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A-kinase anchoring protein 79/150 facilitates the phosphorylation of $GABA_A$ receptors by cAMP-dependent protein kinase via selective interaction with receptor β subunits

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Abstract

$GABA_A$ receptors, the key mediators of fast synaptic inhibition in the brain, are predominantly constructed from α(1–6), β(1–3), γ(1–3), and δ subunit classes. Phosphorylation by cAMP-dependent protein kinase (PKA) differentially regulates receptor function dependent upon β subunit identity, but how this kinase is selectively targeted to $GABA_A$ receptor subtypes remains unresolved. Here we establish that the A-kinase anchoring protein 150 (AKAP150), directly binds to the receptor β1 and β3, but not to α1, α2, α3, α6, β2, γ2, or δ subunits. Furthermore, AKAP79/150 is critical for PKA-mediated phosphorylation of the receptor β3 subunit. Together, our observations suggest a mechanism for the selective targeting of PKA to $GABA_A$ receptor subtypes containing the β1 or β3 subunits dependent upon AKAP150. Therefore, the selective interaction of β subunits with AKAP150 may facilitate $GABA_A$ receptor subtype-specific functional modulation by PKA activity which may have profound local effects on neuronal excitation.

Introduction

$GABA_A$ receptors are the major sites of fast inhibitory neurotransmission in the brain (Rabow et al., 1995). These receptors are heteropentamers that can be assembled from a wide range of receptor subunits: α(1–6), β(1–3), γ(1–3), δ, ε, θ, and π (Rabow et al., 1995; Davies et al., 1997; Hedblom and Kirkness, 1997; Bonnert et al., 1999). Most $GABA_A$ receptor subtypes in the brain however are believed to be composed of α, β, and γ2 subunits (Rabow et al., 1995).

Direct receptor phosphorylation is a mechanism of major significance for controlling the functional properties of $GABA_A$ receptors (Moss and Smart, 2001; Brandon et al., 2002a, 2002b). It is evident that the $GABA_A$ receptor β1–3 and γ2 subunits are the substrates of a number of protein kinases including cAMP-dependent protein kinase (PKA), and protein kinase C (PKC) (Moss et al., 1992a, 1992b; Krishek et al., 1994; McDonald and Moss, 1997; McDonald et al., 1998). $GABA_A$ receptor β subunits are all phosphorylated by PKC on conserved serine (S) residues (S409 in the β1 and β3 subunits and S410 in β2) (Krishek et al., 1994; McDonald et al., 1998). Phosphorylation of these residues has been directly correlated with functional modulation (Kellenberger et al., 1992; Kishek et al., 1994; Lin et al., 1996) for recombinant receptors. Studies in neurons have revealed that receptor β subunits are phosphorylated by PKC activity, and directly interact with both the βII isoform of PKC and the receptor for activated C-kinase (RACK-1; Brandon et al., 1999, 2002a, 2002b).

$GABA_A$ receptor β subunits are also selective substrates of PKA; the β1 subunit is phosphorylated by PKA on serine residue 409 (S409) in vivo while the β3 subunit is phosphorylated on both serine residues 408 and 409 (S409/8) by PKA in vivo (McDonald et al., 1998). In contrast the anal-

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ogous residue in β2, S410, is not phosphorylated by PKA in vivo (McDonald et al., 1998). Functionally, phosphorylation by PKA has differing effects on $GABA_A$ receptors depending upon subunit composition; β1 containing $GABA_A$ receptors are inhibited by phosphorylation of S409 by PKA. In contrast the function of β3 containing receptors is enhanced by PKA-dependent phosphorylation of S408/9 (McDonald et al., 1998). Similar divergent modulation of neuronal $GABA_A$ receptor function by PKA activity has been widely observed (Brandon et al., 2002a). However, the mechanisms underlying this differential regulation of $GABA_A$ receptors by PKA activity remain unknown.

Here we have examined the targeting of PKA to $GABA_A$ receptors, focusing on A-kinase anchoring proteins (AKAPs), which bind the regulatory (RII) subunit dimer of PKA, directing this kinase to specific subcellular sites (Colledge and Scott, 1999). We demonstrate in this report that neuronal AKAP, AKAP79/150, is intimately associated with $GABA_A$ receptor subtypes containing either the β1 or the β3 but not the β2 subunits. In the case of the β3 subunit, AKAP79/150 binding is critical in mediating PKA-dependent phosphorylation of key regulatory sites within the intracellular loop of this protein. Together our results provide a molecular mechanism for the selective modulation of $GABA_A$ receptor subtypes by PKA-dependent signaling pathways via differential association with AKAP79/150.

Results

Phosphorylation of GABAA receptors by PKA activity in neurons

$GABA_A$ receptor regulation by PKA activity has largely been examined in heterologous expression systems or in vitro (Brandon et al., 2002a). To address the phosphorylation of receptor β subunits by PKA in neurons a phosphospecific antiserum against S408/9 in the β3 subunit (ant-pβ3) was utilized in Western blots of cultured striatal neurons treated with a range of kinase activators. This antiserum we have previously established recognizes the β3 subunit when phosphorylated on S408 and S409 (Jovanovic et al., 2000; Brandon et al., 2002). In this system, basal phosphorylation of the β3 subunit on S408/9 was evident (Fig. 1A lane 1), and phosphorylation of these residues could be increased on exposure to two distinct membrane-permeable activators of PKA, forskolin or sp8-bromo-cAMP (Fig. 1B, 184.7 ± 9.9 and 186.4 ± 9.1%, respectively). These values were both significantly different from control ($P < 0.01$; $n = 3$). In addition, phosphorylation of these residues was enhanced upon the activation of PKC using phorbol dibutyrate (PDBu; Fig. 1B) which produced a larger increase in phosphorylation of S408/409 (345.4 ± 22.8%; $P < 0.01$, $n = 3$) than seen with PKA activators. This confirms earlier observations examining PKC-mediated phosphorylation of $GABA_A$ receptors in cortical neurons using labelling with ^{32}P-orthophosphoric acid (Brandon et al., 2000). Together, these results demonstrate that the phosphorylation of functionally important residues within the β3 subunit (McDonald et al., 1998) is stimulated upon the activation of PKA in neurons. This effect may be mediated via enhanced direct receptor phosphorylation by PKA or alternatively via PKA/DARPP-32-mediated inhibition of PP1 (Greengard et al., 1999).

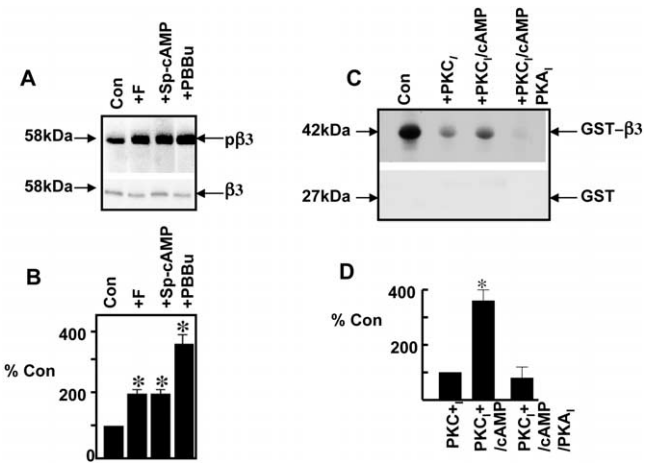

Fig. 1. Phosphorylation of the $GABA_A$ receptor β3 subunit upon activation of PKA in neurons. (A) Cultured striatal neurons (10 DIV) were treated with forskolin (+F, 20 μM), sp8-bromo-cAMP (Sp-cAMP; 10 μM), PDBu (100 nM), or vehicle alone (Con). Cultures were then lysed, subjected to SDS–PAGE, and blotted with anti-pβ3 specific for diphospho 408/9 in the β3 subunit (upper panel) or anti-β3 (lower panel) followed by ^{125}I-Protein A. (B) Phosphorylation of the β3 subunit in striatal neurons was quantified using a phosphoimager with values normalized for total β3 subunit levels. Data were then compared to untreated cultures, which were given a value of 100%. Forskolin (+F) produced a 186.7 + 9.97% increase, sp8-bromo-cAMP (Sp-cAMP) a 186.4 ± 9.1% increase, and PDBu a 345 + 22.8% increase in the phosphorylation of S408/9. * indicates significantly different from control ($P < 0.01$, Student's t-test, $n = 4$). (C) GST-β3 (upper panel) or GST alone (lower panel) was exposed to solubilized neuronal rat brain extracts and after extensive washing bound material was subjected to in vitro kinase assays in the absence or presence of 100 nM PKC_1, 100 nM PKC_1 + 50 μM cAMP, 100 nM PKC_1 + 5 μM cAMP + 50 nM PKI peptide. Phosphorylation of the fusion proteins was then analyzed by SDS–PAGE followed by autoradiography. (D) The phosphorylation of GST-β3 in (C) was quantified using a phosphoimager. Data were normalized to the level of phosphorylation of GST-β3 seen with PKC_1 alone, which was given a value of 100%. * indicates significantly different from control ($P < 0.01$; Student's t-test, $n = 4$).

$GABA_A$ receptors associated with PKA activity in neurons

To begin to address how PKA activity is targeted to $GABA_A$ receptors, affinity purification assays using the intracellular domain of the β3 subunit expressed as a GST fusion protein was utilized (GST-β3; Brandon et al., 1999). GST-β3 was incubated with brain extracts and extensively washed. Bound material was then subject to in vitro kinase assays in the presence of a range of kinase activators or inhibitors. Under basal conditions GST-β3 was rapidly

phosphorylated by an associating kinase activity, which could be largely inhibited by a specific PKC inhibitor peptide (PKC_{19-36}, Fig. 1C). This observation is in agreement with the direct binding of PKC isoforms to $GABA_A$ receptor β subunits (Brandon et al., 1999, 2000, 2002b). However, in the presence of cAMP there was a significant increase in phosphorylation of GST-β3 in the presence of PKC inhibitor (Fig. 1C) which could be blocked by PKI peptide, a specific PKA inhibitor (Fig. 1C). The effects of cAMP on GST-β3 phosphorylation were quantified using a phosphoimager. cAMP produced an enhancement of 350 ± 30%, which was significantly different from control (Fig. 1D; $P > 0.01$, $n = 4$). PKI blocked this enhanced phosphorylation of GST-β3 in the presence of cAMP to below control levels in some experiments (Fig. 1D), suggesting the presence of a free PKA catalytic subunit; however, this effect was not statistically significant (Fig. 1D). There was no detectable phosphorylation of GST under any of these conditions as detailed previously (Brandon et al., 1999). Phosphoamino acid analysis revealed that ^{32}P incorporation occurred only on serine residues in all of these assays (data not shown). The robust enhancement of fusion protein phosphorylation strongly suggests the association of PKA holoenzyme with the β3 subunit intracellular domain.

To further examine the interaction of $GABA_A$ receptors with PKA, we isolated RII subunit–AKAP complexes from rat brain extracts by affinity chromatography on cAMP agarose and looked for a co-purifying $GABA_A$ receptor β3 subunit. Under these conditions binding of the receptor β3 subunits as defined by a band of 58 kDa (Tretter et al., 1997; McDonald et al., 1998) to cAMP agarose was detected in the absence but not in the presence of an excess of cAMP (Fig. 2A). Together these results reveal that PKA activity can bind to and phosphorylate the intracellular domain of the $GABA_A$ receptor β3 subunit in vitro and it is probable that this is mediated by an A-kinase anchoring protein consistent with a predominant role for anchored PKA in controlling the phosphorylation status of the β3 subunit in neurons.

AKAP150 interacts with the intracellular domain of $GABA_A$ receptor β3 subunit

Recently it has become evident that PKA is targeted to specific subcellular sites by interactions with proteins known as A-kinase anchoring proteins (AKAPs; Colledge and Scott, 1999). These proteins can bind a range of signaling molecules, but a common feature is their ability to bind dimers of the regulatory RII subunits of PKA. The isolation of $GABA_A$ receptors with cAMP agarose from brain extracts (Fig. 1) suggests that PKA activity may be targeted to these receptors via an AKAP. Therefore, to identify interactions of $GABA_A$ receptor subunits with AKAPs we utilized a combination of GST affinity purification (Brandon et al., 1999) and RII overlay assays (Hausken et al., 1998). Material binding to either GST-β3 or GST

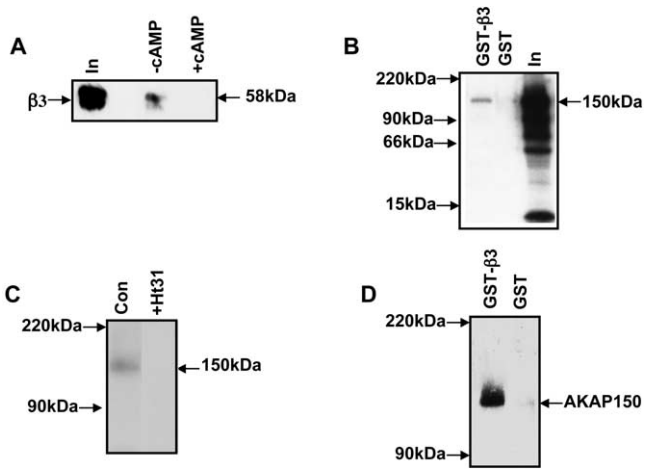

Fig. 2. Interaction of the intracellular domain of the $GABA_A$ receptor with AKAP150. (A) Solubilized neuronal extracts were incubated with cAMP-agarose alone (-cAMP) or in the presence of 5 mM cAMP (+cAMP). After incubation overnight and extensive washing, bound material was separated by SDS–PAGE, transferred to a nitrocellulose membrane, and blotted with anti-β3 antibody to visualize $GABA_A$ receptor binding. "In" represents 1% of the input used. (B) GST- β3 or GST was incubated with neuronal extracts and bound material was resolved by SDS–PAGE, transferred to nitrocellulose, and overlayed with the RII subunit of PKA labeled with ^{32}P. RII binding proteins were then visualized by autoradiography. "In" represents 50 μg of brain extract revealing the presence of multiple AKAPs in this tissue. (C) GST-β3 was exposed to neuronal lysates and transferred to a nitrocellulose membrane as described in (B). The membrane was then overlayed with radiolabeled RII but in the presence of Ht 31 peptide or a scrambled control (Con) peptide as indicated. RII binding proteins were then visualised by autoradiography. (D) GST-β3 or GST was exposed to neuronal lysates. Bound material was separated by SDS–PAGE, transferred to a nitocellulose membrane, probed with antisera against AKAP150 via Western blotting, and visualized by ECL.

from adult rat brain extracts was separated by SDS–PAGE, transferred to a membrane, and overlaid with ^{32}P-radiolabeled RII subunit of PKA. Under these conditions RII bound to a number of proteins in brain lysates (Fig. 2B; Colledge and Scott, 1999). One of these proteins of approximately 150 kDa interacted strongly with GST-β3 but not with GST alone (Fig. 2B). AKAPs bind the RII subunit via a common structural motif and a peptide corresponding to this domain, Ht 31, has been extensively used to block the recruitment of the RII subunit by AKAPs (Colledge and Scott, 1999; Diviani and Scott, 2000). Inclusion of Ht 31 (Carr et al., 1992) in the overlay assays blocked the binding of RII subunit to the 150-kDa protein that binds to GST-β3, but a scrambled control peptide had no effect (Fig. 2C). These results strongly suggest that the RII binding protein that interacts with GST-β3 is an AKAP. An AKAP of this molecular mass (AKAP150) has been shown to play an important role in targeting PKA activity to a number of ion channels and is enriched at excitatory synapses (Carr et al., 1992; Gao et al., 1997; Fraser and Scott, 1999; Colledge et al., 2000). To test the ability of AKAP150 to bind to $GABA_A$ receptor subunits, material bound to GST-β3 was

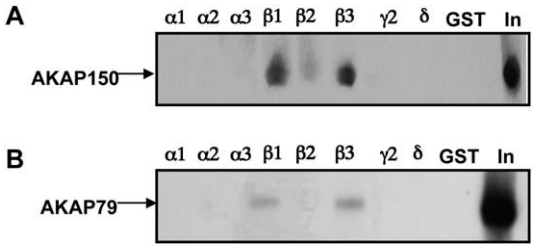

Fig. 3. AKAP150 and AKAP79 selectively interact with the intracellular domains of the $GABA_A$ receptor β1 and β3 subunits. (A) Solubilized neuronal lysates were passed over the respective $GABA_A$ receptor subunit intracellular domains expressed as GST fusion proteins or GST alone immobilized on glutathione agarose. Bound material was subjected to SDS–PAGE and AKAP150 binding was detected via Western blotting and visualized by ECL. "In" represents 10% of the input used. (B) The direct binding of AKAP79 labeled with ^{35}S-methionine with the intracellular domains of the $GABA_A$ receptors was examined. Receptor subunit intracellular domains expressed as GST fusion proteins or GST alone immobilized on GST agarose were exposed to ^{35}S-methionine-radiolabeled AKAP79. Bound material was then separated by SDS-PAGE and visualized by autoradiography. "In" represents 10% of the input used in each experiment.

probed with antisera specific for this protein (Colledge et al., 2000). AKAP150 bound specifically to GST-β3, but not to GST alone (Fig. 2D). Together, our results suggest that AKAP150 may play a specific role in targeting PKA activity to $GABA_A$ receptors.

Direct and specific interaction of AKAP150 with $GABA_A$ receptor β1 and β3 subunits

To examine the subunit specificity of AKAP150 interaction with $GABA_A$ receptors GST-affinity purification assays were utilized with a range of receptor subunit intracellular domains. Brain lysate was passed over these fusion proteins immobilized on agarose and after extensive washing interaction with AKAP150 was tested by immunoblotting. As demonstrated previously (Fig. 2B) AKAP150 bound robustly to GST-β3 but not to GST alone (Fig. 3A). In addition strong binding was also detected to the intracellular domain of the β1 subunit (GST-β1; Fig. 3A). In contrast, AKAP150 did not bind to the intracellular domains of the $GABA_A$ receptor α1, α2, α3, β2, and γ2 subunits or the intracellular domain of the δ subunit (Fig. 3A). Similar results were seen in four separate experiments.

To confirm these observations, we also measured the interaction of AKAP79, the human ortholog of AKAP150, with $GABA_A$ receptor subunits. This protein lacks the rodent-specific repeat sequences in the central domain of AKAP150, but otherwise appears to be functionally identical to AKAP150 (Bergmann et al., 1989; Carr et al., 1992). AKAP79 was translated in vitro and labeled using ^{35}S-methionine, and binding of this protein with $GABA_A$ receptor subunit intracellular domains was then analyzed. Using this approach it was evident that the AKAP79 could only directly bind to the intracellular domains of the $GABA_A$ receptor β1 and β3 subunits, confirming our results with AKAP150 from brain extracts (Fig. 3B). Identical results were seen in three other experiments. Given the ability of AKAP79/150 to selectively interact with receptor β subunits this protein may facilitate the recruitment of PKA to $GABA_A$ receptors. To test this, brain lysates were passed over the intracellular domains of $GABA_A$ receptor β1–3 subunits immobilized on agarose and binding of the PKA catalytic subunit was then measured by Western blotting. This approach revealed that the catalytic subunit of PKA interacts with GST-β3 and GST-β1 but not with GST-β2 or GST alone (Fig. 4A) from brain lysates. In our experiments two bands for PKA catalytic subunit were routinely observed in brain lysates consistent with multiple forms of this protein in the brain (Uhler et al., 1986). However, there was no consistent specific association of either of these bands with individual $GABA_A$ receptor β subunits in three differing experiments. To examine if the catalytic subunit is capable of interacting directly with $GABA_A$ receptors gel overlay assays were used (Fig. 4D). The intracellular domains of receptor β subunits were probed with a biotinylated catalytic subunit of PKA followed by detection with streptavidin–HRP. While binding of the catalytic subunit to low levels of purified RII was easily detected, no interaction with the $GABA_A$ receptor β1, β2, or β3 subunits was observed. Therefore, our results strongly suggest that PKA is targeted indirectly to receptor subunits, most likely via AKAP79/150.

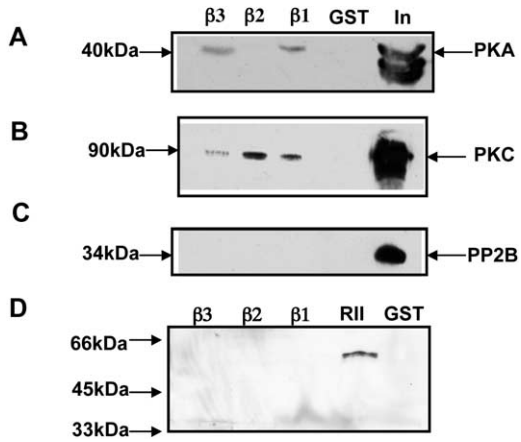

Fig. 4. PKA and PKC but not PP2B interact with $GABA_A$ receptor β subunits. Neuronal lysate was passed over the intracellular domains of the $GABA_A$ receptor β1, β2, and β3 subunits expressed as GST fusion protein or GST alone, immobilized on glutathione agarose. After extensive washing bound material was separated by SDS–PAGE and probed with antibodies against the catalytic subunit of PKA (A), the α isoform of PKC (B) or PP2B (C), and visualized by ECL. "In" represents 10% of the input used in each respective experiment. (D) Five micrograms of the respective $GABA_A$ receptor subunit intracellular domains and 10 ng of purified RII subunit were separated by SDS–PAGE, transferred to a nitocellulose membrane, and incubated with the catalytic subunit of PKA labeled with biotin. The binding of the catalytic subunit of PKA was then visualized using HRP-conjugated streptavidin followed by ECL.

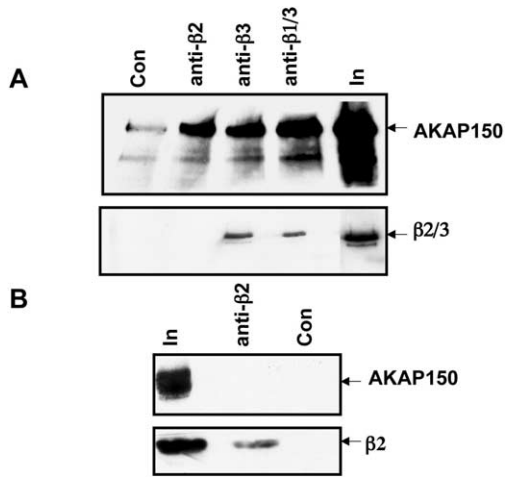

Fig. 5. $GABA_A$ receptors containing the β1 and/or β3 subunits are intimately associated with AKAP150 in neurons. (A) Solubilized neuronal lysates were immunoprecipitated with control IgG, anti-β2, anti-β3, or anti-β1/3 antibodies coupled to Protein A Sepharose. After extensive washing, bound material was separated by SDS–PAGE and transferred to a nitrocellulose membrane. Precipitated material was then Western blotted with anti-AKAP150 antisera (upper panel) or a monoclonal antibody against the β2/3 subunits (lower panel) and visualized by ECL. "In" represents 2% of the material used in the immunoprecipitations. (B) Solubilized neuronal lysates were immunoprecipitated with anti-β2 or control IgG (Con) coupled to Protein A Sepharose, separated by SDS–PAGE, and transferred to a nitrocellulose membrane. Precipitated material was then probed with anti-AKAP150 antisera (upper panel) or monoclonal antisera against the receptor β2/3 subunits (lower panel) and visualized by ECL. Lane 1 represents 2% of the material used for the immunoprecipitations in each case.

It is well characterized that AKAP79/150 provides a scaffold for two other second messenger regulated enzymes, PKC and calcineurin (PP2B) (Coghlan et al., 1995; Klauck et al, 1996; Colledge and Scott, 1999; Diviani and Scott, 2000) in addition to PKA. We tested using GST affinity purification whether the two latter enzymes were able to interact with $GABA_A$ β1/3 subunits. In contrast to our observations with PKA, PKC can robustly interact with the intracellular domains of all three receptor β subunits, (Fig. 4B) consistent with the direct interaction of this kinase with the β1, β2, and β3 subunits as demonstrated previously (Brandon et al., 1999). We were unable to identify an interaction with PP2B, although this protein could be detected in neuronal extracts (Fig. 4C).

Complexes of AKAP150 and $GABA_A$ receptors are found in neurons

To examine the existence of complexes containing both $GABA_A$ receptors and AKAP150 in neurons we utilized immunoprecipitation from detergent-solubilized neuronal lysates. Using antisera against the β1, β3, or β1/β3 subunits resulted in co-immunoprecipitation of AKAP150 as defined by a band of 150 kDa (Fig. 5A). In some of these experiments a weak nonspecific band of similar molecular mass was seen precipitating with control rabbit IgG when probed with the AKAP antibody; however, the intensity of this band did not prevent robust detection of AKAP150 in experimental samples. Precipitated material was also probed for the presence of the β2 and β3 subunits using a selective monoclonal antibody against these two subunits Bd17. Bands representing the correct molecular mass for these receptor subunits could be seen immunoprecipiating with anti-β3, and anti-β1/3 antisera but not with antisera against the β2 subunit or control IgG (Fig. 5A). These results suggest that AKAP150 is able to associate with $GABA_A$ receptors containing either the β1 or the β3 but not the β2 subunits in neurons. To further investigate this we specifically immunoprecipitated receptors containing the β2 subunit (Benke et al., 1994; McDonald et al., 1998) from brain extracts and measured the presence of interaction of AKAP150 via immunoblotting. Using this approach the β2 subunit was efficiently immunoprecipitated with anti-β2 (Fig. 5B), but the levels of AKAP150 co-purifying this subunit were minimal compared to those seen with the β1 or β3 subunits (Fig. 5B).

The subcellular distribution of $GABA_A$ receptors and AKAP150 was compared by immunofluorescence in cultured hippocampal neurons. AKAP150 exhibited a punctate distribution in these neurons with high levels of immunoreactivity being detected in the cell body and neuronal processes (Fig. 6, AKAP150), consistent with earlier studies demonstrating the presence of this protein at excitatory synapses (Colledge et al., 2000). The expression of the $GABA_A$ receptor β2 and β3 subunits, which are components of most CNS receptor subtypes (Benke et al., 1994), was also analyzed using a monoclonal antibody specific for these proteins (Fig. 6, β2/3). We were unable to perform double immunofluorescence using the specific rabbit anti-β3 antisera used for immunoprecipitation as the AKAP150 antibody was also raised in rabbits. $GABA_A$ receptor immunoreactivity was found to have a punctate distribution in these neurons, consistent with previous studies showing enrichment of these receptors at inhibitory synapses (Pirker et al., 2000; Moss and Smart, 2001). To compare the possible co-localization of $GABA_A$ receptors with AKAP150, red puncta were counted representing $GABA_A$ receptor β2/3 subunits and the percentage of these structures that contained green AKAP150 immunofluorescence was scored. This was then expressed as a percentage of the total red puncta counted. From this approach it was evident that 45 $\pm$ 8% (SEM, n = 400) of β2/3 subunit-positive puncta also contained immunoreactivity for AKAP150 (Fig. 6, merge). Given that the $GABA_A$ β2 subunit does not interact with AKAP150, but is abundantly expressed in hippocampal neurons (Pirker et al., 2000), our results strongly suggest that AKAP150 is intimately associated with the $GABA_A$ receptors in hippocampal neurons.

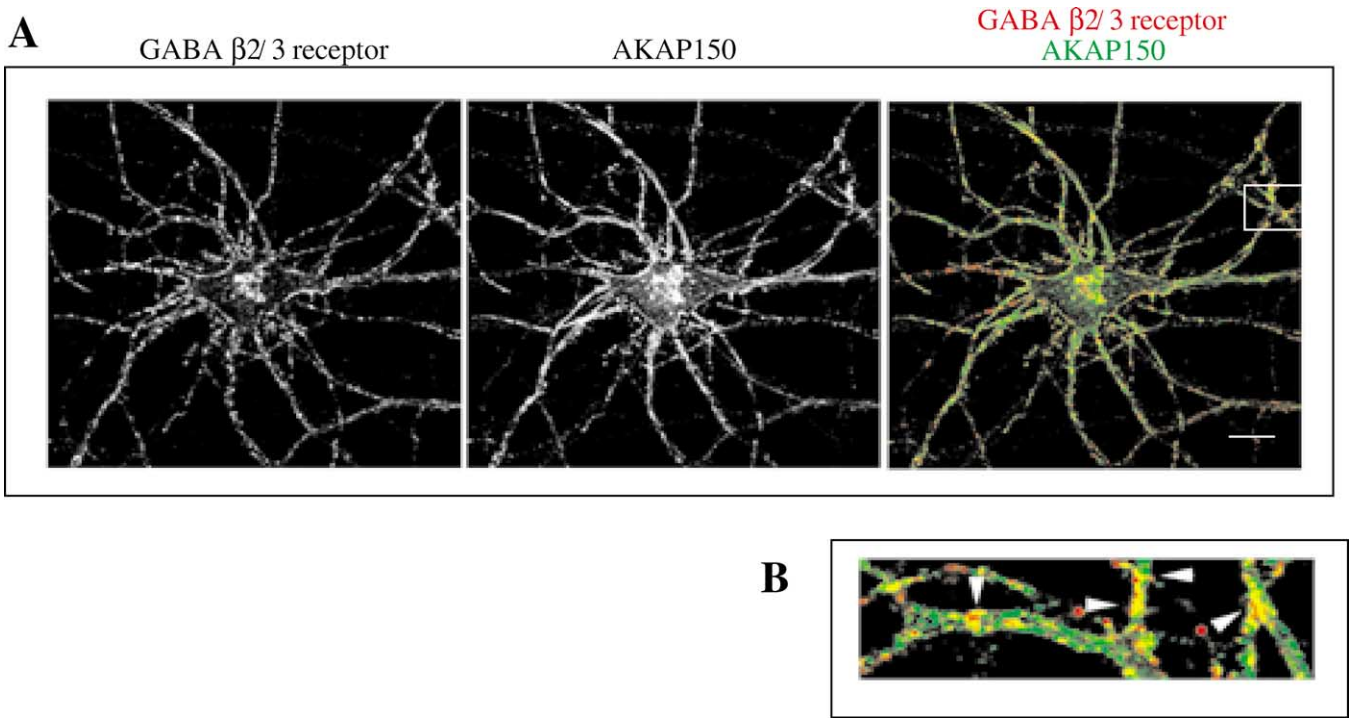

Fig. 6. Localization of AKAP150 and $GABA_A$ receptor β2/3 subunit immunoreactivity in cultured hippocampal neurons. Cultured hippocampal neurons (21 DIV) were fixed, permeabilized in 4% paraformaldehyde, and stained with Bd17 to visualize the $GABA_A$ receptor β2/3 subunits (β2/3) coupled to Texas red and anti-AKAP150 coupled to FITC (AKAP150) as indicated in the respective panels. Images were collected from stained neurons by confocal microscopy and merged in the respective color image. The lower panel represents a higher power image of a selected area (white box) of neuronal processes shown in the merged color image. The arrows indicate puncta that contain both staining for $GABA_A$ receptors and AKAP150. Scale bar, 10 μM.

Functional significance of AKAP79/150 interaction with $GABA_A$ receptors

Heterologous expression in Cos 7 cells was used to examine the role of AKAP79/150 in facilitating $GABA_A$ receptor phosphorylation. Recombinant expression was used for these experiments as it is possible to produce receptors of defined subunit composition; this issue is important as neurons often express multiple numbers of receptor β subunits (Pirker et al., 2000). Therefore $GABA_A$ receptor α1 and β3 subunits were co-expressed in Cos 7 cells as this subunit combination can access the cell surface efficiently producing GABA-activated chloride currents. Moreover, it is possible to overexpress dominant negative reagents to block AKAP79/150 protein–protein interactions (Colledge et al., 2000), facilitating biochemical studies of receptor phosphorylation. Similar experiments are at present difficult to perform in neurons. To test the effects of AKAP79/150 in facilitating $GABA_A$ receptor phosphorylation by PKA, $\alpha1\beta3$ receptors were co-expressed with either wild-type AKAP79 or a mutated version of this protein in which residues 388–409 have been deleted (AKAP79-PKA). This deletion has been previously shown to prevent the binding of the RII subunit, so effectively preventing the recruitment of PKA to AKAP79. The tagging with GFP of these proteins is functionally silent (Gao et al., 1997; DellAcqua et al., 1998; Colledge et al., 2000). The effects of these constructs on forskolin-stimulated phosphorylation of S408/9 in the β3 subunit were assessed using anti-pβ3 antisera. These residues have been previously shown to be the major PKA substrates in $\alpha1\beta3$- or $\alpha1\beta3\gamma2$-containing receptors and are phosphorylated in neurons by PKA activity (McDonald and Moss, 1997; McDonald et al., 1998; Brandon et al., 2000; Jovanovic et al., 2001). While basal levels of phosphorylation of S408/9 were variable between cells expressing differing receptor and AKAP constructs, in cells expressing $\alpha1/\beta3$ receptors alone, forskolin caused a reproducible 40 $\pm$ 4.2% ($P < 0.01$, $n = 4$) enhancement in 408/409 phosphorylation compared to untreated cells (Fig. 7A and B). In cells expressing wild-type AKAP79 with $\alpha1/\beta3$ receptors, forskolin caused a similar enhancement of phosphorylation (Fig. 7A and B). In contrast, co-expression of AKAP79-PKA with $\alpha1/\beta3$ receptors effectively abolished PKA-dependent phosphorylation of S408/9 in the β3 subunit (Fig. 7A and B). The expression levels of the AKAP79 constructs in Cos 7 cells were evaluated by immunoblotting with antisera against GFP, which is present in both AKAP79 constructs. Both constructs appeared to be expressed at similar levels in Cos 7 cells (Fig. 8A). However, a low molecular mass for AKAP79-PKA was observed which is consistent with the deletion of residues 388–402 in this protein compared to wild type. Moreover,

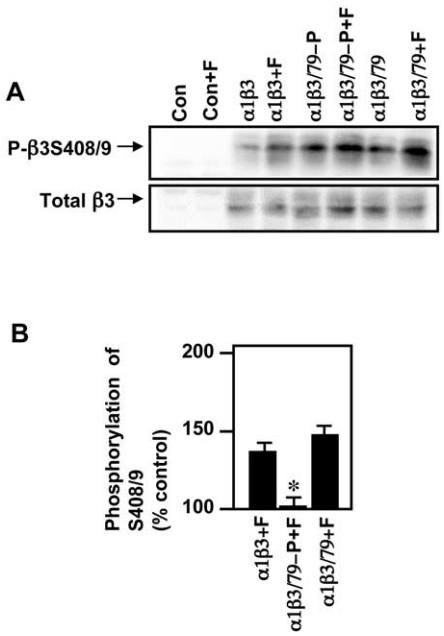

Fig. 7. AKAP79 facilitates PKA-mediated phosphorylation of the $GABA_A$ receptor β3 subunit on S408/9. (A) $GABA_A$ receptor α1 and β3 subunits were expressed in Cos 7 cells alone ($\alpha1\beta3$) with AKAP79 ($\alpha1\beta3/79$) or with the mutant AKAP79-PKA, ($\alpha1\beta3/79$-P) or control untransfected cells (Con). Cells transfected with each set of constructs were treated with the PKA activator forskolin (+F; 20 min 10 μM) 48 h after transfection. Expressing cells were then lysed in 2% SDS and fractionated by SDS–PAGE. Receptor phosphorylation was then evaluated by immunoblot analysis with anti-pβ3 (upper panel) while total β3 subunit levels were measured using anti-β3 (lower panel) followed by incubation with ^{125}I-Protein A and quantification using a phosphoimager. (B) The relative phosphorylation of S408/9 in each experimental condition was then expressed as a percentage of phosphorylation of S408/9 under basal conditions (-forskolin treatment) for cells expressing receptors alone ($\alpha1\beta3$+F) or coexpressed with AKAP79 ($\alpha1\beta3/79$+F) or with AKAP79-PKA ($\alpha1\beta3/79$+F). Data for cells expressing AKAP79 or AKAP79-PKA were then compared to cells expressing receptor subunits alone;* significantly different from control S408/9 phosphorylation ($P < 0.001$, $n = 3$–4).

both wild-type and mutant forms of AKAP79 were equally capable of binding to GST-β3 as judged by affinity purification assays (data not shown). To measure if AKAP79 is endogenously expressed by Cos 7 cells immunoblotting was utilized, which confirmed the expression of AKAP 79 in this cell line (Fig. 8B). Presumably the presence of endogenous AKAP79 in Cos 7 cells is sufficient to promote efficient PKA-mediated phosphorylation of S408/9 in the β3 subunit, explaining the lack of effect of overexpressing AKAP79 in this system. However, AKAP79-PKA appears to act as a dominant negative reagent blocking PKA-mediated phosphorylation of the $GABA_A$ receptor β3 subunit. This action of AKAP79-PKA as a dominant negative is consistent with earlier observations on the PKA-mediated phosphorylation of both glutamate receptors and Ca^{2+} channels when expressed in mammalian cells (Gao et al., 1997; Colledge et al., 2000).

Discussion

While the modulation of $GABA_A$ receptor function has been clinically exploited by exogenous agents such as benzodiazepines and barbiturates (Rabow et al., 1995), the endogenous mechanisms used by neurons to regulate the function of these critically important receptors remains poorly understood. However, accumulating evidence has suggested that direct phosphorylation of the intracellular domains of receptor β and γ2 subunits by both serine/threonine and tyrosine kinases is a diverse and ubiquitous means of regulating receptor function (Brandon et al., 2002a).

In the case of PKA, activation of this kinase can have varying effects of $GABA_A$ receptor function ranging from inhibition to enhancements or no clear effect dependent upon the neuronal preparation studied (Porter et al., 1990; Moss et al., 1992a; Veruki and Yeh, 1994; Kapur and MacDonald, 1996; Yan and Surmeier, 1997; Poisbeau et al., 1999; Brunig et al., 1999; Brandon et al., 2002b). A possible explanation for these observations has been provided by the study of heteromeric recombinant receptors, where receptors containing the β1 subunit have been shown to be negatively regulated by PKA activity via phosphorylation of S409 in this protein (Moss et al., 1992; McDonald et al., 1998). In contrast, receptors containing the β3 subunit are positively regulated via PKA-mediated phosphorylation of both S408 and S409 in this protein (McDonald et al., 1998). Receptors containing the β2 subunit are not PKA substrates (McDonald et al., 1998).

To investigate this possibility, we have examined the targeting of PKA activity to $GABA_A$ receptors. In striatal neurons using phosphospecific antisera it was possible to demonstrate that the β3 subunit was phosphorylated on

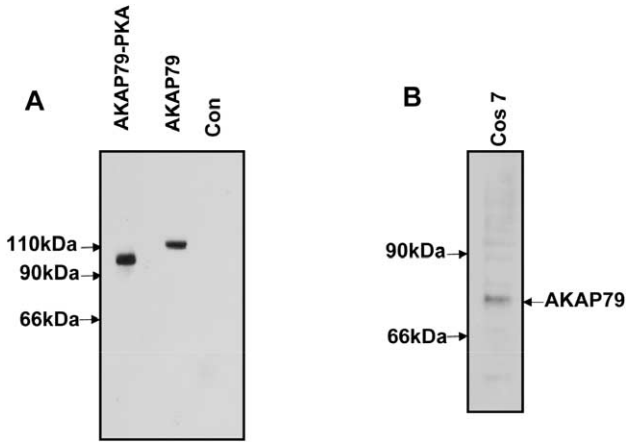

Fig. 8. Expression of recombinant and endogenous AKAPs in Cos 7 cells. (A) Cos 7 cells expressing either AKAP79 or AKAP79-PKA (AKAP79-P) tagged with GFP or control untransfected cells (Con) were lysed and fractionated by SDS–PAGE, transferred to a membrane, blotted with an anti-GFP antibody, and visualized by ECL. (B) SDS-soluble extracts of Cos 7 cells were separated by SDS–PAGE, transferred to a membrane, blotted with anti-AKAP79 antisera, and visualized by ECL.

408/9 by PKA activity (McDonald et al., 1998) in keeping with previous observations showing functional modulation of receptors in these cells by PKA-dependent signaling pathways (Yan and Surmeier, 1997; Flores-Hernandez et al., 2000). Moreover, PKA activity from brain extracts was shown to associate with the intracellular domain of the $GABA_A$ receptor β3 subunit from brain extracts. This ability of $GABA_A$ receptors to interact with PKA RII complexes strongly suggests that PKA activity is targeted to these receptors via an AKAP as all members of this family of scaffold proteins have the ability to bind RII dimers (Colledge and Scott, 1999; Diviani and Scott, 2000). In agreement with this, we were able to detect binding of AKAP150 to the $GABA_A$ receptor β1 and β3 subunits but not the α1–3, β2, or γ2 subunit intracellular domains. Similar results were also obtained with AKAP79, the human ortholog of AKAP150 (Colledge and Scott, 1999; Fraser and Scott, 1999). In keeping with this observation the catalytic subunit of PKA also interacted with the intracellular domains of only the $GABA_A$ receptor β1 and β3 subunits from brain lysates using GST affinity purification. It is likely that PKA is targeted to the β1 and β3 subunits via AKAP150, as we were unable to detect a direct interaction between the catalytic subunit of PKA and either of these receptor subunits. In addition to binding PKA, AKAP79/150 also binds PKC and PP2B (Colledge and Scott, 1999, Diviani and Scott, 2000). PKC was detected binding to all three β subunit intracellular domains. The role of AKAP79/150 in targeting PKC to $GABA_A$ receptors is difficult to ascertain. This is due to the ability of receptor β subunits to directly bind the βII isoform of PKC and RACK-1 (Brandon et al., 1999, 2002b). In contrast to PKC, PP2B could not be detected binding to $GABA_A$ receptor subunits. This observation may suggest that $GABA_A$ receptor β subunits bind to the same site as PP2B on AKAP150. Interestingly this may be similar to the targeting of AKAP150 to glutamate receptors via MAGUK proteins which also exclude the interaction of PP2B to AKAP150 (Colledge et al., 2000). Moreover these results also suggests that PP2B may not play a prominent role in mediating dephosphorylation of $GABA_A$ receptors. In agreement with this we have identified a prominent role for PP2A in dephosphorylation of S408/9 in the receptor β3 subunit (Jovanovic et al., unpublished observation).

Using detergent-solubilized neuronal extracts we were able to demonstrate that AKAP150 co-immunoprecipiated with $GABA_A$ receptors containing the β1 or β3 but not with those containing β2 subunits. Significant co-localization of $GABA_A$ receptors and AKAP150 staining was also evident in cultured hippocampal neurons. Therefore our biochemical and immunohistochemical approaches suggest that AKAP150 selectively binds to $GABA_A$ receptor subtypes containing the β1 and β3 subunits.

To analyze the role that AKAP79/150 plays in controlling $GABA_A$ receptor phosphorylation expression of $GABA_A$ receptors with wild-type and mutated AKAP79 constructs was utilized. Using a mutant AKAP79-PKA construct which is unable to bind PKA (Gao et al., 1997; Colledge et al., 2000) revealed that AKAP79/150 plays a critical role in mediating PKA-dependent phosphorylation of the $GABA_A$ receptor β3 subunit on S408/9, the only PKA phosphorylation sites in this protein (McDonald et al., 1998; Jovanovic et al., 2000). Previous studies have identified S408/9 in the β3 subunit and S409 in the β1 subunit as critical residues for the functional modulation of $GABA_A$ receptors by PKA activity. Therefore, these results strongly suggest that AKAP79/150 plays a critical role in facilitating PKA-dependent phosphorylation and functional regulation of $GABA_A$ receptors containing either the β1 or β3 subunits by specifically targeting this kinase to these receptor complexes at inhibitory synapses. The anatomical distribution of AKAP150 and 79 has been analyzed in rats and humans, respectively, using both in situ hybridization and immunohistochemistry (Glantz et al., 1992). In some brain regions, most notably the olfactory bulb and the forebrain, there is abundant expression of AKAP150 coincident with $GABA_A$ receptor isoforms that contain the β1 or β3 subunits (Glantz et al., 1992; Sik et al., 2000; Pirker et al., 2000). Interestingly, $GABA_A$ receptor function in these structures is modulated by PKA activity (Brandon et al., 2002a). In other brain regions such as the thalamus and hindbrain there are lower levels of AKAP150 expression (Glantz et al., 1992; Sik et al., 2002). Given that AKAP150 is member of a gene superfamily it is possible that another AKAP is responsible for targeting PKA to $GABA_A$ receptors in these brain regions. Another interesting issue is that Sik et al. (2000) reported that AKAP150 is present in only asymmetric excitatory synapses. Whether the co-localization between $GABA_A$ receptors containing the β2 or β3 subunits and AKAP150 seen in our study is at synaptic sites remains to be established. Together, tentatively, these results may suggest that only extrasynaptic $GABA_A$ receptors are being regulated by PKA activity, or alternatively another as yet unidentified AKAP is responsible for controlling PKA-mediated phosphorylation of $GABA_A$ receptors at synaptic sites.

It is evident from our studies that AKAP79/150 can selectively interact with $GABA_A$ receptor β subunits and plays a key role in facilitating the modulation of $GABA_A$ receptor subtypes containing the β1 or β3 subunits by PKA activity. This differential association of receptor subunits with this key signaling scaffold molecule is therefore likely to underlie the variable effects of PKA activity on the function of $GABA_A$ receptors in neurons. Finally, the differential association of $GABA_A$ receptors dependent upon β subunit identity with AKAP79/150 may allow neurons to selectively regulate $GABA_A$ receptor function by PKA-dependent signaling pathways, which may have profound local effects of the efficacy of synaptic inhibition.

Experimental methods

Antibodies

A monoclonal antibody against the $GABA_A$ receptor β2/3 subunits (Bd17) was obtained from Chemicon Ltd. (Harrow, UK). A phosphospecific antiserum that recognizes only diphospho S408/409 within the murine $GABA_A$ receptor β3 subunit (anti-pβ3) and anti-β3 antisera rasied against the intracellular domain of this subunit have been described previously (Tretter et al., 1997; Brandon et al., 1999; Jovanovic et al., 2000; Brandon et al., 2002b), as have antisera specific for the receptor β2, β1, and β1/3 subunits (Benke et al., 1994; McDonald et al., 1998). Antisera against PKC α isoform, catalytic subunit of PKA, calcineurin (PP2B), AKAP150, and AKAP79 have all been detailed previously (Brandon et al., 1999; Colledge et al., 2000; Bedford et al., 2001). Anti-pβ3 specifically recognizes only S408/409 in the β3 subunit when both residues are phosphorylated (Jovanovic et al., 2000; Brandon et al., 2002b). Mouse monoclonal anti-GFP antibody was obtained from Chemicon Ltd.

DNA expression constructs and fusion protein production

GST-fusion protein constructs encoding the intracellular domains of the $GABA_A$ receptor α1–3, β1–3, γ2 and δ subunits in addition to the $GABA_C$ receptor ρ1 and glycine receptor β subunits have been previously described (Hanley et al., 1999; Bedford et al., 2001). Fusion protein expression in *Escherichia coli* BL21 and subsequent purification was performed as described by Smith and Johnson (1988) and Bedford et al. (2001). Expression constructs in PGW1 encoding the murine $GABA_A$ receptor α1 and β3 subunits cDNAS have been described previously. In this vector subunit expression is under the control of the cytomegalovirus promotor (Bedford et al., 2001). The AKAP79 and AKAP79-PKA expression constructs modified with green fluorescent protein (GFP) in pCDNA3 have also been described in previous publications (Gao et al., 1997; DellAcqua et al., 1998; McDonald et al., 1998; Colledge et al., 2000). AKAP-PKA is unable to bind the RII subunit of PKA due to the specific deletion of this protein's binding site between residues 388 and 409 (Carr et al., 1992; DellAcqua et al., 1998).

GST protein affinity purification "pull-down" assays and immunoprecipitation

Brains from adult Sprague–Dawley rats were homogenized in lysis buffer containing 10 mM triethanolamine, pH 7.6, 1% Nonidet P-40, 0.5% deoxycholate, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 1 mM Na orthovanadate, 100 mM PMSF, 10 μg/ml leupeptin, pepstatin, antipain, and aprotinin (Brandon et al., 1999). Insoluble material was removed by centrifugation (1 h, 100,000*g*). Lysates were exposed to GST fusion proteins on glutathione–agarose beads at 4°C for 2 h. Beads were then washed twice in 0.4% NP-40, 500 mM NaCl, 10 mM triethanolamine, pH 7.6, 5 mM EGTA, 5 mM EDTA, 1 mM Na orthovanadate, 1 mM PMSF and then twice in the same buffer supplemented with 150 mM NaCl. Proteins bound to the beads were then separated by SDS–PAGE and analyzed by Western blotting using the appropriate antibodies and followed by detection via enhanced chemiluminescence (ECL Amersham, UK; Brandon et al., 1999).

For immunoprecipitation assays, solubilzed brain lysates in 10 mM triethanolamine, pH 7.6, 1% Nonidet P-40, 0.5% deoxycholate, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 1 mM Na orthovanadate, 100 mM PMSF, 10 μg/ml leupeptin, pepstatin, antipain, and aprotinin (5 μg total protein) were exposed to anti-β3, anti-β1, anti-β1/3, anti-β2 antibodies or control IgG (5 μg) coupled to Protein A Sepharose (Brandon et al., 1999). After 18 h incubation at 4°C, bound material was extensively washed in lysis buffer supplemented with 150 NaCl, separated by SDS–PAGE, and probed with anti-AKAP150 antisera via Western blotting followed by visualization with ECL.

In vitro transcription/translation

AKAP79 in pcDNA was translated in vitro using ^{35}S-methionine (1000 Ci/mmol; Amersham) using the TNT-coupled translation system (Promega Ltd., Southhampton, UK.) For pull-down assays, 10% of a single 50-μl translation assay was mixed with 10 μg of the respective fusion protein immobilized on GST and incubated for 6 h in a buffer containing 10 mM triethanolamine, pH 7.6, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 1 mM Na orthovanadate, 100 mM PMSF, 10 μg/ml leupeptin, pepstatin, antipain, and aprotinin. After extensive washing in the same buffer supplemented with 500 mM NaCl, bound material was separated by SDS–PAGE and visualized by autoradiography.

In vitro kinase assays

To analyze the phosphorylation of $GABA_A$ receptor subunit intracellular domains by associating protein kinases, beads from pull-down assays were washed twice in kinase buffer (20 mM Tris, pH 7.4, 20 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM ouabain, 1 mM Na orthovanadate, 0.1 mM DTT, 2 mM $MnCl_2$) and resuspended finally in 50 μl kinase buffer, containing 3–30 μCi[$^{32}\gamma$P]ATP (3000 mCi/mMol; Amersham). This buffer was supplemented with 100 nM PKC inhibitor peptide (PKC_{19-36}), 50 mM cAMP, and/or 50 nM PKI peptide (a specific PKA inhibitor) The reactions were incubated at 30°C for 15 min. Beads were pelleted and bound material was separated by SDS–PAGE. In some experiments phosphorylation of the intracellular domain of the β3 subunit was quantified using a Bio-Rad phosphoimager. Levels of phosphorylation for PKA stimulation were compared to control seen in the presence of PKC_{18-36} alone and significance was determined using Student's *t*-test.

RII overlay assay

Material binding to GST-β3 or GST from solubilized brain lysates (see section detailing GST affinity purification) was separated by SDS–PAGE (4–15% gradient gels) and transferred to a nitrocellulose membrane. PKA binding proteins were detected by overnight exposure to 5 μg of ^{32}P-labeled RII subunit (220,000 cpm/μg) in 5 ml 0.1% Blotto (TBST, 5% milk, and 0.1% BSA). The membrane was washed extensively with TBST and RII binding proteins were visualized by autoradiography. In some experiments Ht 31 peptide or a scrambled control (Carr et al., 1992; Gao et al., 1997) was also included at final concentrations of 100 μM, respectively.

For the catalytic subunit overlays, purified PKA catalytic subunit (Promega) kinase was biotinylated using a kit from Boehringer (Germany) which adds an average of one biotin molecule per target protein; 1 μg of this protein was incubated overnight with the appropriate membrane in Blotto in which the milk has been replaced by BSA. The membrane was then probed with HRP streptavidin and visualized using ECL. As a positive control 10 ng of purified PKA RII subunit was included in the overlay assays.

cAMP agarose purification

Solubilized rat brain extracts (see section detailing GST affinity purification) in 10 mM triethanolamine, pH 7.6, 100 mM NaCl, , 1% Nonidet P-40, 0.5% deoxycholate, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 1 mM Na orthovanadate, 100 mM PMSF, 10 μg/ml leupeptin, pepstatin, antipain, and aprotinin were incubated with cAMP agarose for 18 h at 4°C in the presence or absence of 5 mM cAMP. The resin was washed twice in the above buffer supplemented with 500 mM NaCl and twice in buffer without NaCl. Proteins were eluted from the resin by incubation with 75 mM cAMP for 1 h at room temperature. Eluted proteins were precipitated with TCA, resuspended in SDS sample buffer, and analyzed by immunoblotting with anti-β3 antisera.

Cell culture experiments

Cos 7 cells were transfected with the appropriate expression constructs in equimolar ratios via electroporation (Connolly et al., 1996; Bedford et al., 2001); 24–48 h after transfection expressing cells were exposed to 20 μM forskolin, 100 nM phorbol dibutyrate (PDBu) or vehicle alone for 10 min prior to lysis in 2% SDS. Five hundred micrograms of protein from each lysate was then resolved by SDS–PAGE, transferred to nitrocellulose membranes, and probed with either anti-pβ3 or anti-β3 followed by ^{125}I-Protein A. Phosphorylation of the β3 subunit on S408/9 was then quantified using a Bio-Rad phosphoimager and corrected for total β3 subunit levels. Cultured striatal neurons were prepared from E17 rat embryos as described by Ventimiglia and Lindsay (1998) and maintained in culture for 7 days prior to treatment with kinase activators. Neurons were then lysed in SDS sample buffer and β3 subunit phosphorylation was assessed using anti-pβ3 as described above.

Neuronal cultures and immunofluorescence

Cultures of hippocampal neurons from E18 rats were prepared essentially as described by Kittler et al. (2000). After 14–21 days in culture, the neurons were fixed in 4% paraformaldehyde and then blocked in phosphate-buffered saline (PBS) containing 10% fetal bovine serum, 0.5% bovine serum albumin, and 0.2% Triton X-100 for 10 min. Striatal neurons were also prepared from E18 rats as described by Yan and Surmeier (1997). Subsequent antibody dilutions were performed in blocking solution and washes were in PBS. Antibodies were used at the following concentrations: anti-$GABA_A$ receptor β2/β3, 10 μg/ml (Bd17), and anti-AKAP150, 2 μg/ml. Texas red- and FITC-conjugated anti-mouse and anti-rabbit secondary antibodies were from Molecular Probes and Jackson and used at 1:400. Coverslips were examined using a confocal microscope (MRC1000, Bio-Rad). To examine the co-localization of AKAP150 with $GABA_A$ receptors, red puncta representing $GABA_A$ receptor β2/3 subunits were counted and the percentage of these structures that contained green AKAP/79/150 immunofluorescence was calculated. This was then expressed as a percentage of the total red puncta counted. We examined 400 $GABA_A$ receptor puncta on the dendrites of eight differing neurons with pyramidal morphology.

Acknowledgments

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References

Bedford, F.K., Kittler, J.T., Muller, E., Thomas, P., Uren, J.M., Merlo, D., Wisden, W., Triller, A., Smart, T.G., Moss, S.J., 2001. $GABA_A$ receptor cell surface number and subunit stability are regulated by the ubiquitin like protein Plic-1. Nat. Neurosci. 4, 917–926.

Benke, D., Fritschy, J.M., Trzeciak, A., Bannwarth, W., Mohler, H., 1994. Distribution, prevalence, and drug binding profile of gamma-aminobutyric acid type A receptor subtypes differing in the beta-subunit variant. J. Biol. Chem. 269, 27100–27107.

Bonnert, T.P., et al., 1999. Theta a novel g-aminobutyric acid type A receptor subunit. Proc. Natl. Acad. Sci. USA 96, 9891–9896.

Brandon, N.J., Uren, J.M., Kittler, J.T., Wang, H., Olsen, R, Parker, P.J., Moss, S.J., 1999. Subunit-specific association of protein kinase C and the receptor for activated C kinase with GABA type A receptors. J. Neurosci. 19, 9228–9234.

Brandon, N.J., Delmas, P., Kittler, J.T., McDonald, B.J., Sieghart, W., Brown, D.A., Smart, T.G., Moss, S.J., 2000. $GABA_A$ receptor phosphorylation and functional modulation in cortical neurons by a protein kinase C dependent pathway. J. Biol. Chem. 275, 38856–38862.

Brandon, N.J., Jovanovic, J.N., Moss, S.J., 2002a. Multiple roles of protein kinases in the modulation of GABAA receptor function and cell surface expression. Exp. Pharmacol. Ther. 94, 113–122.

Brandon, N.J., Jovanovic, J., Smart, T.G., Moss, S.J., 2002b. RACK-1 facilitates PKC phosphorylation and functional modulation of $GABA_A$ receptors upon the activation of G-protein coupled receptors. J Neurosci. 22, 6353–6361.

Brunig, I., Sommer, M., Hatt, H., Bormann, J., 1999. Dopamine receptor subtypes modulate olfactory bulb $GABA_A$ receptors. Proc. Natl. Acad. Sci. USA. 96, 2456–2460.

Carr, D.W., Hausken, Z.E., Fraser, I.D., Stofko-Hahn, R.E., Scott, J.D., 1992. Association of the type II cAMP-dependent protein kinase with a human thyroid RII-anchoring protein. Cloning and characterization of the RII-binding domain. J. Biol. Chem. 267, 13376–13380.

Cho, K.O., Hunt, C.A., Kennedy, M.B., 1992. The rat brain postsynaptic density fraction contains a homolog of the Drosophila discs—Large tumor suppressor protein. Neuron 9, 929–942.

Coghlan, V.M., Perrino, B.A., Howard, M., Langeberg, L.K., Hicks, J.B., Gallatin, W.M., Scott, J.D., 1995. Association of protein kinase A and protein phosphatase 2B with a common anchoring protein. Science. 267, 108–112.

Colledge, M., Scott, J.D., 1999. AKAPs: From structure to function.. Trends Cell Biol. 9, 216–221.

Colledge, M., Dean, R.A., Scott, G.K., Langeberg, L.K., Huganir, R.L., Scott, J.D.., 2000. Targeting of PKA to glutamate receptors through a MAGUK-AKAP complex. Neuron 27, 107.

Connolly, C. N., Krishek, B.J., McDonald, B.J., Smart, T.G., Moss, S.J., 1996. Assembly and cell surface expression of heteromeric and homomeric $GABA_A$Rs. J. Biol. Chem. 271, 89–96.

Davies, P.A., Hanna, M.C., Hales, T.G., Kirkness, E.F., 1997. Insensitivity to anaesthetic agents conferred by a class of GABA(A) receptor subunit. Nature 385, 820–823.

DellAcqua, M.L., Faux, M.C., Thorburn, J., Thorburn, A., Scott, J.D., 1998. Membrane targeting sequences on AKAP79 bind phosphatidylinositol-4,5-bisphosphate. EMBO J. 17, 2246–2260.

Diviani, D., Scott, J.D., 2000. AKAP signaling complexes at the cytoskeleton. J. Cell Sci. 114, 1431–1437.

Flores-Hernandez, J.F., Hernandez, S., Snyder, S., Yan, Z., Fienberg, A., Moss, S.J., Greengard, P., Surmier, J., 2000. D1 dopamine receptor activation reduces $GABA_A$ receptor currents in neostriatal medium spiny neurones through a protein kinase A/DARP-32/PP1 signaling cascade. J. Neurophysiol. 83, 2996–3004.

Fraser, I.D., Scott, J.D., 1999. Modulation of ion channels: a “current” view of AKAPs. Neuron 23 (3), 423–426.

Gao, T., Yatani, A., DellAcqua, M.L., Sako, H., Green, S.A., Dascal, N., Scott, J.D., Hosey, M.M., 1997. cAMP-dependent regulation of cardiac L-type Ca^{2+} channels requires membrane targeting of PKA and phosphorylation of channel subunits. Neuron 19, 185–196.

Glantz, S.B., Amat, J.A., Rubin, C.S., 1992. cAMP signaling in neurons: patterns of neuronal expression and intracellular localization for a novel protein, AKAP 150, that anchors the regulatory subunit of cAMP-dependent protein kinase II beta. Mol. Biol. Cell (11), 1215–1228.

Greengard, P., Allen, P.B., Nairn, A., 1999. Beyond the dopamine receptor: the DARPP-32/protein phosphatase cascade. Neuron 23, 435–447.

Hanley, J.G., Koulen, P., Bedford, F.K., Gordon-Weeks, P.R., Moss, S.J., 1999. Microtubule associated protein MAP-1B links GABAC receptors to the cytoskeleton at retinal synapses. Nature 397, 66–69.

Hausken, Z.E., Coghlan, V.M., Scott, J.D., 1998. Overlay, ligand blotting and band-shift techniques to study kinase anchoring, in: Clegg, R.A. (Ed.), Protein Targeting Protocols, Humana Press, Totowa, NJ.

Hedblom, E., Kirness, E.F., 1997. A novel class of GABAA receptor subunit in tissues of the reproductive system. J. Biol. Chem. 272, 15346–15350.

Jovanovic, J.N., Kittler, J.T., Brandon, N.J., Sieghart, W., Moss, S.J., 2000. Characterization of the specificity *in situ* of the phosphorylation state-specific antibody for $GABA_A$ receptor β3 subunit.. Abstr. Soc. Neurosci. 26, 522.1.

Kapur, J., MacDonald, R.L., 1996. Cyclic AMP-dependent protein kinase enhances hippocampal dentate granule cell $GABA_A$ receptor currents. J. Neurophysiol 76 (4), 2626–2634.

Kellenberger, S., Malherbe, P., Sigel, E., 1992. Function of the $\alpha1\beta2\gamma2S\gamma$-aminobutyric acid type A receptor is modulated by protein kinase C via multiple phosphorylation sites. J. Biol. Chem. 267, 25660–25663.

Kittler, J.T., Delmas, P., Jovanovic, J.N., Brown, D.A., Smart, T.G., Moss, S.J., 2000. Constitutive endocytosis of $GABA_A$ receptors by an association with the adaptin AP2 complex modulates inhibitory synaptic currents in hippocampal neurons. J. Neurosci. 20, 7972–7977.

Klauck, T.M., Faux, M.C., Labudda, K., Langeberg, L.K., Jaken, S., Scott, J.D., 1996. Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. Science 271, 1589–1592.

Krishek, B.J., Xie, X., Blackstone, C., Huganir, R.L., Moss, S.J, Smart, T.G., 1994. Regulation of $GABA_A$ receptor function by protein kinase C phosphorylation. Neuron 12, 1081–1095.

Lin, Y.-F., Angelotti, T.P., Dudek, E.M., Browning, M.D., Macdonald, R.L., 1996. Enhancement of recombinant alpha 1 beta 1 gamma 2LGABA$_A$ receptor whole cell currents by protein kinase C is mediated through phosphorylation of both beta 1 and gamma 2L subunits. Mol. Pharmacol. 50, 185–195.

McDonald, B.J., Amato, A., Connolly, C.N., Benke, D., Moss, S.J., Smart, T.G., 1998. Adjacent phosphorylation sites on $GABA_A$ receptor β subunits determine regulation by cAMP-dependent protein kinase. Nat. Neurosci. 1, 23–28.

McDonald, B.J., Moss, S.J., 1997. Conserved phosphorylation of the intracellular domains of $GABA_A$ receptor β2 and β3 subunits by cAMP-dependent protein kinase, cGMP dependent protein kinase, protein kinase C and Ca+2/calmodulin type 2 dependent protein kinase. Neuropharmacology 36, 1377–1385.

Moss, S. J., Doherty, C.A., Huganir, R.L., 1992a. Identification of the cAMP-dependent protein kinase and protein kinase C phosphorylation sites within the major intracellular domains of the β1, γ2s and γ2L subunits of the $GABA_A$ receptor. J. Biol. Chem. 267, 14470–14476.

Moss, S. J., Smart, T.G., Blackstone, C.D., Huganir, R.L., 1992b. Functional modulation of $GABA_A$ receptors by cAMP-dependent protein phosphorylation. Science 257, 661–665.

Moss, S.J., Smart, T.G., 2001. Constructing inhibitory synapses. Nature Neurosi. Rev. 2, 241–250.

Pirker, S., Schwarzer, C., Wieselthaler, A., Sieghart, W., Sperk, G., 2000. GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101, 815–850.

Poisbeau, P., Cheney, M.C., Browning, M.D., Mody, I., 1999. Modulation of synaptic $GABA_A$ receptor function by PKA and PKC in adult hippocampal neurons. J. Neurosci. 19, 674–683.

Porter, N.M., Twyman, R.E., Uhler, M.D., Macdonald, R.L., 1990. Cyclic AMP-dependent protein kinase decreases $GABA_A$ receptor current in mouse spinal neurons. Neuron 5, 789–796.

Rabow, L.E., Russek, S.J., Farb, D.H., 1995. From ion currents to genomic analysis: recent advances in $GABA_A$ receptor research. Synapse 21, 189–274.

Smith, D.B., Johnson, K.S., 1988. Single step purification of polypeptides expressed in *E. coli* as fusions with glutathione *S*-transferase. Gene 67, 31–40.

Tretter, V., Ehya, N., Fuchs, K., Sieghart, W., 1997. Stoichiometry and assembly of a recombinant $GABA_A$ receptor subtype. J. Neurosci. 8, 2728–2737.

Ventimiglia, R., Lindsay, R. M., 1998. In: Banker, Goslin (Eds.), Culturing Nerve Cells, second ed., MIT Press, Cambridge, MA, pp. 371–995.

Veruki, M.L., Yeh, H.H., 1994. Vasoactive intestinal polypeptide modulates GABAA receptor function through activation of cyclic AMP. Vis. Neurosci. 11, 899–908.

Uhler, M.D., Chrivia, J.C., McKnight, G.S., 1986. Evidence for a second isoform of the catalytic subunit of cAMP-dependent protein kinase. J. Biol. Chem. 261 (33), 15360–15363.

Yan, Z., Surmeier, D. J., 1997. D5 dopamine receptors enhance Zn2+-sensitive GABAA currents in striatal cholinergic interneurons through a PKA/PP1 cascade. Neuron 19, 1115–1125.