Presynaptic Mechanism Underlying Regulation of Transmitter Release by G Protein Coupled Receptors

Tomoyuki Takahashi, Yoshinao Kajikawa, Masahiro Kimura, Naoto Saitoh, and Tetsuhiro Tsujimoto

Department of Neurophysiology, University of Tokyo Graduate School of Medicine, Tokyo 113-0033, Japan

A variety of G protein coupled receptors (GPCRs) are expressed in the presynaptic terminals of central and peripheral synapses and play regulatory roles in transmitter release. The patch-clamp whole-cell recording technique, applied to the calyx of Held presynaptic terminal in brainstem slices of rodents, has made it possible to directly examine intracellular mechanisms underlying the GPCR-mediated presynaptic inhibition. At the calyx of Held, bath-application of agonists for GPCRs such as GABA<sub>B</sub> receptors, group III metabotropic glutamate receptors (mGlur), adenosine A<sub>1</sub> receptors, or adrenaline α<sub>2</sub> receptors, attenuate evoked transmitter release via inhibiting voltage-activated Ca<sup>2+</sup> currents without affecting voltage-activated K<sup>+</sup> currents or inwardly rectifying K<sup>+</sup> currents. Furthermore, inhibition of voltage-activated Ca<sup>2+</sup> currents fully explains the magnitude of GPCR-mediated presynaptic inhibition, indicating no essential involvement of exocytotic mechanisms in the downstream of Ca<sup>2+</sup> influx. Direct loadings of G protein βγ subunit (Gβγ) into the calyceal terminal mimic and occlude the inhibitory effect of a GPCR agonist on presynaptic Ca<sup>2+</sup> currents (I<sub>Psc</sub>), suggesting that Gβγ mediates presynaptic inhibition by GPCRs. Among presynaptic GPCRs glutamate and adenosine autoreceptors play regulatory roles in transmitter release during early postnatal period when the release probability (p) is high, but these functions are lost concomitantly with a decrease in p during postnatal development.

Key Words: GPCR, GABA<sub>B</sub> receptor, mGlur, A<sub>1</sub>R, Voltage-activated calcium channel, Transmitter release, Calyx of Held

INTRODUCTION

The efficacy of synaptic transmission plays a critical role in determining the functional neuronal networks. Synaptic efficacy can be modulated by presynaptic or postsynaptic mechanisms, both of which involve a variety of GPCRs including metabotropic glutamate receptors (mGlur), GABA<sub>B</sub> receptors, acetylcholine receptors, catecholamine receptors, peptide receptors, ATP and adenosine receptors, and lipid receptors such as cannabinoid receptors. Presynaptic GPCRs play roles as autoreceptors, heteroreceptors or receptors for retrograde messengers, on binding with ligands derived from nerve terminals, postsynaptic cells or glia. The presynaptic locus in the inhibitory action of GPCR agonists on the evoked synaptic responses has been deduced from an increase in the coefficient of variation (standard deviation/mean amplitude, Forsythe & Clements, 1990), an increase in the paired-pulse ratio (Baskys & Malenka, 1981), similar magnitude of inhibitions on AMPA and NMDA receptor-mediated EPSCs (Lessa & von Gersdorff, 2001), and unchanged mean amplitude of miniature synaptic responses (Hori et al., 1992; Scanziani et al., 1992). Whereas previous studies on neuronal somata indirectly suggested mechanisms underlying the GPCR-mediated presynaptic inhibition, only recently have the presynaptic mechanism been directly addressed at the nerve terminal. The calyx of Held is a giant glutamatergic synapse in the auditory brainstem (Held, 1893), which can be visually identified in thin slices (Forsythe et al., 1994). At this synapse patch-clamp whole-cell recordings (Fig. 1B) can be made simultaneously from pre- and postsynaptic structures in slices (Borst et al., 1995; Takahashi et al., 1996) from rodents of various postnatal ages, up to one month old (Yamashita et al., 2003). Thus the calyx of Held enables one to directly test hypotheses on synaptic transmission, modulation and development. During the first postnatal month in rodents, the calyx of Held undergoes morphological (Kandler & Friauf, 1993), functional and molecular changes (Iwasaki & Takahashi, 1998, 2001; Tetschenberger & von Gersdorff, 2000; Futai et al., 2001; Joshi & Wang, 2002; Tetschenberger et al., 2002; Kimura et al., 2003; Yamashita et al., 2003) most prominently during the period of hearing onset (Postnatal day 10-12, Friauf & Lohmann, 1999; Futai et al., 2001).

ABBREVIATIONS: GPCR, GTP binding protein coupled receptor; mGlur, metabotropic glutamate receptor; A1R, adenosine A1 receptor; VACC, Voltage-activated Ca<sup>2+</sup> channel; VACK, Voltage-activated K<sup>+</sup> channel; RRP, readily releasable pool; mEPSC, miniature excitatory post synaptic current.
Presynaptic Inhibition via GPCRs

The group III mGluR agonist L-AP4, the GABA$_\beta$ receptor agonist baclofen, $A_1$ receptor agonist adenosine (Barnes-Davies & Forsythe, 1995), and $\alpha2$ receptor agonist noradrenaline (Leao & von Gersdorff, 2001), all attenuate EPSCs at the calyx of Held. The most potent ligand among them is baclofen, which markedly reduces EPSCs without affecting presynaptic action potentials (Fig. 1A). Because baclofen does not affect the mean amplitude or amplitude profile of spontaneous miniature (m) EPSCs (Takahashi et al, 1998) arising mainly from the calyceal terminal (Sahara & Takahashi, 2001), the site of baclofen action is identified as presynaptic. What is the mechanism then underlying the GABA$_\beta$ receptor-mediated presynaptic inhibition? The mean amplitude of EPSCs can be described as $Npq$, where $N$, $p$, and $q$ each represents the size of the readily releasable pool (RRP) of synaptic vesicles, release probability and mean quantal (i.e. mEPSC) amplitude (del Castillo & Katz, 1954; Sahara & Takahashi, 2001). If baclofen inhibits voltage-activated Ca$^{2+}$ channels (VACCs) in the nerve terminal, as reported in neuronal somata (Dolphin & Scott, 1987; Scholz & Miller, 1991), this will reduce $p$. However, a reduction in $p$ might also be caused by activation of voltage-activated K$^+$ channels (VAKCs), which regulate
transmitter release by deactivating VACC (Katz & Miledi, 1968; Ishikawa et al., 2003). GPCR's may also inhibit mechanisms downstream of Ca\(^{2+}\) influx thereby possibly attenuate N or p. Direct answer to this question (Fig. 1C) has been obtained at the calyx of Held by testing the effect of a GPCR agonist upon VACC currents (Fig. 2A) and VACK currents (Fig. 2B), both directly recorded from calyceal presynaptic terminals. Under whole-cell voltage-clamp of the terminal, baclofen (Issaebon 1998; Takahashi et al., 1998), L-AP4 (Fig. 2A, Takahashi et al., 1996), adenosine (Kimura et al., 2003) or noradrenaline (Leao & von Gersdorff, 2001), all attenuates VACC (Fig. 2A), whereas none of them affects VACK (Fig. 2B).

In the hippocampal pyramidal cell somata, multiple GPCRs target the inwardly rectifying potassium channels (GIRK) (Nicoll, 1988). At the calyx of Held terminal Ba\(^{2+}\) (10 \(\mu\)M)-sensitive GIRK currents can be induced by the nonhydrolysable GTP analogue GTP\(\gamma\)S, applied by pharmacology from a caged compound (Takahashi et al., 1998). If GIRQ channels are involved in the GPCR-mediated presynaptic inhibition, GIRK ligands should hyperpolarize nerve terminals, or induce outward currents under voltage clamp (at the holding potential of -70 mV). No such change, however, is observed after application of GIRK ligands (Takahashi et al., 1996, 1998; Kimura et al., 2003). Also blocking GIRK by Ba\(^{2+}\) has no effect on the baclofen-induced presynaptic inhibition (Takahashi et al., 1998).

Thus presynaptic GPCRs are present at the calyx of Held presynaptic terminal, but do not couple with GIRK channels. In many cell systems Go couples with VACC,

whereas Gi couples with GIRK (Kleuss et al., 1991; Campbell et al., 1993; Caufield et al., 1984; De Waard et al., 1997; Takano et al., 1997; Jiang et al., 1998). Consistently the calyx of Held terminal shows positive immuno-reactivity to Go but not to Gi (Fig. 3). Genetic ablation of GIRK channels does not affect the GPCR-mediated presynaptic inhibition at hippocampal synapses (Lascher et al., 1997). Thus, by far, there is no direct evidence to support that GIRK is involved in the GPCR-mediated presynaptic inhibition.

Can the VACC inhibition fully explain the presynaptic inhibitory effect of GPCR ligands then? Answer to this question has been obtained by recording EPSCs evoked by presynaptic Ca\(^{2+}\) currents in simultaneous pre- and postsynaptic recordings (Fig. 4A). Comparison between the effects of GPCR ligands and a reduction in extracellular Ca\(^{2+}\) concentration has revealed that their input (presynaptic Ca\(^{2+}\) current amplitude)-output (EPSC amplitude) relationships are indistinguishable (Fig. 4B), indicating that a reduction of Ca\(^{2+}\) currents fully explains the GPCR-mediated presynaptic inhibition, and therefore that mechanisms downstream of Ca\(^{2+}\) influx are not essentially involved (Takahashi et al., 1996, 1998; Kimura et al., 2003).

Given that the same target is shared by different GPCRs, the inhibitory effects of different GPCR ligands may occlude

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**Fig. 4.** Inhibition of VACC fully explains GPCR-mediated presynaptic inhibition. In simultaneous pre- and postsynaptic whole-cell voltage-clamp recording, EPSCs were evoked by presynaptic VACC currents elicited by a brief (1 ms) depolarizing command pulse. A. Baclofen attenuated I_{PCP} and EPSCs. After washout baclofen, subsequent reduction of Ca\(^{2+}\) concentration in the perfusate also attenuated I_{PCP} and EPSCs. B. The input (I_{PCP}, abscissa)-output (EPSC, ordinate) relationship in double logarithmic co-ordinates. Data points for baclofen application (filled circles) and those during a reduction of external Ca\(^{2+}\) concentration overlapped with each other. Inset graph compares the slope values of the input-output relationships between reduced Ca\(^{2+}\) concentration and baclofen application, at seven synapses. Figure adopted from Takahashi et al. (1998) with permission from the Society for Neuroscience.

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**Fig. 5.** Occlusion between the effects of GPCR agonists. A. The presynaptic inhibitory effect of adenosine on EPSCs (100\(\mu\)M, a/b superimposed on the top column) was occluded by baclofen (20\(\mu\)M, c/d) in a reversible manner (e/f). Figure adapted from Kimura et al. (2003) with permission from The Physiological Society. B. Baclofen (20\(\mu\)M) occluded the presynaptic inhibitory effect of L-AP4 (100\(\mu\)M).
The $\beta\gamma$ subunit of G protein (G $\beta\gamma$) inhibits presynaptic VACC currents. A, G $\beta\gamma$ (100 nM) directly loaded into a presynaptic terminal attenuated Ipc, whereas inactivated (boiled) G $\beta\gamma$ had no such effect (c/d). B, G $\beta\gamma$ blocked the inhibitory effect of baclofen (200 nM) on Ipc. C, The $\alpha$ subunit of G protein affected neither Ipc nor the baclofen-induced Ipc inhibition. Figure adopted from Kajikawa et al (2001) with permission from the National Academy of Sciences.

with each other. Baclofen at its maximal concentration (20 $\mu$M) attenuates EPSCs by 80% (Takahashi et al, 1998), whereas L-AP4 or adenosine attenuates EPSCs maximally by 40% (Takahashi et al, 1996; Kinura et al, 2003). As illustrated in Fig. 5 baclofen (20 $\mu$M) blocks the inhibitory effects of adenosine (Fig. 5A) and L-AP4 (Fig. 5B). Together these results indicate that multiple GPCRs in the calyx of Held presynaptic terminal are converged into a common path for the presynaptic inhibition. Similarly, at the inhibitory synapse of spinal cord in culture, GABA$\beta$ receptors and A1 receptors attenuate transmitter release in a convergent manner (Hugel & Schlichter, 2003). At the hippocampal cell somata, GABA, serotonin and adenosine, all activate GIRK currents (Nicol, 1988). In Xenopus oocytes expressed with multiple recombinant GPCRs, all GPCRs are linked, via inositol 1,4,5-trisphosphate-induced Ca$^{2+}$ release from internal stores, to Ca$^{2+}$-induced CL-channels (Harada et al, 1987; Parker et al, 1987; Takahashi et al, 1987). Thus activations of multiple GPCRs converge into a common mechanism among various cell systems and nerve terminals.

**Intra-terminal Coupling Mechanism between GPCRs and VACCs**

At the cell somata, heterotrimeric G proteins affects their targets either directly via $\beta\gamma$ subunits in a membrane-delimited manner (De Waard et al, 1997), or indirectly via second messengers (for review, see Hille, 1994). What is the intracellular mechanism, which couples GPCRs with VACCs at the nerve terminal? Direct loading of G protein $\beta\gamma$ subunits (G $\beta\gamma$, 100 nM) into the calyceal terminal slows the activation kinetics and reduces the amplitude of Ca$^{2+}$ currents (Fig. 6A, B) (Kajikawa et al, 2001). Heat-inactivated G $\beta\gamma$ in contrast, has no such effect (Fig. 6A). G $\beta\gamma$ also blocks inhibitory effect of baclofen on Ca$^{2+}$ currents (Fig. 6B), whereas G protein $\alpha$ subunit has no such effect (Fig. 6C). Direct loading of cAMP or cGMP at high concentrations (200 $\mu$M) into the calyceal terminal has no effect on the baclofen-induced presynaptic inhibition. Thus,
at the calyx of Held presynaptic terminal, Gβγ dissociated from heterotrimeric G proteins (Gαβγ) seems to directly inhibit VACCs, thereby inhibiting transmitter release (Fig. 7).

At the calyx of Held, PQ-type VACC predominantly mediates transmitter release after postnatal day 14 (P14, Iwasaki & Takahashi, 1998; Iwasaki et al, 2000), whereas N-type (Iwasaki & Takahashi, 1998; Wu et al, 1999; Iwasaki et al, 2000) and R-type (Wu et al, 1998) VACCs also contribute to synaptic transmission at more immature calyces (P8-10). Although N-type VACC is selectively coupled to GPCRs at some synapses (Yao & Chuhma, 1993; Umemiya & Berger, 1994; Momiyama & Koga, 2001), at the immature calyx of Held, GPCR agonists inhibit all three types of VACC (Takahashi et al, 1996, 1998; Wu et al, 1998; Kimura et al, 2003) with no clear selectivity (Kimura et al, 2003).

Physiological Role and Developmental Change of Presynaptic GPCRs

What is the physiological role of presynaptic GPCRs? Transmitter glutamate released from nerve terminals may activate presynaptic mGluR autoreceptors thereby regulating p. In fact the group III mGluR antagonist CPPG (300 μM) significantly reduces the magnitude of synaptic depression during repetitive stimulation at 10 Hz at P7-P11 in rats (Fig. 8A) (von Gersdorff et al, 1997; Iwasaki & Takahashi, 2001). However, this effect becomes undetectable at P14 (Fig. 8A) (Iwasaki & Takahashi, 2001). Because bath-application of L-AP4 inhibits EPSCs at P14 to a similar extent as at P7 (Iwasaki & Takahashi, 2001), amount of released glutamate reaching presynaptic mGluRs seems to decrease with development, possibly because of reduced p or accelerated transmitter clearance. A1 receptors also play auto-regulatory role at immature (P7) calyx of Held, with the A1 receptor antagonist CPT (0.5 μM) slightly attenuating synaptic depression during 10 Hz stimuli (Fig. 8B). ATP released from nerve terminals (and also possibly from postsynaptic cells or glia) is rapidly converted to adenosine (Dunwiddie et al, 1997) and activates A1 receptors. As animals mature, however, A1 receptor expression at the calyceal nerve terminal is down-regulated (Fig. 9C and D). In parallel, adenosine-induced presynaptic inhibition becomes weak (Fig. 9A and B) with CPT no longer attenuating synaptic depression at P14 (Kimura et al, 2003). Thus, auto-receptor function of GPCRs is restricted to the early developmental period at the calyx of Held. Immature synapses show strong synaptic depression during repetitive stimulation because of high p contributing to a depletion of the RRP (Bolshakov & Siegelbaum, 1995; Tutschberger & von Gersdorff, 2000; Iwasaki & Takahashi, 2001). At the immature synapses, autoreceptor inhibition mediated by multiple GPCRs may relieve the nerve terminal from severe depletion of synaptic vesicles.

Another possible role of GPCRs is the regulation of transmitter release via ambient ligands. At the hippocampal mossy fiber synapse A1 receptor antagonist enhances the field EPSP amplitude, suggesting that presynaptic A1 receptor tonically attenuates transmitter release (Moore et al, 2003). A tonic decrease in p caused by ambient GPCR ligands reduces the magnitude of depression during repetitive stimulation thereby gaining higher synaptic efficacy for high frequency inputs (Brenowitz et al, 1998; Moore et al, 2003). At the calyx of Held in slices, however, neither the GABA receptor antagonist SCH 50911 (20 μM, Yamauchi & Takahashi, 2000), mGluR antagonist CPPG (Iwasaki & Takahashi, 2001), nor A1 receptor antagonist CPT (Kimura et al, 2003) enhances EPSCs. The concentration of ambient GPCR agonists might presumably be higher in vivo or in pathological conditions, at which GPCR may tonically regulate transmitter release. Although the exact physiological role of presynaptic GPCRs at the calyx of Held terminal remains unclear, direct evidence obtained at this nerve terminal has elucidated a mechanism underlying

Fig. 8. Autoreceptor inhibitory functions of GPCRs at the calyx of Held. A, The group III mGluR antagonist CPPG rescues synaptic depression during a repetitive (10 Hz) stimulation at P7, but not at P14. B, Adenosine rescues synaptic depression during 10 Hz stimulation at P7. Figures adopted from (A) Iwasaki and Takahashi (2001) and (B) Kimura et al (2003) with permission from The Physiological Society.
GPCR-mediated presynaptic inhibition widely present in the central and peripheral synapses.

Unidentified Intracellular Mechanism Mediated by Presynaptic GPCRs

Blocking G protein activity by intra-terminal loading of GDP βS dramatically slows the recovery time (by >10 s in time constant) from synaptic depression (induced by 10 Hz stimulation) suggesting that monomeric G proteins such as Rab3A may contribute to accelerating vesicle replenishment (Takahashi et al, 2000). More recently, baclofen is shown to slow the recovery of EPSCs from synaptic depression (by about 1 s in half time) at the calyx of Held (P8-10) suggesting that heterotrimeric G proteins may also contribute to recruitment of synaptic vesicles (Sakaba & Neher, 2003). However, this baclofen effect is unlikely to contribute to the baclofen-induced inhibition of EPSCs, because slowing vesicle replenishment by 1 s would not significantly affect transmitter release evoked every 10s (Takahashi, 1998). Thus the physiological role of GPCR-mediated slowing in vesicle replenishment remains elusive.

Although the inhibitory effect of GPCR ligands on evoked synaptic response is mediated by presynaptic VACC, their effect on spontaneous miniature events might be mediated by a different mechanism. Bath-application of GPCR ligands reduces mean frequency of miniature synaptic currents at many synapses (Hori et al, 1992; Hayashi et al, 1993; Sladeczek et al, 1993; Takahashi et al, 1998; Kimura et al, 2003; Liang et al, 2004). These effects cannot be explained by an inhibition of high-voltage-activated VACCs such as N, P/Q and R type channels. In fact, blocking VACC by Cd²⁺ (100 μM) has no effect on the cannabinoid-induced reduction of mEPSC frequency (Liang et al, 2004). However, replacement of extracellular Ca²⁺ by Mg²⁺ abolishes a reduction of mEPSC frequency induced by opiates in spinal cord neurons (Hori et al, 1992) and that induced by mGlur agonist in cerebral cortical neurons (Sladeczek et al, 1993). These results suggest that GPCR-mediated reduction of mEPSC frequency might be caused by a reduction in Ca²⁺ influx through unidentified pathway. At the lamprey spinal cord, Gβγ directly inhibits exocytotic machinery without affecting Ca²⁺ influx (Blackmer et al, 2001). A similar mechanism might also underlie the inhibitory effect of GPCRs on the mEPSC frequency at the mammalian synapses.

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