Phospho-dependent binding of the clathrin AP2 adaptor complex to GABA<sub>A</sub> receptors regulates the efficacy of inhibitory synaptic transmission

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The efficacy of synaptic inhibition depends on the number of γ-aminobutyric acid type A receptors (GABA<sub>A</sub>Rs) expressed on the cell surface of neurons. The clathrin adaptor protein 2 (AP2) complex is a critical regulator of GABA<sub>A</sub>R endocytosis and, hence, surface receptor number. Here, we identify a previously uncharacterized atypical AP2 binding motif conserved within the intracellular domains of all GABA<sub>A</sub>R β subunit isoforms. This AP2 binding motif (KTHLRRRSSQLK in the β3 subunit) incorporates the major sites of serine phosphorylation within receptor β subunits, and phosphorylation within this site inhibits AP2 binding. Furthermore, by using surface plasmon resonance, we establish that a peptide (pepβ3) corresponding to the AP2 binding motif in the GABA<sub>A</sub>R β3 subunit binds to AP2 with high affinity only when dephosphorylated. Moreover, the pepβ3 peptide, but not its phosphorylated equivalent (pepβ3-phos), enhanced the amplitude of miniature inhibitory synaptic current and whole cell GABA<sub>A</sub>R current. These effects of pepβ3 on GABA<sub>A</sub>R current were occluded by inhibitors of dynamin-dependent endocytosis supporting an action of pepβ3 on GABA<sub>A</sub>R endocytosis. Therefore, phospho-dependent regulation of AP2 binding to GABA<sub>A</sub>Rs provides a mechanism to specify receptor cell surface number and the efficacy of inhibitory synaptic transmission.

GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) are the major sites of fast synaptic inhibition in the brain (1). These pentameric ligand-gated ion channels can be constructed from seven subunit classes: α1–6, β 1–3, γ 1–3, δ, ε, π, and θ (2), with the majority of benzodiazepine-sensitive receptor subtypes being assembled from α, β, and γ2 subunits (1,2). A primary determinant for the efficacy of synaptic inhibition and, hence, neuronal excitation is the number of functional GABA<sub>A</sub>Rs expressed on the surface of neurons (3–10). Therefore, there has been considerable interest in understanding the cellular mechanism that neurons use to regulate GABA<sub>A</sub>R cell surface stability and activity. Collectively these studies have revealed that neuronal GABA<sub>A</sub>Rs undergo significant rates of constitutive endocytosis (3, 8, 11–15), a process that has been established to regulate synaptic inhibition (8). GABA<sub>A</sub>Rs enter the endocytic pathway by a clathrin-mediated dynamin-dependent mechanism (8, 11–14), a process that is facilitated by the clathrin adaptor protein 2 (AP2) complex, which is intimately associated with these receptors in neurons (8, 13). Internalized GABA<sub>A</sub>Rs are then subjected to either rapid recycling or targeted for lysosomal degradation, an endocytic sorting decision that is regulated by the Huntingtin associated protein-1 (15). Therefore, changes in the rates of GABA<sub>A</sub>R endocytosis and/or endocytic sorting represent potentially powerful mechanisms to regulate GABA<sub>A</sub>R cell surface number and inhibitory synaptic transmission (8, 15).

A potential mechanism to regulate target protein endocytosis is modulating interaction with the AP2 adaptor protein complex (16–18). This protein complex, which is composed of α, β2, μ2, and α2 adaptin subunits (16–19), binds to defined endocytic motifs in cargo proteins (16–19). There is accumulating evidence that direct phosphorylation of these motifs or adjacent residues can modify AP2 binding and, hence, cargo removal from the cell surface (17–19). It is well established that GABA<sub>A</sub>R intracellular domains are phosphorylated by multiple protein kinases (7, 9, 10, 20–25). Moreover, changes in the stoichiometry of GABA<sub>A</sub>R β-subunit phosphorylation are strongly correlated with modified cell surface receptor number (7, 9), but a molecular mechanism linking these processes remains to be defined.

Here, we identify molecular determinants responsible for binding of the AP2 complex to GABA<sub>A</sub>Rs. We demonstrate that the μ2 subunit of AP2 interacts directly with an atypical sorting motif in GABA<sub>A</sub>R β subunits, which is enriched in lysine and arginine residues. This motif incorporates the major sites of phosphorylation for PKC and protein kinase A (PKA) within this class of receptor subunits, serine residues S408 and S409 in the case of the GABA<sub>A</sub>R β3 subunit peptide; PKA, protein kinase A.

Materials and Methods
Peptides, Antibodies, and cDNA Constructs. Peptides were synthesized corresponding to residues 401–412 of the rat GABA<sub>A</sub>R β3 subunit (pepβ3), and an identical peptide (pepβ3-phos) chemically phosphorylated at S408/S409 (Protein/DNA Technology Center, The Rockefeller University, New York). The dynamin blocking P4 peptide was purchased from Tocris Cookson. Mouse anti-μ2 was from BD Biosciences and was used for immunoblotting at 1:250. Plasmids to the GABA<sub>A</sub>R subunit intracellular domains fused to GST (8, 9, 15) and the subunits of AP2 (16) have been described.

| Abbreviations: AP2, adaptor protein 2; EGFR, epidermal growth factor receptor; GABA<sub>A</sub>R, GABA<sub>A</sub> receptor; ICD, intracellular domain; mIPSC, miniature inhibitory postsynaptic current; pepβ3, GABA<sub>A</sub>R β3 subunit; pepβ3-phos, GABA<sub>A</sub>R β3 subunit peptide; PKA, protein kinase A.

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Affinity Purification Assays and Surface Plasmon Resonance. GST affinity purification assays and surface plasmon resonance assays were performed essentially as described in a number of previous studies (refs. 8, 9, and 26; see also Supporting Materials and Methods, which is published as supporting information on the PNAS web site).

Acute-Dissociation Procedure and Neuronal Culture. Cortical neurons from young adult (3–5 weeks postnatal) rats were acutely dissociated by using procedures similar to those described in ref. 20 and Supporting Materials and Methods. Cultures of cortical neurons were prepared as described in refs. 9 and 15.

Whole-Cell recordings. Whole-cell recordings of currents in isolated and cultured cortical neurons used standard voltage-clamp techniques (8, 15, 20) and are outlined in detail as Supporting Materials and Methods.

Results GABA_A Receptors Associate with the μ2 Subunit of AP2. We have demonstrated that the intracellular domains (ICD) of GABA_ARs β and γ subunits interact with the brain AP2 adaptor complex in vitro, and both we, and more recently others, have shown that these proteins coimmunoprecipitate from neuronal lysates (8, 13). However, it was unclear whether this protein was a direct interaction or mediated by a bridging molecule. The AP2 complex is comprised of four subunits: α, β2, μ2, and σ2 adaptins (16–18). We initially performed affinity purification experiments to establish whether a direct interaction with AP2 is present and, if so, which component(s) of the AP2 complex may mediate interaction with GABA_ARs. A representative member of each of the most common GABA_Ar subunit ICDs were expressed as GST fusion proteins, purified, immobilized on glutathione agarose beads, and exposed to individual [35S]-methionine-labeled AP2 subunits produced by in vitro translation. Using this approach, we found that the ICDs of the GABA_Ar β2 and γ2, but not the ICD of the GABA_Ar α1 subunit or GST, bound specifically to the μ2 subunit of AP2 (Fig. 1a). No significant interaction was observed for any of the fusion proteins of GABA_Ar ICDs with the α, β2 or σ2 subunits of the AP2 complex. We further examined the specificity of μ2 subunit binding by using a range of other GABA_Ar subunit ICDs. The μ2 subunit robustly bound to the ICDs of all GABA_Ar β and γ subunits tested (data for γ1 and γ3 subunits not shown) and to the corresponding domain of the δ subunit (Fig. 1b). In contrast, no binding was observed for the ICDs of the GABA_Ar α1, α3, and α6 subunits. Moreover, the ICD of the β subunit of the glycine receptor did not significantly associate with μ2, and only very weak binding was detected for the ICD of the GABA_Ar π1 subunit (Fig. 1b). Similar results were obtained by using GST pull downs from brain lysates and blotting with a monoclonal antibody to μ2 (Fig. 1c), confirming that μ2 from brain associates with the ICDs of GABA_Ar β and γ subunit isoforms.

To verify the observations obtained with AP2 subunit synthesized by in vitro translation, we examined the ability of bacterially expressed μ2 (His-μ2 residues 156–435) to bind to GABA_Ar ICDs by using affinity purification. After extensive washing and SDS/PAGE, bound μ2 was detected by Coomassie staining. By using this approach, it was evident that stoichiometric levels of His-μ2 156–435 bound directly to both GST-β3 and GST-γ2 (Fig. 1d). In contrast, the C2A domain of synaptotagmin I did not associate with μ2 under our experimental conditions, consistent with previous observations (16). Together, these results demonstrate that the interaction of the AP2 complex with GABA_Ar Rs is mediated via the direct binding of the μ2 subunit of this complex to the ICDs of GABA_Ar receptor β and γ subunits. Moreover, they also suggest that residues 156–435 of the μ2 subunit are sufficient to mediate AP2 binding to GABA_Ar Rs.

Identification of an Atypical μ2 Binding Motif in GABA_Ar β Subunits. Because GABA_Ar β subunits are essential components of most receptor subtypes assembled by neurons (1, 2) and play critical roles in phospho-dependent functional modulation (7, 9, 10, 21–25), we focused on further identifying the amino acid regions in these subunits that mediate binding to μ2 (AP2). Tyrosine (Yxxφ) motifs can signal clathrin-mediated endocytosis and mediate direct binding to the μ2 subunit of AP2 in a variety of proteins, including some ion channels (17–19, 27, 28); however, there are no classical Yxxφ motifs in GABA_Ar β-subunit ICDs. To identify binding sites for μ2 in GABA_Ar β subunits, we used GST fusion protein constructs encoding ICDs of the β1 (residues 302–425) and β3 (residues 302–426) subunits and examined their ability to bind [35S]-labeled μ2. Using this approach, we were able to establish that a conserved region between amino acids 395–415 in the β1 subunit (Fig. 2a) and 395–410 in the β3 subunit (Fig. 2b) mediate binding to μ2. Surprisingly, this region does not contain any classical internaliza-
Fig. 2. Identification of the μ2 binding domain on GABAAR β subunits and GABAAR β subunit-binding domain within μ2. (a–c) Identification of the μ2 binding site in GABAAR β subunits. GST fusion protein deletion constructs of GABAAR β1 ICD (a) and β3 ICD (b) were tested for binding to 35S-labeled μ2. Bound material was separated by SDS-PAGE and visualized by autoradiography. (a) The different GST-β1 ICD deletion constructs are represented in Upper. Lanes: 1, GST; 2, GST-β1 whole ICD residues 302–425; 3, GST-β1 residues 302–365; 4, GST-β1 residues 302–332; 5, GST-β1 residues 333–365; 6, GST-β1 residues 333–395; 7, GST-β1 residues 366–425; 8, GST-β1 residues 366–395; 9, GST-β1 residues 365–404; 10, GST-β1 residues 366–415. Binding of these constructs to 35S μ2 shown in Lower. (b) The different GST-β3 ICD deletion constructs are represented in Upper. Lanes: 1, GST-β3 whole ICD residues 302–426; 2, GST-β3 residues 366–426; 3, GST-β3 residues 366–395; 4, GST-β3 residues 395–426; 5, GST-β3 residues 345–408. Binding of these constructs to 35S μ2 is shown in Lower. (c) Alignment showing the identified μ2 binding domain in GABAAR β subunits and homology to the μ2 binding motif of synaptotagmin, and the sequence of β3 peptides (pepβ3 and pepβ3-phos) used in later experiments. Note also that conserved serine phosphorylation sites in GABAAR β subunit (S408 in β1, S410 in β2, and S408/S409 in β3) S408/S409 in pepβ3-phos are marked in red to denote phosphorylation. (d–f) Identification of the GABAAR β subunits binding site within μ2. (d) Diagram of 35S-labeled in vitro translated μ2 deletion constructs: A, full length μ2, residues 1–435; B, residues 158–435; C, residues 158–407; D, residues 151–157, E, residues 283–394. Note the carboxy-terminal region (residues 407–435) contains the tyrosine motif binding domain and is present in constructs A (full length μ2, residues 1–435) and B (residues 156–435), whereas the core domain of μ2 (residues 283–394) present in A–C and E has been previously shown to bind to synaptotagmin 1 AP2 binding basic domain. (e) Binding of GST β1 and β3 to all constructs containing the core domain (residues 283–394) of μ2, whereas GST-EGFR (f), which contains a tyrosine type motif, binds to full length μ2 (A) and construct C (residues 158–435) containing the carboxy-terminal domain but not to a μ2 construct containing the core domain but lacking the carboxy domain (residues 158–407).

Role of Receptor Phosphorylation in AP2 Binding. We noted that the GABAAR β subunit μ2 binding sites overlap with conserved sites of receptor phosphorylation; S409 in β1, S410 in β2, and S408 and S409 in β3, respectively (Fig. 2c). These residues are substrates for several kinases, including PKA and PKC, in vitro, when expressed in expression systems and for native receptors in cultured neurons (7, 9, 10, 23–25). Because phosphorylation of receptor intracellular domains at S408 and S409 will dramatically modify the charge environment of the β-subunit AP2 binding domain, we hypothesized that phosphorylation of S408 and S409 may regulate the interaction of GABAAR with the AP2 complex. To test this hypothesis, we repeated the in vitro pull down assay by using in vitro translation 35S-labeled μ2 and GST-β3 that had been subjected to prior in vitro phosphorylation with either PKA or PKC. Critically,
we have established that under these conditions, the sole sites of phosphorylation in the GABA$_{AR}$ β subunit are S408 and S409 (9, 23). Samples containing equal amounts of phosphorylated GST-β3 (P-GST-β3, 0.8 mol phosphate/mole protein) or unphosphorylated GST-β3 (mock P-GSTβ3) were then analyzed for $\mu_2$ binding. In vitro phosphorylation of GST-β3 by PKA or PKC dramatically inhibited $\mu_2$ binding (Fig. 3 a–d).

To further investigate the role of phosphorylation, we synthesized a peptide (pepβ3) representing the minimal $\mu_2$-binding region in the GABA$_{AR}$ β3 subunit, KTHLRRRSSQLK (residues 401–412, see Fig. 2c). For these studies, we immobilized either pepβ3 or a version of this peptide that had been chemically phosphorylated on S408/S409 (pepβ3-phos) to beads and looked at binding to $\mu_2$. Although pepβ3 exhibited robust binding to $\mu_2$ in this assay, binding was dramatically decreased for pepβ3-phos (Fig. 3e). In addition, we used surface plasmon resonance to measure the relative affinities of pepβ3 and pepβ3-phos for the AP2 complex. This approach revealed that pepβ3 bound with a high affinity to AP2 ($K_d = 300$ nM; Fig. 3f; see also Table 1, which is published as supporting information on the PNAS web site), which is similar to values reported for other $\mu_2$ binding signals (33). In contrast pepβ3-phos bound AP2 with a 6.3-fold lower affinity (1,900 nM). We also used surface plasmon resonance to confirm that bacterially expressed $\mu_2$ (residues 156–435) also binds directly to this peptide and that mutating the tyrosine motif binding domain of $\mu_2$ (W421A) does not affect binding to pepβ3, in agreement with the pull-down approach described in Fig. 2e (data not shown). Together, these results suggest that phosphorylation of conserved serine residues within the GABA$_{AR}$ β subunits may serve as a regulatory mechanism to control the interaction of these receptors with the AP2 complex.

**Phospho-Dependent Inhibition of GABA$_{A}$ Receptor AP2 Binding Modifies mIPSCs.** To test the functional consequences of disrupting AP2 recruitment to the GABA$_{AR}$, we carried out whole-cell patch clamp electrophysiological experiments to monitor the effects of pepβ3 on inhibitory synaptic transmission. We have reported that blocking clathrin-dependent endocytosis of GABA$_{AR}$s with a peptide that targets the function of the GTPase dynamin (P4 peptide) results in an increase in the amplitude and frequency of mIPSCs in cultured cortical neurons (8). We predicted that the dephosphorylated version of pepβ3, which binds AP2 with high affinity, would block receptor internalization and similarly cause an increase in mIPSC amplitude and/or frequency. As shown in Fig. 4a and b, control cultured cortical neurons showed a stable mIPSC amplitude within 30 min from the onset of recording. In contrast, dialysis of pepβ3 peptide via the patch pipette caused a sustained increase in mIPSC amplitude over the same 30 min time course (pepβ3: 119.5 ± 6.2%, n = 5; control: 97.4 ± 6.8%, n = 4). Dialysis of the dephosphorylated pepβ3-phos peptide also caused a significant enhancement in mIPSC frequency within 30 min (pepβ3: 138.8 ± 7.6%, n = 5; control: 96.7 ± 8.6%, n = 4). The phosphorylated version of pepβ3 (pepβ3-phos), which differs from pepβ3 only in phosphorylation of S408 and S409 on this peptide, had no effect on either mIPSC amplitude (pepβ3-phos: 94.5 ± 2.7%, n = 6; control: 97.4 ± 6.8%, n = 4) or mIPSC frequency (pepβ3-phos: 95.1 ± 6.8%, n = 6; control: 96.7 ± 8.6%, n = 4). In contrast to the striking difference in the effects of pepβ3 and pepβ3-phos on mIPSC amplitude and frequency, no significant difference between these two peptides was observed on the mIPSC rise time (pepβ3: 4.42 ± 0.52 ms, n = 8; pepβ3-phos: 4.65 ± 0.73 ms, n = 6) or the mIPSC decay kinetics (pepβ3: 7.46 ± 0.35 ms, n = 8; pepβ3-phos: 7.35 ± 0.42 ms, n = 6), suggesting it is unlikely that the effects of pepβ3 are due to modulation of channel gating. In addition, neither pepβ3 nor pepβ3-phos had any effect on the amplitude or frequency of miniature excitatory postsynaptic currents (mEPSCs), an important control for the specificity of pepβ3 action on inhibitory synapses (Fig. 4c and d). Representative cells showing the different effects of pepβ3 and pepβ3-phos on mIPSCs are illustrated in Fig. 4e. It is evident that pepβ3, but not pepβ3-phos, caused a significant increase in the mIPSC frequency, as indicated by a leftward shift of the distribution of mIPSC inter-event intervals. Presumably it is due to the recruitment of mIPSCs previously below the threshold of detection, owing to an increased number of surface active GABA$_{AR}$ receptors.

**Phospho-Dependent Modulation of GABA$_{A}$ Receptor Function Is Occluded by Inhibitors of Dynamin.** We also tested the effect of pepβ3 on whole-cell GABA$_{AR}$-mediated currents in dissociated prefrontal cortical neurons. As shown in Fig. 5a and b, dialysis with pepβ3 caused a significant increase in the size of the GABA$_{AR}$ current over 15 min of recording compared with dialysis with pepβ3-phos or control internals (pepβ3: 113.0 ± 4.1%, n = 11; pepβ3-phos: 90.0 ± 4.1%, n = 15; control: 94.1 ± 3.6%, n = 4), which is in correspondence with the increase in mIPSC amplitude seen in cultured cortical neurons. The observed effects of pepβ3 on whole-cell GABA$_{AR}$-mediated currents and mIPSCs are most likely due to increased cell surface and synaptic GABA$_{AR}$ number through the inhibition of GABA$_{AR}$ endocytosis. If so, then blocking GABA$_{AR}$ endocytosis by another method would occlude the effect of pepβ3. We have shown that blocking GABA$_{AR}$ endocytosis with a dynamin function blocking peptide (P4 peptide) increases synaptic GABA$_{AR}$ receptor numbers in cultured cortical neurons (8). If both the P4 peptide and pepβ3 mediate their effects by targeting different steps of the same endocytic pathway to block
GABA<sub>A</sub>R internalization, then we would not expect an additive effect on the GABA<sub>A</sub>R-mediated current of the two peptides. In agreement with this hypothesis, no additive effect of coidalysis with the P4 peptide and pepβ3 peptide could be detected (Fig. 5c). As summarized in Fig. 5f, injecting both P4 and pepβ3 peptides caused a similar enhancement of the GABA<sub>A</sub>R current during 15 min of recording compared with injecting P4 peptide alone (P4: 118.5 ± 5.0%, n = 17; P4 + pepβ3: 120.2 ± 6.3%, n = 7), suggesting that P4 peptide occluded any effects of pepβ3. This result supports the conclusion that pepβ3 is mediating its effect by blocking GABA<sub>A</sub>R endocytosis.

Discussion

Here, we have begun to analyze the molecular determinants that regulate the association of GABA<sub>A</sub>Rs with the AP2 complex, a critical regulator of endocytosis. Our studies have identified an atypical AP2 binding motif conserved in GABA<sub>A</sub>R β-subunits (between residues 401–412) that mediates the direct binding to the µ2 subunit of the AP2 complex. Importantly, we show that a peptide corresponding to these residues is sufficient to mediate high affinity binding to AP2. This GABA<sub>A</sub>R β-subunit AP2 binding motif is enriched in basic amino acids and does not have any similarity with known classical tyrosine or dileucine based AP2 binding motifs. Moreover, this motif does not bind µ2 via the carboxyl terminal region of µ2 (residues 407–435; containing the critical W421) necessary for association with tyrosine type sorting signals (32) or the N-amino terminal region of µ2 (residues 111–148) implicated in direct association of some dileucine type signals with µ2 (17). In contrast, the GABA<sub>A</sub>R β-subunit µ2 binding motif associates directly with a core domain in µ2 (residues 283–394). Interestingly the arginine and lysine rich GABA<sub>A</sub>R β-subunit µ2 (AP2) binding motif has significant similarity in amino acid content to an atypical AP2 binding motif recently identified in several other receptors and membrane proteins, including Syt 1 (16), the α<sub>1b</sub> adrenergic receptor (29) and AMPA receptors (30), supporting a conserved mechanism for AP2 binding in neuronal membrane proteins and neurotransmitter receptors.

In addition, the GABA<sub>A</sub>R β subunit µ2 (AP2) binding motif contains conserved serine residues (S408 in β1, S410 in β2, and S408 and 409 in β3) that are substrates for several serine/threonine protein kinases.
kinases, and we have previously established that S408 and S409 in the β3 subunit are phosphorylated by both PKA and PKC (9, 10, 23–25). In this study, we found that phosphorylation of these conserved serines in GABAAR β3 subunits dramatically reduced association of the α1 subunit with the AP2 complex, suggesting a potential post-synaptic signaling mechanism to regulate receptor endocytosis and cell surface stability. Importantly, this result is an example of phosphorylation regulating the interaction between AP2 and an atypical μ2 binding motif. This mechanism may also be important for regulating endocytosis of other membrane proteins and neurotransmitter receptors. In this report, we also identify a direct interaction of μ2 with GABAAR γ and δ subunit ICDs. Inspection of the amino acid sequence of the ICDs of these subunits revealed that they contain both potential classical tyrosine-type motifs and sequences similar to the basic amino acid rich atypical μ2 binding motif. These additional potential sites for μ2 binding may allow neurons to regulate the rates of endocytosis for distinct cell surface populations of GABAARs with differing functional properties. It will therefore also be important to fully characterize the determinants for μ2 binding in γ and δ subunits. In particular, δ-containing extrasynaptic GABAARs form an important subtype of GABAAR, thought to specifically mediate tonic, rather than phasic inhibition (34) and that are implicated in regulation of inhibitory control by several important physiologically relevant modulators, including ethanol and neurosteroids (34–37).

To test the functional significance of the phosphorylation-dependent interaction of AP2 with GABAARs, we examined the ability of phosphorylated and nonphosphorylated peptides corresponding to the μ2 subunit binding site in the GABAAR receptor β3 subunit to modulate the efficacy of synaptic inhibition. Dialysis of pepβ3 peptide (with high affinity for AP2) into cultured cortical neurons increased both the amplitude and frequency of mIPSCs. This effect is very similar to that previously observed on mIPSCs upon blocking GABAAR internalization by using a peptide that targets the function of the GTPase dynamin (8). In contrast, a version of pepβ3, in which serines 408 and 409 are phosphorylated (pepβ3-phos; with low affinity for AP2) did not modulate either mIPSC amplitude or frequency. In addition, in dissociated prefrontal cortical neurons, a similar difference in the effect of pepβ3 and pepβ3-phos could be observed on whole-cell GABAAR currents. The markedly contrasting effects of pepβ3 and pepβ3-phos on mIPSCs, coupled with our observations that pepβ3-phos has dramatically reduced affinity for the AP2 complex, strongly suggest that modified rates of GABAAR endocytosis underlie the functional modulation seen in our experiments. In agreement with this observation, the effect of pepβ3 on whole-cell GABAAR response was occluded by codialysis with the dynamin inhibitory P4 peptide, suggesting these agents both modulate GABAAR receptor internalization.

There is accumulating evidence that phosphorylation of GABAAR ICDs may regulate receptor cell surface number. Both insulin and BDNF have been demonstrated to enhance GABAAR cell surface numbers and the stoichiometry of phosphorylation of S410 in the β2 subunit and S408/S409 in the β3 subunits by AKT and PKC-dependent mechanisms, respectively (7, 9). It should be noted that studies in HEK293 cells and Xenopus Oocytes have shown that PKC activity can also decrease surface GABAAR levels by an indirect mechanism that is independent of the major GABAAR phosphorylation sites by inhibiting the recycling of internalized receptors, but the significance of this mechanism for regulation of the cell surface stability of neuronal GABAARs remains to be established (12, 38). It is also evident that direct receptor phosphorylation can regulate both GABAAR receptor desensitization and channel kinetics (21, 25, 39). However, to date, a mechanism linking GABAAR receptor phosphorylation and modified cell surface expression levels remains to be established. Here, we provide both biochemical and functional evidence to support the notion that phosphorylation at conserved serine residues in the GABAAR β3 subunits can act as a molecular switch to regulate AP2 clathrin adaptor recruitment, thus modifying receptor endocytosis and the number of these receptors at inhibitory synapses. This process provides a significant previously uncharacterized mechanism for multiple intracellular signaling pathways to regulate GABAAR cell surface number by controlling the stoichiometry of β-subunit phosphorylation and, therefore, receptor endocytosis, with critical consequences on the efficacy of inhibitory synaptic transmission.

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