SA1

Subunit composition of nicotinic acetylcholine receptors in the neurons of autonomic ganglia

Vladimir Skok

A.A. Bogolometz Institute of Physiology, Kiev

SA2

Glycine receptor channels: structure, function and modulation

P. Bregestovski*, Y. Jacob† and E. Real†

**INSERM U29, Institute of Neurobiology, Marseille and †Pasteur Institute, Paris, France*

Glycine receptors (GlyRs) belong to the family of ligand-gated ion channels which includes receptors for gamma-aminobutyric acid (GABA), serotonin and acetylcholine. GlyRs provide inhibitory neurotransmission mainly in spinal cord and brainstem synapses of vertebrates. They are implicated in the coordination of reflex responses, processing of sensory signals and pain sensation. Dysfunction of these receptors results in hypertonic motor disorders. One of them, hyperkplexia (or startle disease) is a genetic neurological disorder of humans. To struggle with these disorders it is important to develop strategies for increasing an activatory capability of GlyR channels. Function of GlyRs is known to be regulated by protein phosphorylation and several other pathways (Betz *et al.* 1999). We cloned two GlyR subunits from zebrafish and determined their functional properties. Fusing of the inhibitory GlyR with green fluorescent protein (GFP) allowed us visualization and analysis of distribution of this protein in living cells.

Recently we discovered a novel mechanism of GlyR modulation: rapid potentiation by intracellular Ca^{2+} (Fucile *et al.* 2000). Using a patch-clamp and imaging techniques we demonstrated that in spinal cord neurons and in the HEK cells expressing homomeric GlyRs: (i) Ca^{2+} influx through receptor-operated or voltage-gated Ca^{2+}-permeable channels causes rapid and transient augmentation the amplitude of GlyR currents; (ii) the minimal interval necessary for GlyR channel potentiation is less than 100 ms; (iii) phosphorylation and G-protein pathways do not underlie this phenomenon; (iv) elevation of intracellular Ca^{2+} results in prolongation of single channel burst kinetics; (v) Ca^{2+} potentiates GlyR by increasing its apparent affinity to glycine; (vi) in inside-out patches, exposure of the cytoplasmic side of the membrane to Ca^{2+} had no effect on activity of GlyR channels, suggesting involvement of diffusible factor, presumably a Ca^{2+}-binding protein.

To identify proteins interacting with GlyR, we used the cytoplasmic loop of human α1 subunit (GlyRh1) as a bait for two-hybrid screening of a human brain cDNA library. This approach allowed us to identify five new interactors which were then individually co-expressed with GlyRh1 in human cell lines (HEK-293 and CHO cells). Analysis of the concentration dependencies of glycine-induced whole-cell currents revealed that overexpression of one of these proteins results in a 3–5-fold decrease of GlyR sensitivity to agonist.

Our results suggests that Ca^{2+} ions trigger a powerful and rapid modulation of neurotransmission at glycinergic synapses controlling a gating of GlyR channels through a diffusible Ca^{2+}-sensitive cytoplasmic intermediate.

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SA3

Getting more from channel silence: implications for the regulation of AMPA receptors by phosphorylation and synaptic plasticity

Victor A. Derkach

Vollum Institute, Oregon Health and Science University, Portland, Oregon, USA

Regulation of AMPA-type glutamate receptors by phosphorylation is central to the problem of synaptic plasticity at CNS glutamatergic synapses. Our understanding of molecular mechanisms of plasticity critically depends on measurements of the three channel properties – the number of functional receptors in the synapse, their open probability and single-channel conductance – because their product ultimately determines the amplitude of synaptic response and thus the synaptic strength. Two techniques, fluctuation analysis (Katz & Miledy, 1970; Sigworth, 1980; Traynelis *et al.* 1993) and single-channel recordings (Hamill *et al.* 1981; Colquhoun & Hawkes, 1995), have been broadly used so far to measure these fundamental parameters. I will describe in my talk a new approach to channel behaviour, silence analysis, which allows assaying these parameters in an alternative way, by analysing the probability of channels to be simultaneously closed (silent). New opportunities brought by silence analysis to the field of ion channels and receptors will be discussed. In particular, silence analysis was applied to the GluR1 AMPA receptor mutated at the S831, the site phosphorylated by calcium–calmodulin-dependent protein kinase II (CaM-KII) during long-term potentiation in the CA1 area of the hippocampus. Results indicate that a negative charge at S831 is a critical determinant for the enhanced channel function of this receptor as a charge carrier. Silence analysis provides independent evidence for the mechanism of AMPA receptor regulation by CaM-KII and further strengthens the idea how calcium-dependent phosphorylation of AMPA receptors can contribute to the plasticity at central glutamatergic synapses.

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SA4

Molecular insights into the function of the $GABA_A$ receptor

A.M. Hosie*, E.L. Dunne*, R.J. Harvey† and T.G. Smart*

**Department of Pharmacology, University College London, Gower Street, London WC1E 6BT and †Department of Pharmacology, School of Pharmacy, London WC1N 1AX, UK*

The $GABA_A$ receptor is the main inhibitory neurotransmitter receptor in the brain and is a member of the cysteine loop ligand-gated ion channel family. It has featured as a target for the action of many drugs and is thought to play a pivotal role in particular neurological diseases, including epilepsy and anxiety. In addition, these receptors are also subject to modulation by numerous endogenous agents in the brain, including Zn^{2+}, H^+ and neurosteroids as well as by intracellular regulatory processes

such as protein phosphorylation. $GABA_A$ receptors are now accepted to be hetero-pentamers, composed of core subunit members from the $\alpha(1–6)$ and $\beta(1–3)$ families, which are usually co-expressed with $\gamma(1–3)$ subunit family members to a stoichiometry of $2\alpha{:}2\beta{:}1\gamma$. There are also minor receptor populations where the γ subunit is believed to be replaced in the receptor by either δ or ϵ subunits, and a π subunit has also been recently cloned.

To date, trying to elucidate the underlying mechanisms by which these receptors operate has relied largely upon the use of site-directed mutagenesis which has outlined those domains on the receptor that are mostly responsible for GABA activation and modulation by benzodiazepines. In the absence of any crystalline structures for the $GABA_A$ receptor, it has proved difficult to obtain precise information on the location of ligand binding sites and on the identity of residues involved in signal transduction. This is particularly true when the agent under investigation exhibits distinct subtype selectivity such as Zn^{2+}, which is a potent inhibitor on $\alpha\beta$ $GABA_A$ receptors but quite weak on $\alpha\beta\gamma$ subunit receptors.

We have utilised two approaches to probing the molecular structure of $GABA_A$ receptors. A molecular modelling comparison was enabled between the $GABA_A$ receptor and the acetylcholine binding protein (AChBP) coupled with a rationale site-directed mutagenesis programme to completely resolve the molecular determinants involved in Zn^{2+} regulation of the receptor. Such an approach, coupled with patch-clamp electrophysiology, identified two main but discrete binding domains for Zn^{2+}. These are coupled with residues proximal to the GABA binding site that act as signal moderators. When the binding domains are mutated, they totally accounted for the Zn^{2+} inhibitory effect. The presumed binding domains are located in the N-terminal domain, at an interfacial site between α and β subunits, whilst the other, functionally dominant site, is located in the mouth of the anion-selective ion channel. We can now demonstrate that the inclusion of the γ subunit, which markedly reduces the sensitivity to Zn^{2+}, arises largely from disruption of the ion channel and N-terminal domain sites.

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SA5

Structure and function of P2X receptors

Alan North

University of Sheffield, Sheffield, UK

SA6

A mechanism for BK channel inactivation

Neil V. Marrion

Department of Pharmacology and MRC Centre for Synaptic Plasticity, University of Bristol, Bristol BS8 1TD, UK

Large conductance Ca^{2+}-activated K^+ (BK) channels recorded from acutely dissociated rat CA1 hippocampal neurons exhibit a slow (41 ± 9 s in 10 μM Ca^{2+}, mean ± S.E.M., $n = 12$) Ca^{2+}-dependent inactivation (Hicks & Marrion, 1998). Inactivation was reversed by membrane hyperpolarization and when $[Ca^{2+}]_i$ was reduced to resting levels (100 nM), and permanently removed by the application of intracellular trypsin. These data suggest that inactivation of BK channels results from the intracellular block of the channel pore by an associated particle (Hicks & Marrion, 1998).

An inhibitor of Ca^{2+}/calmodulin-dependent protein phosphatase 2B (PP2B), calcineurin, significantly delayed this slow inactivation behaviour. However, this appeared to be independent of protein dephosphorylation as application of ATP alone, or in combination with cAMP and the catalytic subunit of PKA did not alter the channel's inactivation properties. Application of a pre-activated Ca^{2+}/calmodulin-independent form of calcineurin (CaN_{420}) to inside-out patches excised from cultured hippocampal neurons mimicked the kinetic properties of BK channel inactivation. This effect was reversible in the absence of ATP, further underlying the dephosphorylation-independent nature of this activity. Calcineurin would appear to be regulating BK channel inactivation by a mechanism independent of protein dephosphorylation.

A direct association of the BK channel with calcineurin was therefore investigated in rat brain. A polyclonal antiserum raised against the C-terminal residues 1118–1135 of mSlo; a region conserved in rSlo (rat BK channel) immunoprecipitated a number of different proteins from solubilized rat brain. This antiserum immunoprecipitated the A subunit of calcineurin (58 kDa) in addition to the BK channel. Conversely, an antibody directed against the carboxyl terminus of the calcineurin Aα subunit immunoprecipitated the BK channel (125 kDa) from solubilized rat brain membranes. These data demonstrate a direct protein–protein interaction between the BK channel and calcineurin in rat brain, suggesting that constitutively bound calcineurin promotes BK channel inactivation by a mechanism independent of protein dephosphorylation. All animals were humanely killed.

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SA7

Two-pore domain potassium channels in mammalian neurons

Alistair Mathie, Catherine Clarke, Kishani Ranatunga and Emma Veale

Department of Biological Sciences, Imperial College London, Prince Consort Road, London SW7 2BW, UK

Background potassium (K) channels control the resting membrane potential and excitability of many mammalian neurons. They are modulated by a range of compounds, which include neurotransmitters and general anaesthetic agents. The two-pore domain K (2-PK) channel superfamily has been proposed to underlie these background K channels (Goldstein *et al.* 2001). As their name implies, the individual subunits of this superfamily have two pore regions in the amino acid sequence, which both contribute to the single pore of the functional channel. In mammals, each subunit consists of four putative transmembrane domains as opposed to six for K_V channels or two for K_{IR} channels. Currently there are known to be fourteen mammalian channels in this 2-PK channel superfamily.

We have been considering the functional properties of four of these 2-PK channels, TASK-1, TASK-2, TASK-3 and TREK-1. Our goal is to establish a functional fingerprint for each of these channels to help us to determine which of them are the most important contributors to the background currents recorded from particular native neurons. Interestingly, although these channels are open at all potentials, of the four, only TASK-1 channels show no voltage dependence of activation or inactivation. TASK-2 channels and TREK-1 channels both

display voltage-dependent activation, while TASK-3 channels display both voltage-dependent activation then inactivation. We are investigating the mechanisms underlying these voltage-dependent components.

It has not been established whether 2-PK channels can form heterodimers in native neurons. This is an important general issue since many cells are proposed to express a number of different 2-PK channels (Talley *et al.* 2001). For example, TASK-1 and TASK-3 channels are both functionally expressed in cerebellar granule neurons (Han *et al.* 2002). Recent studies provide information on this issue that is equivocal, with evidence both for and against the formation of heterodimers of 2-PK channels (Karschin *et al.* 2001, Czirjak & Enyedi, 2002). We have created a fixed pair tandem heterodimer of TASK-1 and TASK-3. We have found that this fixed tandem expresses functionally and we have used it to help us to predict whether TASK-1 and TASK-3 channels do, in fact, form heterodimers in native neurons.

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SA8

A melange of channels involved in brain and pain transmission

Brian Robertson

University of Strathclyde, Glasgow, UK

SA9

Gradients of ion channel expression in the ventricle

David McKinnon

SUNY, USA

SA10

Calmodulin involvement in Ca^{2+}-mediated modulation of M-type K^+ channels

Mark S. Shapiro and Nikita Gamper

Department of Physiology, MS 7756, UT Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78230, USA

To quantify the modulation of KCNQ2/3 current by $[Ca^{2+}]_i$ and to test if calmodulin (CaM) mediates this action, simultaneous whole-cell recording and Ca^{2+} imaging was performed on CHO cells expressing KCNQ2/3 channels, either alone, or together with wild-type (wt) CaM, or dominant-negative CaM (DN CaM). We varied $[Ca^{2+}]_i$ from < 10 to > 400 nM with ionomycin (5 μM) and either 2 mM Ca^{2+} or EGTA-buffered 0 Ca^{2+}. wt CaM made KCNQ2/3 currents highly sensitive to $[Ca^{2+}]_i$ (IC_{50} 70 ± 20 nM, maximum inhibition 73 %, $n = 10$). However, DN CaM rendered KCNQ2/3 currents largely $[Ca^{2+}]_i$ insensitive (maximum inhibition 8 ± 3 %, $n = 10$). In cells without co-transfected CaM, the Ca^{2+} sensitivity was very variable but generally weak. Co-immunoprecipitations showed binding of CaM to KCNQ3–5 that was similar in the presence of 0.5 mM Ca^{2+} or 0.5 mM EGTA. Gel-shift analyses suggested Ca^{2+}-dependent CaM binding to an 'IQ-like' motif in the carboxy-terminus of KCNQ3. We tested whether bradykinin modulation of M-current in superior cervical ganglion (SCG) neurons uses CaM. wt or DN CaM was exogenously expressed in SCG neurons using pseudovirions. With EGFP only, the inhibition was 76 ± 9 % ($n = 6$); with DN CaM, it was 31 ± 10 % ($n = 9$), and with wt CaM, it was 33 ± 9 % ($n = 8$). In all three groups, muscarinic inhibition of M-current was normal. We observed similar $[Ca^{2+}]_i$ rises by bradykinin in the three groups, indicating that CaM did not affect Ca^{2+} release from stores. Finally, $[Ca^{2+}]_i$-modulation of M-current in non-pseudovirus-infected SCG cells followed the same pattern as in CHO cells overexpressing KCNQ2/3 and wt CaM, suggesting that endogenous M-current in neurons is sensitive to $[Ca^{2+}]_i$ as well. We conclude that M-type currents are highly sensitive to $[Ca^{2+}]_i$ and that calmodulin may act as their Ca^{2+} sensor.

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SA11

Chasing the elusive second messenger for agonist-induced M-current suppression

P.A. Smith, C.P. Ford, P.L. Stemkowski and P.E. Light

Department of Pharmacology, University of Alberta, Edmonton, Canada, T6G 2H7

Neurotransmitter suppression of the voltage-dependent K^+ conductance (M-conductance, g_M) is a ubiquitous mechanism for increasing neuronal excitability (Adams *et al.* 1982). We found that ATP (P2Y)-induced g_M suppression in bullfrog sympathetic ganglion (BFSG) neurons involves phospholipase C (PLC) but not the downstream messengers, Ca^{2+}, inositol 1,4,5-trisphosphate or protein kinase C. Because the PLC inhibitor, U73122 (EC_{50} ~3 μM) promoted a transient increase in g_M, we suggested that preservation of membrane levels of phosphatidyl-inositol 4, 5 bisphosphate (PIP_2) may be conducive to M-channel opening (Stemkowski *et al.* 2002), see also Suh & Hille (2002). The transduction mechanism for ATP-induced g_M suppression may therefore involve PLC depletion of PIP_2 rather than the production of downstream products. To test this, we used standard whole-cell recording from BFSG neurons isolated from bullfrogs that were humanely killed according to locally and nationally approved protocols (Selyanko *et al.* 1990). Data are expressed as means ± S.E.M. and compared with Student's two-tailed t test for paired or unpaired data. The phosphatidyl-inositol-4-kinase (PtdIns4K) inhibitor, wortmannin (10 μM), significantly slowed the rate of recovery of g_M suppression induced by 250 μM ATP. The time for 50 % recovery increased from 19.7 ± 8.3 to 198.1 ± 33.5 s after 5 min in wortmannin, ($n = 11$, $P < 0.0004$, paired t test). Because this effect was not seen with LY294002 (10 μM, an inhibitor of phosphatidylinositol 3-kinase) or ML-7 (10 μM, an inhibitor of myosin light chain kinase), the effect of wortmannin probably reflects an action on PtdIns4K. This would impair re-synthesis of PIP_2 following its depletion during the action of ATP. Interruption of the lipid cycle at an earlier point, by inhibition of diacylglycerol kinase with R59022 (40 μM), also slowed recovery of ATP responses (half-time of recovery increased from 22.6 ± 6.0 to 56.7 ± 8.1 s, $n = 6$, $P < 0.01$, paired t test). This effect required more than one

application of agonist to deplete the levels of phospholipid intermediates within the cycle. When a 'PIP_2 neutralising antibody' (Huang *et al.* 1998) was included (1:100) in the patch pipette, ATP initially suppressed g_M by 85.6 ± 3.1 % but after 25 min of recording, the agonist effectiveness was reduced so that only 42.9 ± 15.2 % suppression was seen ($n = 4$, $P = 0.05$, paired t test). Inclusion of a similar concentration of horse serum as a control failed to affect ATP-induced g_M suppression. When KCNQ2/3 channels were expressed in tsA201 cells, the resulting 'M-like' current ran down to 48.7 ± 4.8 % ($n = 21$) of control in 5 min. Inclusion of PIP_2 (40 μM) in the pipette significantly attenuated rundown to only 78.3 ± 15.7 % of control ($n = 10$, $P < 0.05$, unpaired t test). Currents recorded in inside-out, excised patches were reduced by 47.0 ± 6.7 % ($n = 5$) by 50 μM Al^{3+} which disrupts PIP_2–ion channel interactions (Hilgemann & Ball, 1996). Although the PLC inhibitor U73122 (10 μM) antagonised g_M suppression produced by 2 μM muscarine, a similar effect was observed with the inactive isomer, U73343. It was therefore difficult to demonstrate a role for PLC in the effect of muscarine. Despite this, the time for 50 % recovery of g_M from muscarine inhibition was consistently increased after 5 min in 10 μM wortmannin. The PIP_2 antibody reduced muscarine-induced g_M suppression from 87.0 ± 6.3 to 64.0 ± 8.7 % ($n = 5$, $P < 0.002$, paired t test). These results are largely consistent with the hypothesis that ATP- and muscarine-induced g_M suppression involves PLC depletion of PIP_2.

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SA12

M-currents in sensory neurons: significance for pain suppression

G.M. Passmore*, the late A.A. Selyanko*, M. Mistry*, M. Alqatari*, S.J. Marsh*, E.A. Matthews*, A.H. Dickenson*, T.A. Brown†, S.A. Burbidge†, M. Main‡ and D.A. Brown

**Department of Pharmacology, University College London, Gower Street, London WC1E 6BT, †Neurology and CEDD, GlaxoSmithKline, North Frontiers Science Park, Harlow CM19 5AW and ‡Systems Research, GlaxoSmithKline, Medicines Research Centre, Stevenage SG1 2NY, UK*

K^+ channels play an essential role in setting the resting membrane potential and controlling the excitability of neurons, and so represent potentially attractive targets for the treatment of pain.

Recently, the novel anticovulsant retigabine was shown to have anti-neuropathic activity in two models of chronic pain, whilst having no effect in a model of acute pain (Rostock *et al.* 2000). The action of retigabine is mediated through enhancement of currents generated by neuronal KCNQ channels (KCNQ2–5) (Main *et al.* 2000; Wickenden *et al.* 2000; Tatulian *et al.* 2001). These channels are the molecular correlates of the M-channel (Wang *et al.* 1998), which carries a slowly activating, non-inactivating, voltage-dependent K^+ current (I_M) that dampens excitability (Brown, 1988).

In this study, using whole-cell perforated-patch recording, we sought the presence of M-current in cultured dorsal root ganglion (DRG) neurons from rats (17 days old, humanely killed according to approved Schedule 1 methods). M-current was the dominant subthreshold sustained current in all small cells tested (capacitance 20.4 ± 1.1 pF, $n = 30$; numbers are means and S.E.M. throughout), of which 16/22 cells were sensitive to capsaicin. M-current was also present in the majority (9) of large cells (capacitance > 100 pF, $n = 10$) tested, but in contrast to small cells, large dendrotoxin-sensitive (DTX, 100 nM) and Cs^+-sensitive (1 mM) currents were also observed.

The kinetics and pharmacology of M-current in small DRG neurons were further characterized. M-current activated at ~−60 mV and deactivated slowly (t_{fast} = 76.4 ± 9.9 ms, t_{slow} = 583 ± 134 ms, $n = 9$). I_M was inhibited by the M-channel blocker linopirdine (IC_{50} 2.1 ± 0.2 μM; $n = 8$), its analogue XE991 (IC_{50} 0.26 ± 0.01 μM; $n = 6$), Ba^{2+} (IC_{50} 0.3 ± 0.04 mM; $n = 4$) and TEA (IC_{50} 1.1 ± 0.08 mM; $n = 7$). As expected, retigabine (10 μM) enhanced I_M in a voltage-dependent manner (EC_{50} values: 0.18 ± 0.02 and 1.19 ± 0.07 μM at −20 and −50 mV, respectively, $n = 7$). Furthermore, linopirdine (10 μM) and retigabine (10 μM) reduced and increased the threshold of firing, respectively.

RT-PCR confirmed the presence of all four neuronal KCNQ subunits in whole DRG, though KCNQ4 was absent at the single cell level. Immunocytochemical data provided further evidence for the presence of KCNQ subunits in both small and large DRG neurons.

The role of I_M in the processing of neuropathic pain was investigated by recording the responses of dorsal horn spinal neurones in both naive and neuropathic (spinal nerve ligation) rats (halothane/nitrous oxide-anaesthetized). Spinal retigabine (10–90 μg) exerted dose-related inhibition of both the electrically and low- and high-intensity mechanical and thermal evoked neuronal responses in naive animals. Nociceptive primary afferent C-fibre responses and measures of spinal cord hyperexcitability were most susceptible to retigabine, whereas Aβ-fibre evoked responses were spared.

Finally, the effects of activating M-current were examined in a behavioural model of inflammatory hyperalgesia. Hyperalgesia was assessed using a dual weight averager (Clayton *et al.* 1997). Following intraplantar administration of carrageenan (2 %, 100 μl), animals treated with vehicle only distributed 21 ± 3 % of their hindleg load onto the inflamed paw. Retigabine (5 mg kg^{-1} P.O.) produced a reversal of the decrease in weight bearing on the inflamed pore (41 ± 2 %), while animals treated with both retigabine and XE991 (5 mg kg^{-1} P.O.) distributed a similar weight to those treated with vehicle alone (28 ± 3 %).

Together, these findings suggest that I_M helps to control excitability in nociceptors and that it may represent a novel therapeutic target for the treatment of pain.

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