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Activation and Modulation of Ligand-Gated Ion Channels

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Summary

Ligand-gated ionic channels are integral membrane proteins that enable rapid and selective ion fluxes across biological membranes. In excitable cells, their role is crucial for generation and propagation of electrical signals. This survey describes recent results from studies performed in the Department of Cellular Neurophysiology, Institute of Physiology ASCR, aimed at exploring the conformational dynamics of the acetylcholine, glutamate and vanilloid receptors during their activation, inactivation and desensitization. Distinct families of ion channels were selected to illustrate a rich complexity of the functional states and conformational transitions these proteins undergo. Particular attention is focused on structure-function studies and allosteric modulation of their activity. Comprehension of the fundamental principles of mechanisms involved in the operation of ligand-gated ion channels at the cellular and molecular level is an essential prerequisite for gaining an insight into the pathogenesis of many psychiatric and neurological disorders and for efficient development of novel specifically targeted drugs.

Key words

Acetylcholine receptor • GABA receptor • glutamate receptor • NMDA receptor • Vanilloid receptor • TRP receptor • Ionic channel

Introduction

The aim of this review is to outline the current research interests of the members of the Department of Cellular Neurophysiology of the Institute of Physiology ASCR celebrating its fiftieth anniversary of foundation. The intellectual roots of the Department can be traced to early sixties of the past century when an electrophysiological laboratory was founded as a subdivision of the Department for the study of neurotrophic functions headed by Prof. Ernest Gutmann. Three young scientists Drs. Radan Beránek, Pavel Hník and Ladislav Vyklický Sn. became founders of the laboratory. Their stays in the prominent laboratories abroad (Dept. of Biophysics, University College London, Prof. B. Katz; Dept. of Physiology, Aberdeen, Prof. Malcolm; Dept. of Physiology, University Gothenburg, Prof. A. Lundberg) greatly influenced further scope of the Department http://www2.biomed.cas.cz/d331/index.html that soon became a part of the world scientific community. The advent of new electrophysiological techniques, mostly the "patch clamp technique", complemented with the original techniques for fast application of drugs made it possible to study the

mechanisms of glutamate, acetylcholine and vanilloid receptor channel activation and modulation that represent the major research projects of the Department at present.

Ion channels are macromolecular protein pores in cell membranes. Although the precise evolutionary past of different groups of ionic channels is not known, they are widespread in virtually all organisms. Ion channels mediate many physiologically important processes e.g. receptor signaling, membrane potential maintenance, accumulation and transduction of electrochemical energy, and generation and spreading of action potential. Similarly to other macromolecules, they can exist in various states e.g. resting, desensitized, inactivated, activated and different states induced by various allosteric modulators.

Individual classes of channels differ according to the stimulus required for the ion channel to undergo transition from the closed into the open state. Ligand-gated ion channels represent a specific group that is activated by signal molecules and are involved in chemical signaling. The role of chemically activated channels is crucial in synaptic transmission in the nervous system and understanding their function and pharmacology has been in the focus of the neuroscience research for many years (for review see Hille 2001).

Receptor channel activation

Del Castillo and Katz (1957) were the first who proposed a simple scheme to describe activation of ligand-gated ion channels (Colquhoun and Sakmann 1985, Ogden and Colquhoun 1983, 1985, Vyklický *et al.* 1988). The scheme originally proposed for activation of nicotinic acetylcholine (nACh) receptors is useful in revealing fundamental principles of receptor channel activation and particularities of their biophysical properties that often have biological consequences.

The muscle nACh receptor is activated from two nonequivalent binding sites localized on the boundary between α subunit and neighboring δ, γ or ε subunits (for review see Arias 1997). It is still a matter of speculation whether the apparent cooperativity of cholinergic activation is inherent to a nonequivalence of binding sites or to allosteric coupling of both binding sites. However, the difference between the binding sites of embryonic and adult receptors known from the literature could partially help to understand the observed difference in the Hill coefficient (Krůšek and Vyskočil 2003). Identical affinities of both binding sites in the adult receptor (Akk and Auerbach 1996) enable to achieve theoretically the highest values of Hill coefficient (H = 2) to be attained. In the embryonic receptor with two different binding sites (Blount and Merlie 1989), the Hill coefficient decreases as the affinities of both sites differ (Fig. 1).

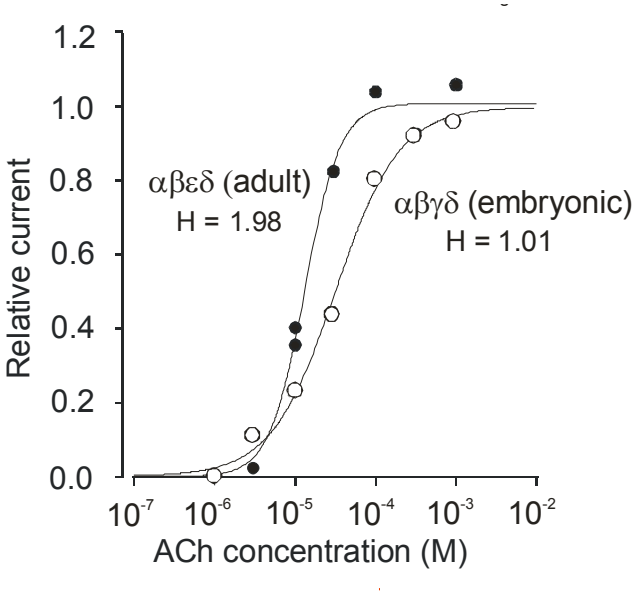

Fig. 1. The degree of cooperation between nACh binding sites underlies differences in the dose response relationship between the adult (filled circles) and embryonic (open circles) form of the receptor. Dose-response graph of relative membrane currents from COS cells clamped at –40 mV expressing adult ($\alpha_2\beta\varepsilon\delta$) and embryonic type of nACh receptor ($\alpha_2\beta\gamma\delta$). Experimental points were fitted by the Hill equation ($I(C)=C^H/(C^H+K^H)$ where C is agonist concentration, I(C) is the relative amplitude of membrane current at concentration C, K is the apparent dissociation constant for the agonist, and H is the Hill coefficient). Note a steep curve with a Hill coefficient near to 2 for adult receptor and gradually rising curve with a Hill coefficient near to 1 for embryonic receptor. Higher value of the Hill coefficient in the adult receptor corresponds to more equivalent agonist binding sites than in the embryonic receptor.

N-methyl-D-aspartate (NMDA) receptors are ligand-gated channels that upon activation are permeable for Ca^{2+} and monovalent cations. They are fundamental for excitatory neurotransmission and normal CNS function (for review see Kemp and McKernan 2002). They share the same activation principles with other ionotropic neurotransmitter receptors. Binding of the neurotransmitter – glutamate, increases the probability of channel opening, however, this is not absolutely required because the channel can open spontaneously even in the absence of glutamate (Tureček *et al.* 1997). Similar spontaneous openings were described for the nACh receptor channels (Jackson 1984, 1989, Jackson *et al.*

990). In this respect it is important to note that even in the presence of saturating glutamate concentration, the probability of NMDA receptor channel opening is rather low, in the range of ~10 %, depending on the receptor subunit composition (Chen *et al.* 1999). This is in contrast to the probability of opening of nACh receptors that is ~90 %.

A specific feature of NMDA receptor channel is that it is gated by two different agonists – the glutamate (or NMDA; selective synthetic agonist) and glycine. Simultaneous binding of both agonists to specific recognition sites at the receptor is required for an increase in the probability of channel opening (Benveniste *et al.* 1990, Johnson and Ascher 1987, Mayer *et al.* 1989a, Vyklický Jr. *et al.* 1990a). Although the binding sites for each agonist are localized at different subunits, the presence of one agonist influences binding of the other. The binding of glutamate (or NMDA) to the receptor reduces the affinity for glycine and *vice versa*. Therefore the application of glutamate and glycine results in a transient receptor activation followed by apparent receptor desensitization because one or the other molecule steadily dissociates from its binding site. The molecular mechanism underlying this process could be explained by negative allosteric coupling between glutamate and glycine binding sites (Benveniste *et al.* 1990, Vyklický Jr. *et al.* 1990a).

Temperature affects function of all ion channels, including their single channel conductance, gating, permeability and ligand-binding affinities. Generally, a reaction with temperature coefficient Q_{10} > 2-3 is suggestive of a significant temperature-dependent event (Hille 2001), but some have been demonstrated to be as high as 5-10 and even above (DeCoursey and Cherny 1998, Nobile *et al.* 1997, Pusch *et al.* 1997). Although these channels exhibit a high temperature dependence, only few of them can be directly activated by a change in temperature alone. The first cloned heat-activated channel, vanilloid receptor 1 (TRPV1, formerly VR1), has been suggested to function as a polymodal signal transducer of noxious stimuli in the mammalian somatosensory system (Caterina *et al.* 1997). A prominent characteristic for this non-selective cation channel is a rapid, nonlinear change of the response to linear temperature changes above 43 °C that is noxious for humans (Dittert *et al.* 1998). This unusually strong temperature dependence, with a high temperature coefficient (Q_{10} ~ 26), is believed to be correlated with some unusual and large-scale structural rearrangements within the homotetrameric channel complex (Vyklický *et al.* 1999). However, the critical structural domains and the mechanisms by which thermal stimuli translate into TRPV1 channel gating were revealed recently (Vlachová *et al.* 2003).

Noxious heat above ~43 °C, acidic pH (<6.8) or the irritant alkaloid capsaicin are required to open the TRPV1 channel and these stimuli act strongly synergistically: capsaicin and acids lower the threshold for heat activation, while capsaicin-activated currents are augmented by heat or by lowering pH (Tominaga *et al.* 1998, Vlachová *et al.* 2001). Moreover, at room temperature and pH 7.3, TRPV1 behaves as a voltage-gated outwardly rectifying channel since it can be activated by depolarizing voltage steps in the absence of any agonist (Vlachová *et al.* 2003). The feature of multimodality is well-characterized at the level of nociceptor, in particular, the nociceptive C fibers and it is remarkable that such multimodal function is also present at a single molecular level. Several distinct ligand recognition domains are apparently required for vanilloid-induced activation of TRPV1 channel (Jordt and Julius 2002, Jung *et al.* 2002, Vlachová *et al.* 2003, Vlachová and Vyklický 1993, Vyklický *et al.* 2003). Therefore, it seems likely that common gating machinery is involved in the activation pathways induced by various stimuli and that distinct stimuli interact in an allosteric manner.

Structure to function relationship

Brief exposure to temperatures above 52-55 °C exerts irreversible changes in vanilloid receptor TRPV1 channel activity by decreasing activation threshold to room temperatures and Q_{10} to < 5 (Lyfenko *et al.* 2002). The receptor retains its sensitivity to capsaicin and to acidic pH, supporting the conclusion that the heat-sensing protein suffers a certain degree of denaturation, while its ligand recognition site remains preserved and effectively coupled to the channel gating. TRPV1 channel heat sensitivity is strongly regulated by cytoplasmic COOH-terminal tail. The truncated receptor lacking the distal half of this structural domain (72 residues; Fig. 2) exhibits a progressive reduction of the activation thermal threshold (from 41.5 to 28.6 °C) and slowing of the activation rate of heat-evoked membrane currents (Q_{10} from 25.6 to 4.7 (Vlachová *et al.* 2003). In this context, species differences in vanilloid sensitivity are of particular importance in searching for structural domains

that confer the responsiveness of TRPV1 receptor to the specific activators. The recent findings suggest that the molecular structure of the noxious heat sensors in the frog (Kuffler *et al.* 2002) and in the chicken (Jordt and Julius 2002) are lacking the domain for binding vanilloids.

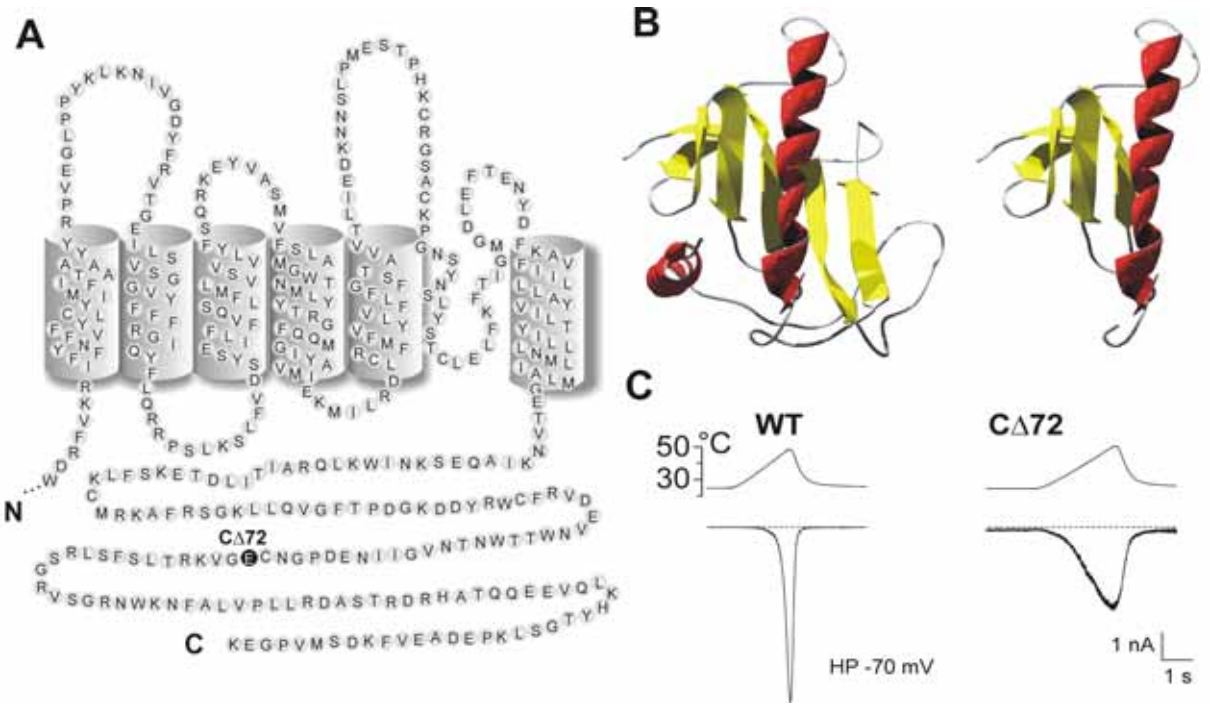

Fig. 2. The cytoplasmic C-terminal domain regulates the heat sensitivity of the vanilloid receptor TRPV1. (**A**) Putative membrane topology of the C-terminal half of the rat vanilloid receptor TRPV1 with indicated location (CΔ72) at which the stop codon has been introduced. (**B**) Proposed molecular model of the C-terminal tail of the wild type (*left*) and the truncated mutant (*right).* Homology modeling of the wild-type C terminus predicts two alpha-helices and seven beta-strands. In truncated receptor, CΔ72, one helix and two beta-strands are missing. (**C**) Whole cell heat-induced responses recorded from HEK293T cells transiently transfected with wild-type TRPV1 (*left*) and CΔ72 (*right*) mutant. Heat-evoked currents were induced by a 3 s temperature ramp (24-49 °C) in control extracellular solution. The temperature applied is shown above the records. Deletion of 72 residues from the C-terminal end reduced the amplitude of the heat induced currents (by 60 %) and the thermal threshold was significantly shifted from 42.5 °C to 28.6 °C, in average (according to Vlachová *et al.* 2003).

Recent studies from several laboratories indicate that functional and pharmacological properties of NMDA receptor channels are dependent on the receptor subunit composition. It is therefore important to understand the structural functional consequences of this receptor channel complex to reveal the role which they may play in the central nervous system under physiological and pathological conditions. NMDA receptors are heterotetramers composed of two NR1 subunits in combination with NR2A-D and/or NR3A-B subunits (Dingledine *et al.* 1999). Based on combination function it could be expected that hundreds of NMDA receptors may exist in the central nervous system that differ according to their subunit composition.

Since NMDA receptors were proposed to play a role in axotomy-induced motoneuron cell death, an experimental model of neurodegeneration, we made an attempt to characterize properties of NMDA receptors in this specific neuronal population. Unexpectedly, we found that the single NMDA receptor channel conductance is much higher (70 pS) in motoneurons than that in the spinal cord interneurons and that described for the same receptor channel type in other brain structures (Paleček *et al.* 1999). The results of non-stationary noise analysis indicated that in motoneurons the NMDA receptor channels with high conductance are activated synaptically and that their functional properties underlie an exceptionally fast deactivation of NMDA receptor mediated component of excitatory postsynaptic currents (EPSC) (Abdrachmanova *et al.* 2000a). We also found that in axotomized motoneurons destined to die, single NMDA receptor channel properties were similar to those in control motoneurons, however the time course of deactivation of the NMDA receptor EPSCs became faster

with increasing time after injury (Abdrachmanova *et al.* 2002). Single-cell RT-PCR analysis of mRNA revealed that NR1, NR2A-D and NR3A transcripts were expressed both in the control and in the axotomized motoneurons (Abdrachmanova *et al.* 2000a, 2002). These results suggest that specific assembly of NMDA receptor subunits expressed in motoneurons determine the functional and pharmacological properties of NMDA receptors in these cells.

Similarly to NMDA receptors, the (S)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of glutamate receptors are likely to be heterotetramers that in a distinct subunit composition, are also permeable to Ca^{2+}. Our results indicate that the current induced by activation of synaptic and extrasynaptic AMPA receptors in motoneurons is mediated by opening of both Ca^{2+}-permeant and Ca^{2+}-impermeant channels. As a result of axotomy, the current mediated by Ca^{2+}-permeant AMPA receptor channels is significantly reduced (Abdrachmanova *et al.* 2000b).

The nACh receptor is a transmembrane pentameric complex composed of four partially homologous polypeptide subunits (with the stoichiometry $\alpha_2\beta\varepsilon\delta$ for the adult receptor, and $\alpha_2\beta\gamma\delta$ for the embryonic receptor) surrounding a central pore region. Adult receptors are present in the postsynaptic membrane of innervated neuromuscular junctions and are crucial for safe quantal transmission (Bucharaeva *et al.* 1999, 2002, Giniatullin *et al.* 2001) and non-quantal modulation (Galkin *et al.* 2001, Malomouzh *et al.* 2003, Vyskočil 2003a), whereas the embryonic receptors are scattered in the cell membrane of embryonic and denervated muscles (Beránek and Vyskočil 1967) as well as in the *Torpedo* electric organ (Hall and Sanes 1993). Nicotinic agonists and competitive antagonists are supposed to interact with two binding sites which are positioned at the boundary between the αγ and αδ subunits in the embryonic muscle and in *Torpedo* receptors, and between the αε and αδ subunits in the adult muscle receptor. The role of the secondary (non-α) parts of the nicotinic receptor binding sites is not yet fully understood. It is supposed that these parts of binding sites interact with positively charged quarternary ammonium parts of cholinergic ligands and that they contribute to different affinities of binding sites to agonists and curariform antagonists (Bren and Sine 1997). Both binding sites in adult muscle receptors are equivalent (Akk and Auerbach 1996), while the binding sites in embryonic receptors are non-equivalent. The αδ have a greater affinity for agonists than the αγ site, but the latter has a greater affinity for antagonists than the former (Blount and Merlie 1989, Sine and Taylor 1980).

Our mutational experiments on nACh receptors indicate that selected negatively charged amino acids in δ subunit of the embryonic receptor do not control directly agonist binding, but more probably influence allosteric interactions between receptor subunits. The role of homologous amino acids in γ subunit and δ subunit in the adult receptor is less clear. The differences in the effects exerted on agonist binding and on receptor allostery could not be distinguished from whole-cell experiments in this case (Krůšek and Vyskočil 2003).

Different properties of nACh receptors in different species could be caused not only by molecular structure of receptors but could also be a subject of modulation by different microenvironment of the receptor. It was found for two close frog species (*Rana temporaria* and *Rana ridibunda*) that postsynaptic endplate currents have different onset, time course and intensity of desensitization (Giniatullin *et al.* 2001). This determines not only the safety factor of synaptic transmission but also the efficacy of drugs which are designed and used for inhibition of acetylcholinesterase (e.g. Guillou *et al.* 2000, Kovyazina *et al.* 2003, Mukhtarov *et al.* 2000) or which can promote desensitization (verapamil; Sharifullina *et al.* 2002).

Allosteric modulation

Most ligand-gated ion channels, in addition to neurotransmitter (or agonist) binding sites, also have other sites that when activated by appropriate compounds can alter the activity of the receptor channel complex. These allosteric binding sites may be located anywhere at the molecule of the receptor or ion channel pore and their activation results in a change in the affinity for the agonist (neurotransmitter) or efficacy by which the channel is opened (Mayer *et al.* 1992).

NMDA receptors possess a number of distinct recognition sites for allosteric exogenous and endogenous modulators (for review see Dingledine *et al.* 1999). The results of our experiments contributed to characterization of the pharmacological properties and action of small ions like protons (Vyklický Jr. *et al.* 1990b), Zn^{2+} (Mayer and Vyklický Jr. 1989a, Mayer *et al.* 1989b), Ca^{2+} (Vyklický Jr. 1993) and Cu^{2+} (Vlachová *et al.* 1996). The results indicate that these ions act through separate binding sites to affect (usually to decrease) the efficacy of the NMDA receptor channel opening and, therefore, to

affect excitatory synaptic transmission mediated by these receptors (Mayer *et al.* 1989b). Subsequent studies performed at recombinant NMDA receptors indicated that the action of allosteric modulators is often influenced by the receptor subunit composition. In contrast to NMDA receptors whose activity is controlled by several allosteric binding sites, the AMPA receptors appear to be less susceptible to pharmacological modulation. We identified and characterized three classes of compounds – lectins, nootropic drug aniracetam and cyclothiazide that act *via* distinct binding sites to reduce desensitization of AMPA receptor channels (Mayer and Vyklický Jr. 1989b, Patneau *et al.* 1993, Vyklický Jr. *et al.* 1991).

$GABA_A$ receptor represents the main inhibitory receptor in the brain. This chloride ion conducting channel is activated by γ-amino butyric acid and allosterically modulated by benzodiazepines and many other compounds e.g the group of antihelmintic drugs avermectins. A member of this group, ivermectin, acts at distinct binding sites to enhance affinity of the receptor for GABA and to accelerate desensitization of the $GABA_A$ receptor channel complex (Krůšek and Zemková 1994). Potentiation of $GABA_A$ receptors is of high clinical importance because it likely mediates nootropic effects of brain-derived peptide mixture Cerebrolysin. The molecular mechanism of the complex beneficial action has not been fully revealed, however, a part of the mechanism could be associated with direct activation of the $GABA_A$ and different types of glutamate receptors. Higher molecular weight fraction of Cerebrolysine is without direct activating effects, but it allosterically potentiates $GABA_A$ receptors (Zemková *et al.* 1995).

The ability of inflammatory mediators, bradykinin, prostaglandin E_2 and serotonin to interact or converge to activate/sensitize the TRPV1 receptor (Vyklický *et al.* 1998) is attributable to a synergistic action involving increased phosphorylation and decreased dephosphorylation at several protein kinase C and protein kinase A consensus sites (Bhave *et al.* 2002, Kuffler *et al.* 2002, Numazaki *et al.* 2002, Vlachová *et al.* 2002). Among the mechanisms by which the vanilloid receptor TRPV1 can be regulated from the extracellular side are also those related to alterations in local oxygen level (Szallasi and Blumberg 1993, Vyklický *et al.* 2002). Each of the four identical subunits contains 18 cysteine residues of which three, C616, C621 and C634, are located on the extracellular side of the receptor, in the pore-forming loop and the region flanking it. The finding that the reducing agent dithiothreitol (DTT) robustly increases the activation induced by capsaicin and noxious heat in both heterologously-expressed and native TRPV1 channels (Vyklický *et al.* 2002) suggests that the reduced state of sulfhydryl groups of the cysteine residues underlies the enhancing effects of DTT. On the contrary, oxidation of closely placed cysteine thiols to a disulfide bridge might be an important factor of physiological relevance for regulating the function of TRPV1.

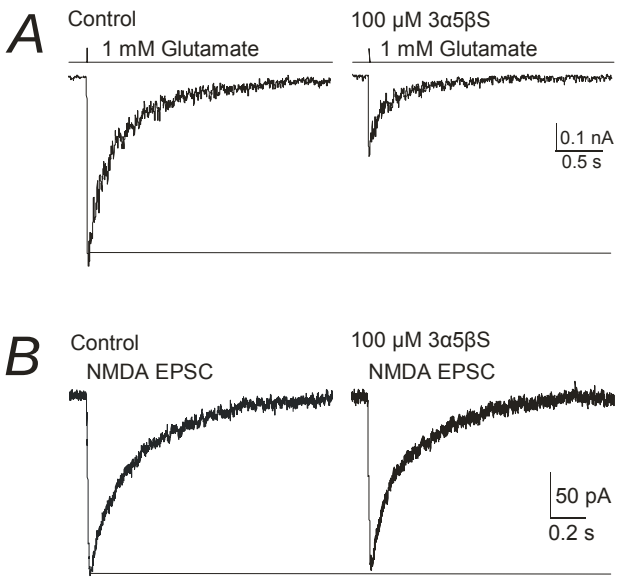

Fig. 3. Neurosteroid 20-oxo-5β-pregnan-3α-yl sulfate (3α5βS) inhibits recombinant NMDA receptors however fails to inhibit synaptically activated NMDA receptors. (**A**) Examples of traces obtained from HEK293 cells transfected by NR1-1a and NR2B subunits of the NMDA receptor. Control responses were recorded by rapid application of 1 mM glutamate for 20 ms at –60 mV in the absence of Mg^{2+} and presence of 10 μM glycine. Responses made in the presence of 100 μM 3α5βS were inhibited by 65 %. The same degree of inhibition was also obtained for NR1-1a/NR2A receptors and native mainly the extrasynaptic NMDA receptors in cultured hippocampal neurons. (**B**) Pharmacologically isolated NMDA receptor component of excitatory postsynaptic currents (NMDA receptor EPSC) recorded from layer II/III pyramidal neurons in thin brain slices from rat cerebral cortex and induced by focal electric stimulation. Recordings were made at +40 mV in the presence of Mg^{2+}. Perfusion of the slice for 5 min with extracellular solution containing 100 μM 3α5βS had only a negligible effect on the amplitude of NMDA receptor EPSC.

Conclusions

To reveal fundamental principles of operation and modulation of ligand-gated ion channels – structures vital for fast signal transduction within the central and peripheral nervous system – is essential for better understanding of complex functions like cognition and

emotions, and forms a basis for the design of new therapeutic drugs effective in the treatment of psychiatric and neurologic disorders (Vyskočil 2003b). During the last decades an enormous scientific effort revealed new exciting insights into the role of receptor channels and their clinical importance in understanding of many human diseases is just emerging. This is also true for the ionotropic glutamate receptors that mediate most of the fast excitatory synaptic transmission in the brain and spinal cord, although their excessive activation causes neurodegeneration. The results of experiments in several laboratories including ours indicate the existence of specific binding sites at NMDA receptors for neurosteroids (Abdrachmanova *et al.* 2001, Bowlby 1993, Park-Chung *et al.* 1994, Yaghoubi *et al.* 1998). Activation of these sites by various neurosteroids results in potentiation or inhibition that appears to be use-dependent. The inhibitory effect of a neurosteroid 20-oxo-5β-pregnan-3α-yl sulfate (3α5βS) appears to be restricted to extrasynaptic receptors only (Fig. 3; Sedláček, Petrović, Horák and Vyklický Jr., unpublished results) and the results are promising in the search for new drugs potentially useful in the treatment of glutamate-induced neurodegeneration e.g. in vascular and Alzheimer dementia.

The implication of TRPV1 in chemical and thermal nociception makes this receptor potentially important for pharmacological intervention. Over the last several years, an increasing number of novel compounds have been discovered that show a distinct relationship between the chemical structure and pharmacological potency to effectively block the TRPV1 channel (Garcia-Martinez *et al.* 2002, Planells-Cases *et al.* 2000, Toušová *et al.* 2004, Wang *et al.* 2002). Advancements in the knowledge of mechanisms involved in the modulation of the TRPV1 channel may provide clues in searching for potentially useful drugs.

In vitro electrophysiological analysis of ion channels connected with neuromuscular transmission and other synapses, patch-clamp recordings of currents passing through single acetylcholine, glutamate and vanilloid receptor channels, and detailed functional and molecular biology examination of the synapse can often point to candidate genes or proteins responsible for serious diseases. The expression studies that followed in the wake of mutation analysis not only afforded proofs of pathogenicity but also provided clues for rational therapy, led to precise structure – function correlations, and highlighted functionally significant molecular domains.

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