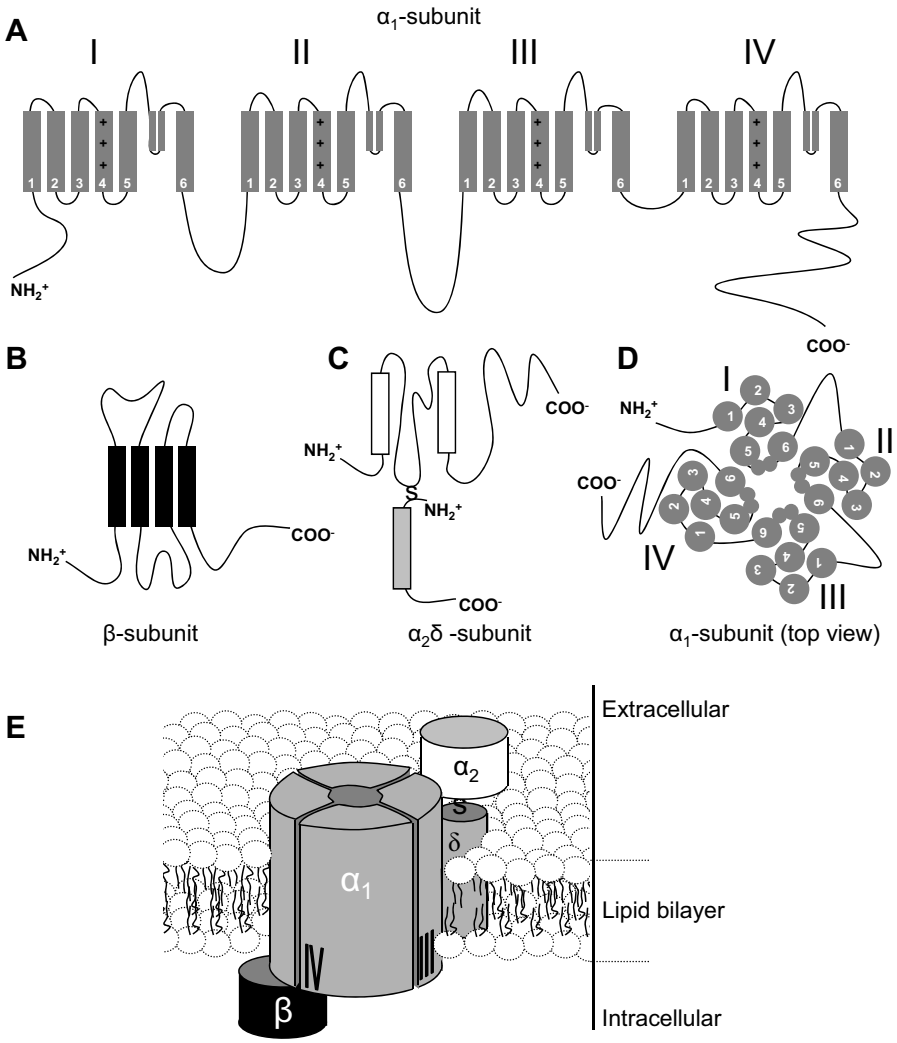

Figure 2: The structure of voltage gated calcium channels. (A) Transmembrane looping of the α1-subunit. The repeated domains are labeled with I, II, III and IV. (B) Looping of the cytoplasmic β-subunit. (C) Structure of the α2δ-subunit. The extracellular α2 and membrane spanning δ part are linked with a sulfide bridge. (D) Top view of the presumed configuration of the α1-subunit. (E) Cartoon of an assembled voltage gated calcium channel 'floating' in the lipid bilayer.

the channels can be, but are not necessarily, coupled.

In this study we looked mainly at the effect of the local anesthetic butamben on voltage-gated calcium and potassium channels, which will be discussed in the following sections.

Calcium channels

Under normal conditions intracellular calcium concentrations are kept very low, typically ~100-200 nM in neurons (Ganong, 2003). This is roughly 10^4 times lower than the extracellular calcium concentration. This steep gradient allows the cell to raise the intracellular calcium concentration quickly by an order of magnitude. The increased intracellular calcium concentration is used in the cell as a signal to trigger various kinds of actions. For instance, upon an increase in the calcium concentration close to a docked transmitter-containing vesicle in a presynaptic terminal, the vesicle will release its transmitter content in the extracellular space facing the postsynaptic membrane with its receptors for the transmitter. The resting calcium concentration is tightly regulated by calcium pumps and exchangers, removing the redundant calcium, while calcium channels serve to generate intracellular calcium signals by allowing a calcium inflow in order to (transiently) raise the calcium concentration. A prolonged increased intracellular calcium concentration is damaging and can even lead to cell death. Calcium channels are present in almost every excitable cell where they serve a variety of functions (Hille, 2001). Prominent among these are neurotransmitter release, gene transcription and, especially in the heart, action potential shaping and excitation contraction coupling.

Functional (voltage-gated) calcium channels consist of several subunits (Fig. 2). The α_1-subunit is the pore forming subunit that consists of four homologous domains, each with 6 transmembrane segments. The α_1-subunit can interact with several other noncovalently associated subunits, among which are the cytoplasmic β- and the transmembrane $\alpha_2\delta$-subunit. The β-subunit increases the current density and modulates both activation and inactivation kinetics (Varadi et al., 1991; Shistik et al., 1995). Furthermore, it is involved in second-messenger regulation and influences the pharmacological properties of the α_1-subunit (Moreno et al., 1997; Roche and Treistman, 1998). At this moment, four types of β-subunits have been identified. The $\alpha_2\delta$-subunit consists of two parts from a

single gene that are linked via disulfide bonds. It too has modulatory effects on the calcium current kinetics as well as pharmacological properties (Klugbauer et al., 1999).

Voltage-gated calcium currents have been divided into subtypes (Hille, 2001). These subtypes vary in their properties of activation, deactivation, inactivation and recovery from inactivation. The current that can be evoked at slightly depolarized potentials (~-40 mV) is called low-voltage-activated (LVA) or T-type. The high-voltage activated (HVA) calcium current is activated at strongly depolarized potentials (~ 0 mV). These currents can be pharmacologically separated into subtypes with the use of drugs and specific toxins. Examples of toxins inhibiting HVA calcium currents are ω-conotoxin-GVIA (N-type currents) and dihydropyridines (L-type). Recently, the different subtypes have been matched with several genes that encode for the α-subunit of the different subtypes. At this moment, three main families have been identified: Ca_V1-Ca_V3, each consisting of 3 or 4 members. Table 1 shows the different subtypes.

α_1 name	former name	Specific blocker	Current type
$Ca_V1.1$	α_{1S}	dihydropyridines	L-type
$Ca_V1.2$	α_{1C}	dihydropyridines	L-type
$Ca_V1.3$	α_{1D}	dihydropyridines	L-type
$Ca_V1.4$	α_{1F}	dihydropyridines	L-type
$Ca_V2.1$	α_{1A}	ω-Agatoxin-IVA	P/Q-type
$Ca_V2.2$	α_{1B}	ω-conotoxin-GVIA	N-type
$Ca_V2.3$	α_{1E}	-	R-type
$Ca_V3.1$	α_{1G}	Kurtoxin, mibefradil	T-type
$Ca_V3.2$	α_{1H}	Kurtoxin, mibefradil	T-type
$Ca_V3.3$	α_{1I}	Kurtoxin, mibefradil	T-type

Table 1: Voltage gated calcium channel genes and the associated current subtype.

Action potentials in fast myelinated nerve fibers are carried by sodium channels. The nodes of Ranvier contain, besides the sodium channels, also potassium channels, but calcium channels are absent (Waxman and Ritchie, 1993). However, the slow unmyelinated C-fibers do contain calcium channels (Quasthoff et al., 1996; Mayer et al., 1999). Several lines of research indicate a role for calcium channels in pain transmission, in particular the N- and T-type. Modifying the T-type currents in vivo has shown that they are involved in pain transmission. Agents that selectively enhance T-type currents result in exaggerated thermal and mechanical nociception, whereas T-type current reducing agents do the opposite (Todorovic et al., 2001). Apparent contradictory results were found in mice lacking one of the T-type channels (Kim et al., 2003). There T-type currents were shown to have an anti-nociceptive role, albeit in the central nervous system rather than peripheral. The N-type, which is the main subtype of calcium current present in dorsal root ganglia, plays a role too. Mice lacking the N-type calcium channel gene $Ca_V2.2$ show suppressed responses to painful stimuli (Saegusa et al., 2001). Furthermore, intrathecally applied ziconotide (synthetic form of ω-conotoxin-MVIIA), an N-type calcium current blocker, has been shown to have analgesic effects in humans (Cox, 2000). Although the exact roles remain to be determined, it is clear that calcium channels are involved in pain transmission.

Potassium channels

Potassium channels are the most diverse group of ion channels (Gutman et al., 2003). Their functions in excitable cells range from setting the membrane potential to shaping the action potential and modulating firing patterns (Hille, 2001).

The voltage gated potassium channels (K_V) are structurally similar to the voltage gated calcium channels. However, the α-subunit of the K_V channels is homologous to a single domain in the Ca_V-α1-subunit. With both the NH_2- and the COOH-terminal in the cytoplasm, it has six transmembrane segments with a pore loop between the fifth and the sixth segment. Four of these subunits together form a tetramere, which acts as a functional channel.

Kv1		Shaker-related	
	K_V1.1	Delayed rectifier	drg
	K_V1.2	Delayed rectifier	drg
	K_V1.3	Delayed rectifier	drg
	K_V1.4	A-type current	drg
	K_V1.5	Delayed rectifier	drg
	K_V1.6	Delayed rectifier	drg
	K_V1.7	Delayed rectifier	
	K_V1.8	Delayed rectifier	
K_V2		Shab-related	
	K_V2.1	Delayed rectifier	drg
	K_V2.2	Delayed rectifier	drg
K_V3		Shaw-related	
	K_V3.1	Delayed rectifier	drg
	K_V3.2	Delayed rectifier	drg
	K_V3.3	A-type current	
	K_V3.4	A-type current	drg
K_V4		Shal-related	
	K_V4.1	A-type current	drg
	K_V4.2	A-type current	drg
	K_V4.3	A-type current	drg
K_V5			
	K_V5.1	Modifier of Kv2 channels	
K_V6			
	K_V6.1	Modifier of Kv2 channels	
	K_V6.2	Modifier	
	K_V6.3	Modifier	
K_V7			
	K_V7.1	Delayed rectifier	
	K_V7.2	Delayed rectifier, M-current	drg
	K_V7.3	Delayed rectifier, M-current	drg
	K_V7.4	Delayed rectifier	drg
	K_V7.5	Delayed rectifier, M-current	drg
K_V8			
	K_V8.1	Modifier	
K_V9			
	K_V9.1	Modifier	drg
	K_V9.2	Modifier	drg
	K_V9.3	Modifier	

K_V10		Ether-a-go-go (EAG)	
	$K_V10.1$	Delayed rectifier, also conducts Ca^{2+}	
	$K_V10.2$	Outward rectifying	
K_V11		Ether-a-go-go-related (ERG)	
	$K_V11.1$	ERG, inward rectifier	drg
	$K_V11.2$	ERG	drg
	$K_V11.3$	ERG	drg
K_V12		Ether-a-go-go-like	
	$K_V12.1$	ELK, slow activation/deactivation	
	$K_V12.2$	ELK	
	$K_V12.3$	ELK, slow activation	

Table 2: Family of voltage activated potassium channels (K_V) with a short description and demonstrated presence in dorsal root ganglia (drg).

The K_V channels can be divided into several families (Coetzee et al., 1999). Table 2 shows the known K_V channels and their possible prevalence in dorsal root ganglions. The most common K_V channels are homologous to the channel families found in the insect genus *Drosophila*, which were identified first: Shaker ($K_V1.x$), Shab ($K_V2.x$), Shaw ($K_V3.x$) and Shal ($K_V4.x$). In addition, in mammals several other families have been found (K_V5-K_V12).

The Shaker potassium currents display two kinds of inactivation, N- and C-type inactivation, referring to the N- and C-terminus, respectively. N-type inactivation involves a ball-and-chain mechanism, where the N-terminus of each of the four subunits forms an inactivation particle that can reversibly occlude the channel pore (Hoshi et al., 1990). Independent of this N-type inactivation is C-type inactivation. The latter type of inactivation is a result of conformational changes in the selectivity filter and the outer pore mouth (Liu et al., 1996).

Different types of these K_V-channels can form heteromultimeric channels with properties distinct from homomultimeric channels (Isacoff et al., 1990; Ruppersberg et al., 1990). Although not all combinations seem to be found in vivo, this results in an enormous number of possible channel variants that allow cells to 'mold' their own potassium currents according to the required functions.

Like for calcium channels, auxiliary subunits can be added to modify the current. The most well defined group of these subunits is the Kvβ-subunits. This β-

subunit has been found to bind to the α-subunit of the Shaker-related Kv1 family (Sewing et al., 1996). It lacks transmembrane segments and binds noncovalently to the cytoplasmic N-terminus of the α-subunit in a 1:1 stochiometry. So, functional channels contain four α- and four β-subunits. At this moment, three Kvβ-genes have been identified, each with several splice variants. Although the effects that the β-subunits have on the ion currents seem to depend on the α-subunit composition of the channels, in general they accelerate N-type inactivation (Pongs et al., 1999). Another modulatory protein associated with a subgroup of the K_V channels is the KchAP. Its role has not been clarified, but there are indications that it acts as a chaperone protein (Kuryshev et al., 2000).

The combination with the different auxiliary subunits gives rise to an extra increase in diversity of the already highly diverse group of voltage gated potassium channels.

Kv1.1 is one of the major K_V-subunits and it plays an important role in different functional areas. Kv1.1 is present in developing neurons where it has been suggested to be involved in migration of neurons (Hallows and Tempel, 1998). Furthermore, several human disorders, like epilepsy and episodic ataxia, have been linked to Kv1.1 channels (Browne et al., 1994; Smart et al., 1998). And most relevant to this thesis, it plays an important role in pain transmission. Mice treated with antisense oligonucleotide of the Kv1.1 gene lack central analgesia induced by morphine and baclofen (Galeotti et al., 1997). Studies with mice lacking the Kv1.1 gene showed that the mice had hyperalgesia compared to the wildtype mice (Clark and Tempel, 1998). Also, a decreased efficacy of morphine was found in these null mutant mice. These studies show that Kv1.1 plays and important role in nociceptive and antinociceptive signaling pathways.

A special form of voltage gated potassium channels are the *erg* or Kv11 channels. *Erg* channels are homologous to the Drosophila *ether-a-go-go* channels. This name was derived from the mutant behavior, which displayed 'go-go-dancing' upon exposure to ether. In humans, the *erg* channels were originally thought to mainly play a role in the heart. There they are responsible for the action potential repolarizing current (Curran et al., 1995). Certain mutations in these channels are responsible for long QT syndrome, causing cardiac arrhythmias. However, more recent studies have revealed three different genes encoding for

erg in mammals (Shi et al., 1997), two of which (*erg2* and *erg3*) are specific to the nervous system. In mice it has been shown that all three variations are expressed in the dorsal root ganglia (Polvani et al., 2003). In neurons, the *erg* channels are linked to neuro-excitablility (Sacco et al., 2003).

Currents conducted by *erg* channels are characterized by a slow activation gate. A relatively fast C-type inactivation gate prevents a large current upon depolarization. However, subsequent repolarizing results in a fast relieve of inactivation, leading to an increase in current, despite a smaller driving force. This is due to a drastic increase in conductance by a fast recovery from inactivation, whereas the deactivation process takes much more time. It is this last feature that makes the *erg* channels excellent models for studying deactivation.

LOCAL ANESTHETICS

Local anesthetics have important functional properties, since regional application to nerve tissue results in a local block of nerve impulse conduction, which is reversible leaving no damage. These properties make the local anesthetics invaluable for surgical or dental procedures, which do not require or even cannot stand general anesthesia. After discovery of the first local anesthetic cocaine, from the leaves of the coca shrub, many would follow; all with slightly different properties (cf. Fig. 3). The molecular structure they share consists of hydrophilic and hydrophobic domains separated by an intermediate ester (e.g. butamben, Fig. 3) or amide linkage (e.g. lidocaine). It is generally accepted that the major mechanism of action of local anesthetics involves their interaction with one or more specific binding sites on or inside the voltage gated sodium channel (Ragsdale et al., 1994; Wang et al., 2000), the predominant ion channel type causing excitability in nerve and muscle cells. The resulting inhibition of the sodium current prevents the generation and conduction of the action potential. At least, this seems to be the case for peripheral nerve block. In epidural and spinal anesthesia, however, the mechanism may be more complex.

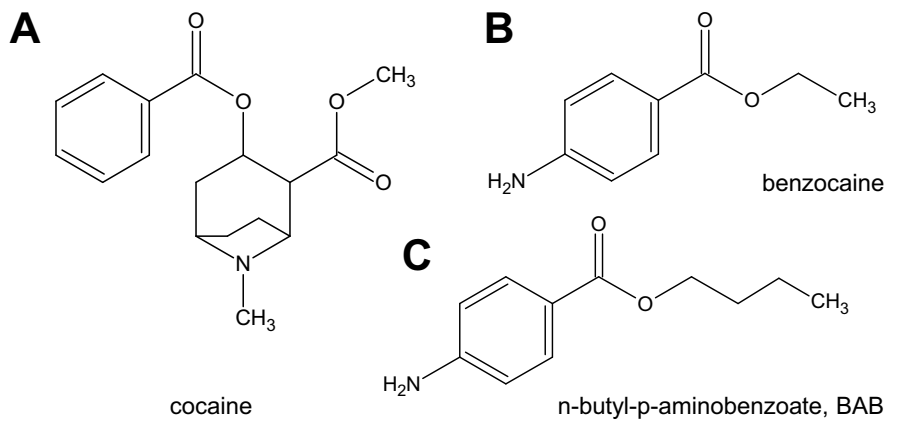

Figure 3: Comparison of the molecular structures of the local anesthetic esters cocaine, benzocaine and butamben (n-butyl-p-aminobenzoate, BAB).

It is likely to involve, besides sodium channels, other targets, such as calcium channels (Butterworth and Strichartz, 1990).

The local anesthetic n-butyl-p-aminobenzoate (abbreviated as BAB), the object of study of the present thesis, consists of a butyl ester-linked to an aminobenzoate (Fig. 3). This makes it very similar to the widely used local anesthetic benzocaine, which is an ethyl ester-linked to an aminobenzoate. The ester linkage ensures that the local anesthetic can be broken down by cholinesterase. BAB, also known as butamben, was considered of low usability, since its use was limited to topical anesthesia, due to very low water solubility (~140 mg/l at room temperature). So, soon after its development in the early twentieth century it was almost forgotten. More recently however, a renewed interest in this drug came when Shulman described ultra-long lasting selective analgesia in his patients with a 10 % aqueous suspension of BAB (Shulman, 1987). A suspension is a condition of a substance whose particles are dispersed through a fluid but not dissolved in it. Epidural injections of the BAB suspension lead to reduction of the pain for up to several months without impairing motor function. These observations have been confirmed by Korsten et al. (1991) in the

early 90s and they could even improve conditions for preparing the suspension (Grouls et al., 1991).

The long lasting effect of BAB on patients can be explained by the slow release of BAB from the suspension particles to their surroundings. The suspension serves as a depot. The question remains why BAB shows better results in selectively suppressing pain than other local anesthetics. The low octanol/water partition coefficient and the low permeability of the dura-arachnoid barrier are the unique parameters that are likely to be involved (Grouls et al., 1997; Grouls et al., 2000). However, the actual mechanism by which BAB displays its action is still unknown, but ion channels are likely targets, because ion channel block would directly affect signal transduction and transmission in pain transmitting neurons.

METHODS AND TECHNIQUES

The patch–clamp technique

The main technique used in this thesis is the patch-clamp technique in its whole-cell configuration (Hamill et al., 1981). The principle on which it is based is very simple and is known as Ohm's law: V=I*R, where V is the voltage (in Volt, V), I is the current (in Ampere, A) and R the resistance (in Ohm, Ω), the inverse of conductance G (in Siemens, S; thus, R=1/G). The idea is to get one electrode at the intracellular side of the cell membrane and another on the extracellular side and measure the resistance (or conductance) of the membrane (Fig. 4). In that configuration the bilayered membrane is the largest resistor between the two electrodes, thus any leak of current through transmembrane ion channels can easily be measured upon applying voltage across the membrane. The ions in the intra- and extracellular solution act as charge carriers. The charge and the direction of flow of the ions determine the sign of the current. For example, positive ions moving from the intracellular recording electrode through the membrane to the outside of the cell constitute positive (outward) current. The variable membrane resistance (R_V) or conductance (G_V) can be measured with

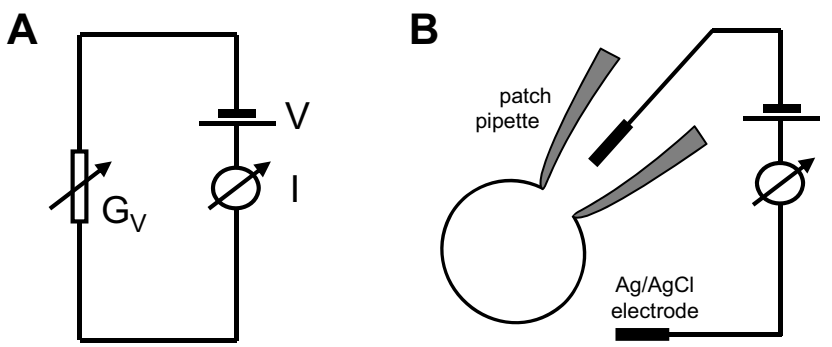

Figure 4: Schematic presentation of the voltage clamp set-up. (A) With a constant voltage source (V) and a current meter (I) the variable membrane conductance Gv can be measured. (B) Voltage-clamp conductance measurement as in (A), but with the variable conductance Gv exchanged by the membrane in the whole-cell configuration. Both the electrodes in the bath and pipette are Ag/AgCl-electrodes.

either a voltage source and a current meter in series or a current source and a voltage meter in parallel. The first method is called voltage clamp (Fig. 4A) and the latter current clamp (not shown).

To get the two electrodes at both sides of the membrane a glass pipette filled with a conducting ion solution and an inserted electrode is brought to the cell. The tip of this pipette seals with its opening to the cell membrane. With the second reference electrode in the extracellular bath solution, the configuration obtained is called the cell-attached mode. This mode allows the recording of single channel currents (in voltage-clamp) in a membrane patch of an undisrupted cell. By applying a suction pulse one can open the cell from the cell-attached mode. The membrane in the pipette mouth then breaks locally and the pipette solution will flow freely into the intracellular space, now to replace the intracellular solution. The electrode in the pipette is then in direct electrical contact with the inside of the cell membrane. This is the whole-cell configuration used in this thesis (Fig. 4B). Other possible patch-clamp measurement configurations (See Hamill et al., 1981) are not described here. The currents obtained in the whole-cell are a sum of all the currents through individual channels. When measuring from a patch containing only a small number of channels, it is possible to see the channel opening and closing of individual

channels. These so-called single channel measurements allow you to measure the actions of single protein molecules. Few techniques allow you to look at the functional behavior of single molecules on such a fast time scale. Therefore, patch clamping is a very useful technique that is implemented in a wide variety of research areas.

Other than using regular voltage or current clamp it is possible to make a combination of these techniques, the so-called 'action potential clamp' (Doerr et al., 1989). By recording an action potential in current clamp mode and applying the measured action potential shape in the voltage clamp mode to the cell, one can see the ion current flow during an action potential. During a normal action potential the individual ion channels can be considered as 'voltage-clamped' by the majority of the other channels. The 'applied' voltage in that case also has the shape of the action potential. By applying the same action potential to an isolated current type, like calcium currents, to be obtained by blocking all other channels, it is possible to see the calcium current in a more natural way.

Drug application and perfusion

Several problems or possible artifacts accompany drug application. When investigating the effect of a certain drug on ionic currents it is important to make sure that the effects measured are caused by the drug and not by other events. For instance, the ionic currents should be measured with a constant flow of extracellular solution. Changes in the velocity or direction of the flow may have direct effects on the current amplitude and kinetics (Bouskila and Bostock, 1998).

Another problem can be that the actual concentration reaching the cell is not the same as the dissolved concentration. This is important for substances that can be degraded, or be absorbed by materials present in the experimental setup. Notorious is the tubing that often is used for perfusion. 'Loading' the tubes with the used concentration before the actual experiment can prevent a lot of trouble. In all cases it is important to check the actually applied concentration with other methods where possible. The hydrophobic BAB has at room temperature a maximum solubility of ~700 µM. Making solutions with concentrations close to this maximum should be done very carefully. Ethanol, in which BAB dissolves very easily, can be used as an intermediate solvent, but

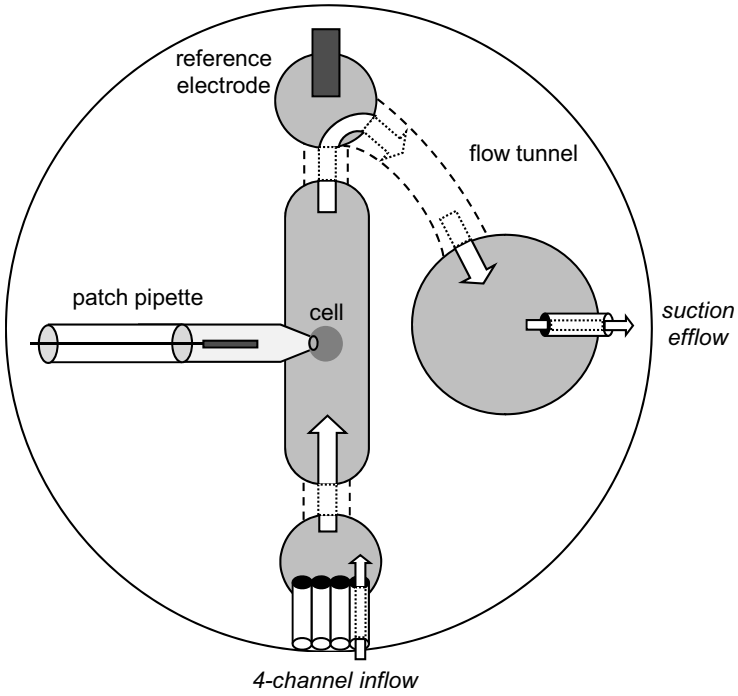

Figure 5: Schematic presentation of the micro-superfusion system used in this thesis, showing a stainless steel multi-chambered disk (19 mm diameter, 1 mm height), which could be placed inside a culture dish. Four micro-baths had been excavated in the disk and were connected through submerged tunnels, as shown in the figure (not on scale). The recording bath (~3x11 mm), drawn with cell with connected patch pipette, has a volume of ~32 µl. The shape results in a constant laminar flow feeded by one of the four superfusion inlets. The separation of the inflow and outflow from the recording bath limits the disturbing effects of vibration of the liquid. The flow is driven by gravity and was set in our experiments at ~300 µl/min. As a result the medium is fully replaced within approximately 30 s.

implies that control experiments have to be done in order to check whether the solvent or vehicle is responsible for any of the measured effects. Preheating the solutions (not too high, keeping in mind that BAB has its melting point at 58˚C) and constant stirring are necessary to prevent the formation of BAB crystals, which can take a long time to dissolve again. Furthermore, BAB binds very easily to polyethylene tubing as well as filters. BAB concentration can be checked using a spectrophotometer at a wavelength of 292 nm (Grouls et al., 1991). Absorption should be directly correlated with BAB concentration.

A schematic presentation of the perfusion system is shown in Figure 5. It consists of a coin-like piece of metal with an excavated elongated micro-bath (~32 µl), which can be inserted into the cell chamber in the set-up. Access holes for the perfusion tubes and the reference electrode and connecting micro-perfusion tunnels are also illustrated. Further explanations are provided in the legend.

In the present thesis all these precautions have been taken to study the effects of BAB.

Ion current isolation by subtraction

Highly specific blockers of ion channels can be used to identify currents from a single type of ion channel. Examples in this thesis are dendrotoxin-K and ω-conotoxin-GVIA. Dendrotoxin-K is a component of the fast and dangerous black mamba (*Dendroaspis polylepis*) venom (Harvey, 2001). Untreated envenomation by a black mamba causes death by paralysis of the respiration muscles. The 7-kDa dendrotoxin-K peptide has a high specificity for Kv1.1 subunits. Only homomultimeric channels with Kv1.1 subunits and heteromultimeric channels with two adjacent Kv1.1 subunits are blocked by dendrotoxin-K in the nanomolar range (Wang et al., 1999). The marine cone snail *Conus geographus* produces among other toxins ω-conotoxin-GVIA in its venom. With a harpoon-like tooth it injects its venom, which causes fish to become paralyzed (Olivera et al., 1991). Although the snails hunt mainly fish, the stinging can be fatal to humans as well. The ω-conotoxin-GVIA is specific for the $Ca_v2.2/\alpha_{1B}$- subunit or N-type calcium channels (Regan et al., 1991).

In our experiments the specificity of these peptides is used to discriminate between different types of current. For ω-conotoxin-GVIA that would be the N-type calcium current and for dendrotoxin-K, this is the Kv1.1 potassium current. If a certain toxin blocks only channels of interest, then the remaining current represents all channels insensitive to the drug. By subtracting the remaining current from the control or total current, the drug-sensitive current can be obtained.

It is difficult to look at effects of other drugs, e.g. BAB, on the subtracted currents, though not impossible as shown in the present thesis. Since the toxins block the channels irreversibly, the toxin-sensitive current can only be obtained

once in every cell. So, one cannot obtain paired data from the same cell (control and drug-affected currents) on the effects of BAB on the subtracted current. However, the subtraction can be done in the presence of different concentrations of BAB. If the whole toxin application and subtraction is done in the presence of concentration x, the subtracted current will represent the toxin-sensitive component in the presence of x µM BAB. This results in unpaired toxin-sensitive currents, which can be analyzed as a function of BAB concentration x.

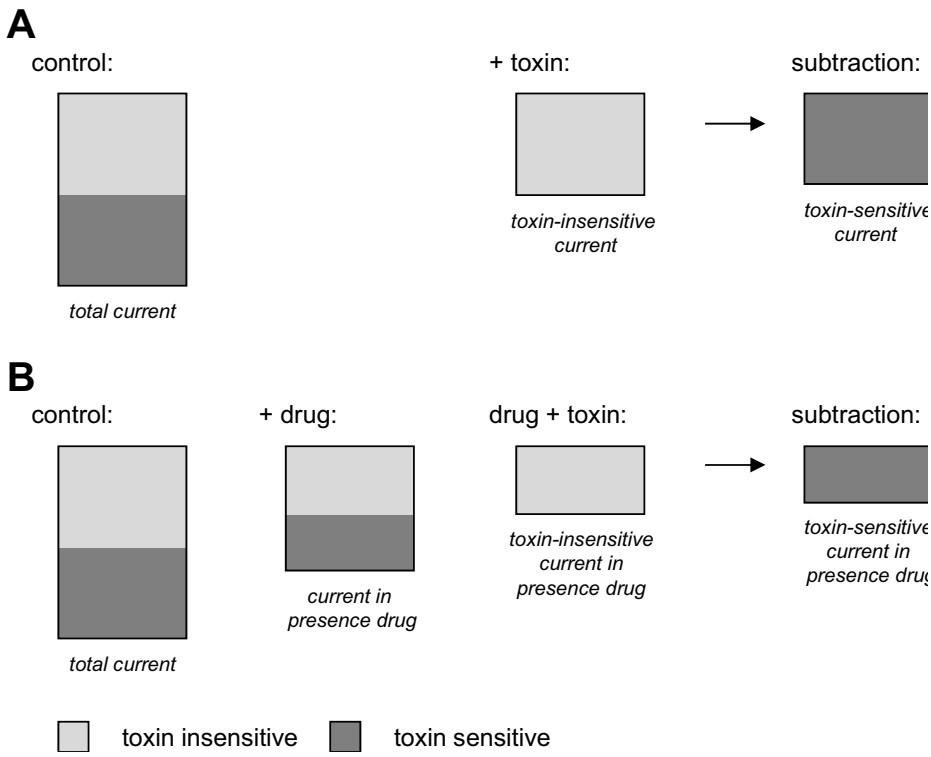

Figure 6: Schematic presentation of combined subtraction method. (A) Control subtraction procedure where the remaining current after toxin application is subtracted from the control current. The resulting current represents the toxin-sensitive current. (B) Before toxin application a drug (e.g. BAB) is applied resulting in a reduction of the total current. In the presence of this drug the toxin is applied. By subtracting the remaining current after drug- and toxin application from the current after drug-, but before toxin application one obtains the toxin-sensitive current in the presence of the drug. The drug concentration can be varied in a population of cells. So, the resulting subtracted currents can be used as unpaired data for constructing a concentration response relation.

This subtraction method is illustrated in Figure 6. Since the specific blockers bind irreversibly to the channels, as opposed to BAB, the BAB application is unlikely to interfere with the steady-state toxin binding and vice versa. Effects of variations between individual cells are diminished by an increasing number of experiments. With the same idea, the kinetics of the obtained subtracted currents can also be analyzed.

The method of subtraction demands stable conditions. Whole-cell parameters, like membrane capacity and series resistance, should stay constant during the application of the toxin. Therefore, the subtracted current traces should not show capacitive transients or membrane leak, since these would reflect changes in at least one of the parameters.

The method of subtraction is a valuable tool, but has an increased number of possible artifacts and misinterpretations, so the researcher should be extra alert.

Cloned versus native ion channels

Since the introduction of the patch clamp technique, ion currents have been measured in cells in primary culture, i.e. cells directly isolated from a tissue from the living animal. The channels conducting the ionic currents have been expressed by the cells under physiological conditions in a functional organism. With the identification of the genes encoding the ion channel proteins it became possible to bring the appropriate coding DNA or RNA into other cell systems where the channels can be expressed by the 'host' cell. Using expression systems with relatively small own 'native' conductances, it is possible to create uniform currents encoded by a known single gene. The two methods working with either cloned or native ion channels have both their advantages and supplement each other.

In the present study we used primary cultures of dorsal root ganglion (DRG) neurons (Figs 1, 7A) obtained from neonatal mice. We also used cultered undifferentiated PC12 cells (Fig. 7B). This cancer cell line has been established in the 70s of the 20th century from a transplantable rat adrenal pheochromocytoma. PC12 cells grow slowly, have features resembling chromaffin cells and have functional voltage gated calcium channels (Avidor et al., 1994). Finally, we used the HEK-tsA201 cell line as an artificial ion channel

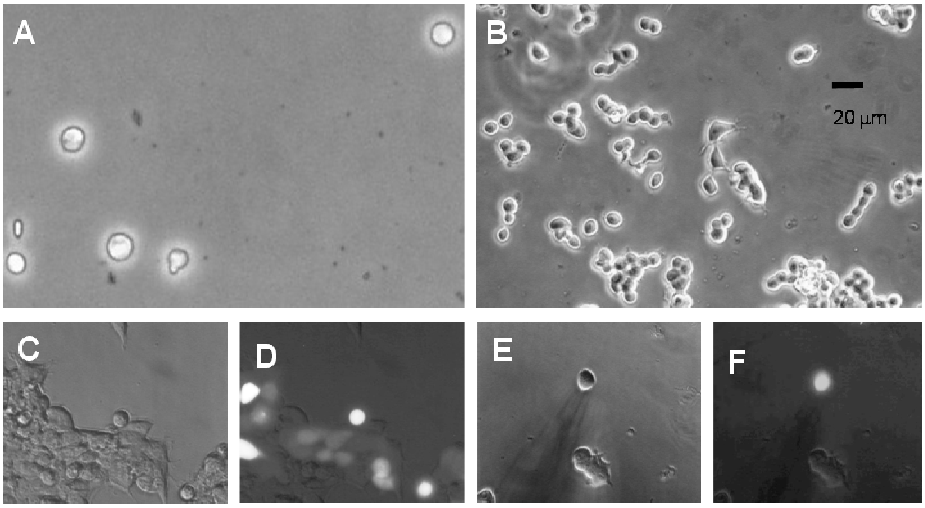

Figure 7: (A) Primary culture of acutely dissociated neonatal mouse DRG neurons within a few hours after dissociation, as seen with phase contrast microscopy. The phase-bright round sensory neuron somata are ~20 µm in diameter. The neurons have not yet grown neuronal processes at this stage, which is a requirement for good voltage-clamp recording of membrane currents. (B) phase-contrast photo of low density culture of undifferentiated PC12 cells, as used in the experiments of Chapter 5. Note the rounded morphology of the small dividing cells and the flattened polygonal cells, spreading on the dish. (C) Transfected ~50% confluent tsA-201 cell cultures (one to a few days old), as used in this study, photographed with phase contrast microscopy. The cDNA encoding for cytoplasmic Green Fluorescent Protein (GFP) is coexpressed with the cDNA of the chosen ion channel, but the transfected cells cannot be recognized with phase contrast illumination. (D) The culture in 'B' is shown with fluorescent illumination in order to identify the transfected cells. The emission at a wavelength of 508 nm during excitation with 395 nm is shown. A high correlation exists between cells expressing cytoplasmic GFP (green fluorescing at various intensities) and expressing the cotransfected ion channel. (E,F) An isolated single cell in a culture just prior to the cell-attached configuration is shown with phase contrast (E) and with fluorescence (F) illumination. The shadows in panels 'E' and 'F' in the bottom left of the pictures are the patch pipette. Isolated single cells were chosen, because coupling to neighbour cells spoils the quality of the voltage-clamp recording of whole-cell membrane currents. Cell diameter of the rounded cells in panels 'C' to 'F' is ~20 µm.

expression system (Fig. 7C-F). The advantage of using native cells is that it reveals the currents to be studied in the presence of its naturally associated processes. The membrane- or cytoplasm composition can be different between cells. This difference can have effects on the functioning of the expressed ion

channels. Furthermore, ion channels can be part of a bigger (yet unidentified) complex affecting the functioning of the channels. Finally, modulatory factors are present in these native dissociated neurons. The advantages of using cell line cultures like PC12 is that they are easily available and do not require sacrificing laboratory animals. Although their properties may have changed during long term culturing, they are often useful for specific questions. In conclusion, in native cells ion channels can be studied in their physiological environment.

The tsA201 cell line (Margolskee et al., 1993) is a subclone of HEK293 cells (Human Embryonic Kidney). tsA201 cells have a limited number of native ion channels, but have the possibility to incorporate cDNA and express the encoded protein in vast amounts. If the cells express a protein they normally lack, it is called heterologous expression (Fig. 7C,E). In order to get the DNA in the cell cytoplasm, several techniques have been developed. A well known method to transfer DNA into the cells uses a calcium-phosphate/plasmid precipitate, which enters the cell via endocytosis (Graham and van der Eb, 1973). In Chapter 2 of this thesis we used DOTAP, a cationic liposome, which associates in a complex manner with the DNA, in order to transfer it into the cell. The complex has been characterized as a "spaghetti-and-meatball" structure (Lasic, 1997; Zuidam and Barenholz, 1998). In Chapter 6 of this thesis we used a similar alternative, the commercially available Lipofectamine 2000, which is also a cationic lipid-based transfection reagent.

The advantage of studying expressed cloned ionic currents versus native ion currents is that the gene encoding the ion channel is known and that its product can be studied in isolation. When studying ion channels in cellular or multicellular processes it is beneficiary to combine the two methods, since the results will complement each other, making it easier to come to solid conclusions. Where the expressed ion channels give information about isolated interactions, the native channels allow you to study properties of ion channels in their physiological environment and therefore investigate the physiological roles of these properties.

The mechanism of selective analgesia by BAB is still unknown. Effects of BAB on voltage-gated sodium channels in sensory neurons have been described before (Van den Berg et al., 1995; Van den Berg et al., 1996). However, the effects observed in the treated patients are unlikely to be caused by effects on sodium channels alone (Butterworth and Strichartz, 1990). So, in order to explain how BAB works, it is important to look at the effects of BAB on all ion channel types involved in pain signal transmission. The obtained results do not only give information about the mechanism of selective analgesia, but they also provide information about local anesthetic action in general and lead to more insight in the physiology of the studied ion channels.

In the present thesis study we have investigated the effects of BAB on non-sodium voltage-activated channels expected to be important in pain fiber excitability, e.g. potassium and calcium channels. In Chapter 2 we first report on the effect of BAB on native total K_V and Kv1.1 current in DRG neurons and on cloned Kv1.1 channels expressed in tsA cells. BAB turned out to reduce both native total K_V currents (including Kv1.1) and cloned Kv1.1 currents while at the same time accelerating activation, deactivation and inactivation. In Chapter 3 we explored the effect of BAB on calcium channels and on N-type channels in particular. These currents were also reduced with accelerated kinetics. In Chapter 4 we focus on BAB-effects on the low-voltage activated T-type calcium currents in dorsal root ganglion neurons and see similar effects. Chapter 5 reveals that L-type calcium currents in PC12 cells are sensitive to BAB as well and in Chapter 6 we take a look at effects of BAB on the hERG potassium current. Because the simultaneous current reduction and kinetics acceleration by BAB were key features of the effects of BAB on all channels investigated in this thesis, they were put into several mathematical models in order to shed light on the mechanism behind the BAB effects at the ion channel level.

All investigated channels are present in the dorsal root ganglia in considerable numbers (Carbone and Lux, 1984; Doerr et al., 1989; Beckh and Pongs, 1990; Polvani et al., 2003) and may be involved in pain physiology or have already been shown to do so (Galeotti et al., 1997; Clark and Tempel, 1998; Hatakeyama et al.,

2001; Kim et al., 2001; Saegusa et al., 2001; Todorovic et al., 2001). Finally, we integrate in a general discussion (Chapter 7) all the results in a general picture of the mechanism of action of BAB on voltage activated cation channels and of possible mechanisms of the specific analgesic action of epidural BAB suspensions in the treatment of intractable pain.

References

Avidor B, Avidor T, Schwartz L, De Jongh KS, Atlas D (1994) Cardiac L-type Ca2+ channel triggers transmitter release in PC12 cells. FEBS Lett 342:209-213.

Beckh S, Pongs O (1990) Members of the RCK potassium channel family are differentially expressed in the rat nervous system. EMBO J 9:777-782.

Bouskila Y, Bostock H (1998) Modulation of voltage-activated calcium currents by mechanical stimulation in rat sensory neurons. Journal Of Neurophysiology 80:1647-1652.

Browne DL, Gancher ST, Nutt JG, Brunt ER, Smith EA, Kramer P, Litt M (1994) Episodic ataxia/myokymia syndrome is associated with point mutations in the human potassium channel gene, KCNA1. Nat Genet 8:136-140.

Butterworth JFt, Strichartz GR (1990) Molecular mechanisms of local anesthesia: a review. Anesthesiology 72:711-734.

Candido K, Stevens RA (2003) Intrathecal neurolytic blocks for the relief of cancer pain. Best Pract Res Clin Anaesthesiol 17:407-428.

Carbone E, Lux HD (1984) A low voltage-activated, fully inactivating Ca channel in vertebrate sensory neurones. Nature 310:501-502.

Clark JD, Tempel BL (1998) Hyperalgesia in mice lacking the Kv1.1 potassium channel gene. Neurosci Lett 251:121-124.

Coetzee WA, Amarillo Y, Chiu J, Chow A, Lau D, McCormack T, Moreno H, Nadal MS, Ozaita A, Pountney D, Saganich M, Vega-Saenz dM, Rudy B (1999) Molecular diversity of K+ channels. Ann NY AcadSci 868:233-285.

Cox B (2000) Calcium channel blockers and pain therapy. Curr Rev Pain 4:488-498.

Curran ME, Splawski I, Timothy KW, Vincent GM, Green ED, Keating MT (1995) A molecular basis for cardiac arrhythmia: HERG mutations cause long QT syndrome. Cell 80:795-803.

Djouhri L, Lawson SN (2004) Abeta-fiber nociceptive primary afferent neurons: a review of incidence and properties in relation to other afferent A-fiber neurons in mammals. Brain Res Brain Res Rev 46:131-145.

Doerr T, Denger R, Trautwein W (1989) Calcium currents in single SA nodal cells of the rabbit heart studied with action potential clamp. Pflugers Arch 413:599-603.

Donnelly S, Davis MP, Walsh D, Naughton M (2002) Morphine in cancer pain management: a practical guide. Support Care Cancer 10:13-35.

Galeotti N, Ghelardini C, Papucci L, Capaccioli S, Quattrone A, Bartolini A (1997) An antisense oligonucleotide on the mouse Shaker-like potassium channel Kv1.1 gene prevents antinociception induced by morphine and baclofen. J Pharmacol Exp Ther 281:941-949.

Ganong WF (2003) Review of Medical Physiology. , 21st Edition. New York: McGraw-Hill.

Graham FL, van der Eb AJ (1973) Transformation of rat cells by DNA of human adenovirus 5. Virology 54:536-539.

Grouls R, Korsten E, Ackerman E, Hellebrekers L, van Zundert A, Breimer D (2000) Diffusion of n-butyl-p-aminobenzoate (BAB), lidocaine and bupivacaine through the human dura-arachnoid mater in vitro. Eur J Pharm Sci 12:125-131.

Grouls RJ, Ackerman EW, Machielsen EJ, Korsten HH (1991) Butyl-p-aminobenzoate. Preparation, characterization and quality control of a suspension injection for epidural analgesia. Pharm Weekbl Sci 13:13-17.

Grouls RJ, Ackerman EW, Korsten HH, Hellebrekers LJ, Breimer DD (1997) Partition coefficients (n-octanol/water) of N-butyl-p-aminobenzoate and other local anesthetics measured by reversed-phase high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl 694:421-425.

Gutman GA, Chandy KG, Adelman JP, Aiyar J, Bayliss DA, Clapham DE, Covarriubias M, Desir GV, Furuichi K, Ganetzky B, Garcia ML, Grissmer S, Jan LY, Karschin A, Kim D, Kuperschmidt S, Kurachi Y, Lazdunski M, Lesage F, Lester HA, McKinnon D, Nichols CG, O'Kelly I, Robbins J, Robertson GA, Rudy B, Sanguinetti M, Seino S, Stuehmer W, Tamkun MM, Vandenberg CA, Wei A, Wulff H, Wymore RS (2003) International Union of Pharmacology. XLI. Compendium of voltage-gated ion channels: potassium channels. Pharmacol Rev 55:583-586.

Hallows JL, Tempel BL (1998) Expression of Kv1.1, a Shaker-like potassium channel, is temporally regulated in embryonic neurons and glia. J Neurosci 18:5682-5691.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 391:85-100.

Harvey AL (2001) Twenty years of dendrotoxins. Toxicon 39:15-26.

Hatakeyama S, Wakamori M, Ino M, Miyamoto N, Takahashi E, Yoshinaga T, Sawada K, Imoto K, Tanaka I, Yoshizawa T, Nishizawa Y, Mori Y, Niidome T, Shoji S (2001) Differential nociceptive responses in mice lacking the alpha(1B) subunit of N-type Ca(2+) channels. Neuroreport 12:2423-2427.

Hille B (2001) Ionic Channels of Excitable Membranes. Sunderland MA: Sinauer Associates.

Hoshi T, Zagotta WN, Aldrich RW (1990) Biophysical and molecular mechanisms of Shaker potassium channel inactivation. Science 250:533-538.

Isacoff EY, Jan YN, Jan LY (1990) Evidence for the formation of heteromultimeric potassium channels in Xenopus oocytes. Nature 345:530-534.

Kim C, Jun K, Lee T, Kim SS, McEnery MW, Chin H, Kim HL, Park JM, Kim DK, Jung SJ, Kim J, Shin HS (2001) Altered nociceptive response in mice deficient in the alpha(1B) subunit of the voltage-dependent calcium channel. Mol Cell Neurosci 18:235-245.

Kim D, Park D, Choi S, Lee S, Sun M, Kim C, Shin HS (2003) Thalamic control of visceral nociception mediated by T-type Ca2+ channels. Science 302:117-119.

Klugbauer N, Lacinová L, Marais E, Hobom M, Hofmann F (1999) Molecular diversity of the calcium channel alpha2delta subunit. J Neurosci 19:684-691.

Korsten HH, Ackerman EW, Grouls RJ, van Zundert AA, Boon WF, Bal F, Crommelin MA, Ribot JG, Hoefsloot F, Slooff JL (1991) Long-lasting epidural sensory blockade by n-butyl-p-aminobenzoate in the terminally ill intractable cancer pain patient. Anesthesiology 75:950-960.

Kuryshev YA, Gudz TI, Brown AM, Wible BA (2000) KChAP as a chaperone for specific K(+) channels. Am J Physiol Cell Physiol 278:C931-C941.

Lasic DD (1997) Liposomes in Gene Delivery: CRC Press.

Liu Y, Jurman ME, Yellen G (1996) Dynamic rearrangement of the outer mouth of a K+ channel during gating. Neuron 16:859-867.

Margolskee RF, McHendry-Rinde B, Horn R (1993) Panning transfected cells for electrophysiological studies. Biotechniques:906-911.

Mayer C, Quasthoff S, Grafe P (1999) Confocal imaging reveals activity-dependent intracellular Ca2+ transients in nociceptive human C fibres. Pain 81:317-322.

Moreno H, Rudy B, Llinas R (1997) beta subunits influence the biophysical and pharmacological differences between P- and Q-type calcium currents expressed in a mammalian cell line. PNAS 94 (25):14042-14047.

Olivera BM, Rivier J, Scott JK, Hillyard DR, Cruz LJ (1991) Conotoxins. J Biol Chem 266:22067-22070.

Polvani S, Masi A, Pillozzi S, Gragnani L, Crociani O, Olivotto M, Becchetti A, Wanke E, Arcangeli A (2003) Developmentally regulated expression of the mouse homologues of the potassium channel encoding genes m-erg1, m-erg2 and m-erg3. Gene Expr Patterns 3:767-776.

Pongs O, Leicher T, Berger M, Roeper J, Bahring R, Wray D, Giese KP, Silva AJ, Storm JF (1999) Functional and molecular aspects of voltage-gated K+ channel beta subunits. Ann NY Acad Sci 868:344-355.

Quasthoff S, Adelsberger H, Grosskreutz J, Arzberger T, Schroder JM (1996) Immunohistochemical and electrophysiological evidence for omega- conotoxin-sensitive calcium channels in unmyelinated C-fibres of biopsied human sural nerve. Brain Res 723:29-36.

Ragsdale DS, McPhee JC, Scheuer T, Catterall WA (1994) Molecular determinants of state-dependent block of Na+ channels by local anesthetics. Science 265:1724-1728.

Regan LJ, Sah DW, Bean BP (1991) Ca2+ channels in rat central and peripheral neurons: high-threshold current resistant to dihydropyridine blockers and omega-conotoxin. Neuron 6:269-280.

Roche JP, Treistman SN (1998) The Ca2+ channel beta(3) subunit differentially modulates G-protein sensitivity of alpha(1A) and alpha(1B) Ca2+ channels. J Neurosci 18 (3):878-886.

Ruppersberg JP, Schroter KH, Sakmann B, Stocker M, Sewing S, Pongs O (1990) Heteromultimeric channels formed by rat brain potassium-channel proteins. Nature 345:535-537.

Sacco T, Bruno A, Wanke E, Tempia F (2003) Functional roles of an ERG current isolated in cerebellar Purkinje neurons. J Neurophysiol 90:1817-1828.

Saegusa H, Kurihara T, Zong S, Kazuno A, Matsuda Y, Nonaka T, Han W, Toriyama H, Tanabe T (2001) Suppression of inflammatory and neuropathic pain symptoms in mice lacking the N-type Ca2+ channel. EMBO J 20:2349-2356.

Sewing S, Roeper J, Pongs O (1996) Kv beta 1 subunit binding specific for shaker-related potassium channel alpha subunits. Neuron 16:455-463.

Shi W, Wymore RS, Wang HS, Pan Z, Cohen IS, McKinnon D, Dixon JE (1997) Identification of two nervous system-specific members of the erg potassium channel gene family. J Neurosci 17:9423-9432.

Shistik E, Ivanina T, Puri T, Hosey M, Dascal N (1995) Ca2+ current enhancement by alpha 2/delta and beta subunits in Xenopus oocytes: contribution of changes in channel gating and alpha 1 protein level. J Physiol 489 (Pt 1):55-62.

Shulman M (1987) Treatment of cancer pain with epidural butyl-amino-benzoate suspension. Regional Anesth 12:1-4.

Smart SL, Lopantsev V, Zhang CL, Robbins CA, Wang H, Chiu SY, Schwartzkroin PA, Messing A, Tempel BL (1998) Deletion of the K(V)1.1 potassium channel causes epilepsy in mice. Neuron 20:809-819.

Todorovic SM, Jevtovic-Todorovic V, Meyenburg A, Mennerick S, Perez-Reyes E, Romano C, Olney JW, Zorumski CF (2001) Redox modulation of T-type calcium channels in rat peripheral nociceptors. Neuron 31:75-85.

Van den Berg RJ, Wang Z, Grouls RJ, Korsten HH (1996) The local anesthetic, n-butyl-p-aminobenzoate, reduces rat sensory neuron excitability by differential actions on fast and slow Na+ current components. Eur J Pharmacol 316:87-95.

Van den Berg RJ, Van Soest PF, Wang Z, Grouls RJ, Korsten HH (1995) The local anesthetic n-butyl-p-aminobenzoate selectively affects inactivation of fast sodium currents in cultured rat sensory neurons. Anesthesiology 82:1463-1473.

Varadi G, Lory P, Schultz D, Varadi M, Schwartz A (1991) Acceleration of activation and inactivation by the beta subunit of the skeletal muscle calcium channel. Nature 352:159-162.

Wang FC, Bell N, Reid P, Smith LA, McIntosh P, Robertson B, Dolly JO (1999) Identification of residues in dendrotoxin K responsible for its discrimination between neuronal K+ channels containing Kv1.1 and 1.2 alpha subunits. Eur J Biochem 263:222-229.

Wang SY, Nau C, Wang GK (2000) Residues in Na(+) channel D3-S6 segment modulate both batrachotoxin and local anesthetic affinities. Biophys J 79:1379-1387.

Waxman SG, Ritchie JM (1993) Molecular dissection of the myelinated axon. Ann Neurol 33:121-136.

Zuidam NJ, Barenholz Y (1998) Electrostatic and structural properties of complexes involving plasmid DNA and cationic lipids commonly used for gene delivery. Biochim Biophys Acta 1368:115-128.