Modulation of GABA<sub>A</sub> Receptor Function by Tyrosine Phosphorylation of β Subunits

Qi Wan, 1,2 Heng Ye Man, 1,2 Jodi Braunton, 1,2 Wei Wang, 1,2 M. W. Salter, 2,3 L. Becker, 1 and Yu Tian Wang 1,2

Divisions of 1 Pathology and 2 Neuroscience, Research Institute of Hospital for Sick Children, and Departments of 1 Pathology and 3 Physiology, University of Toronto, Toronto, Ontario M5G 1X8, Canada

Protein tyrosine phosphorylation is a key event in diverse intracellular signaling pathways and has been implicated in modification of neuronal functioning. We investigated the role of tyrosine phosphorylation in regulating type A GABA (GABA<sub>A</sub>) receptors in cultured CNS neurons. Extracellular application of genistein (50 μM), a membrane-permeable inhibitor of protein tyrosine kinases (PTKs), produced a reversible reduction in the amplitude of GABA<sub>A</sub> receptor-mediated whole-cell currents, and this effect was not reproduced by daidzein (50 μM), an inactive analog of genistein. In contrast, intracellular application of the PTK pp60<sup>c-src</sup> (30 U/ml) resulted in a progressive increase in current amplitude, and this potentiation was prevented by pretreatment of the neurons with genistein. Immunoprecipitation and immunoblotting of cultured neuronal homogenates indicated that the β2/β3 subunit(s) of the GABA<sub>A</sub> receptor are tyrosine phosphorylated in situ. Moreover, genistein (50 μM) was found to be capable of decreasing GABA<sub>A</sub> currents in human embryonic kidney 293 cells transiently expressing functional GABA<sub>A</sub> receptors containing the β2 subunit. Thus, the present work provides the first evidence that native GABA<sub>A</sub> receptors are phosphorylated and modulated in situ by endogenous PTKs in cultured CNS neurons and that phosphorylation of the β subunits may be sufficient to support such a modulation. Given the prominent role of GABA<sub>A</sub> receptors in mediating many brain functions and dysfunctions, modulation of these receptors by PTKs may be important in a wide range of physiological and pathological processes in the CNS.

Key words: GABA<sub>A</sub> receptor; protein tyrosine phosphorylation; protein tyrosine kinase; cultured neurons; recombinant GABA<sub>A</sub> receptor; HEK 293 cell
(Valenzuela et al., 1995) and in cultured sympathetic neurons (Moss et al., 1995), suggesting that GABA$_A$ receptors are dynamically regulated by a balance between activities of endogenous protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). There has been no evidence, however, for in situ tyrosine phosphorylation of any subunit of native GABA$_A$ receptors by endogenous PTKs, and consequently the molecular substrate(s) for functional modulation of the receptor by protein tyrosine phosphorylation in the CNS remains unknown.

Given the prominent role of GABA$_A$ receptors in brain functions and dysfunctions and the ubiquitous signaling pathways using PTKs and PTPs in the brain, in the present study we set out to examine whether the native GABA$_A$ receptor expressed in CNS neurons is functionally modulated and phosphorylated by endogenous PTKs, and if so, to determine which subunit(s) is the most likely substrate.

Parts of this paper have been published previously in abstract form (Wang and Wang, 1995; Wan et al., 1996).

**MATERIALS AND METHODS**

**Preparation of neuronal cultures.** Methods for preparing cultures from embryonic rat spinal dorsal horn have been described in detail (Saltar and Hicks, 1994). For primary cultures of dorsal medulla neurons, fetal Wistar rats (embryonic day 17–19) were decapitated, and their brainstems were removed surgically under a dissection microscope. The dorsal part of the medulla containing the solitary complex was dissected out using block dissection (Yu, 1989). The tissue was treated with trypsin and triturated using a Pasteur pipette. The cells were then plated onto collagen-coated 25 mm glass coverslips and set into a standard 35 mm culture dish. The cultures were maintained in minimum essential medium supplemented with 10% fetal bovine serum, 10% heat-inactivated horse serum, and 1 U/ml insulin. Cells were used for recording 1–3 weeks after plating.

**Plasmids and transient transfections.** All of the GABA$_A$ receptor subunit cDNAs were gifts of Drs. C. Kaufman and D. Gunnersen (Laboratory of Neuroscience, National Institute of Diabetes and Digestive and Kidney Diseases). CMV$a$ containing the rat $\alpha 1$ cDNA was cloned into the expression vector pRc/CMV (Invitrogen, San Diego, CA); CMV$b$, the rat $\beta 2$ subunit cDNA, was cloned into pcDNAI (Invitrogen); and CMV$\gamma 2$, the short form of the rat $\gamma 2$ subunit, was cloned into pcDNA3 (Invitrogen). To facilitate identification of the transfected cells for electrophysiological recordings, a cDNA encoding the jellyfish green fluorescent protein (GFP) inserted into pcDNA3 (Marshall et al., 1995) (a gift from Drs. J. R. Howe and T. E. Hughes, Yale University) was used as an expression marker and cotransfected with GABA$_A$ receptor subunit cDNAs. Human embryonic kidney (HEK) 293 cells were plated onto collagen-coated 22 mm glass coverslips set in a standard 35 mm culture dish and maintained in minimum essential medium supplemented with 10% fetal calf serum. Cells were used for recording 30–48 hr after transfection.

**Electrophysiological recordings.** For electrophysiological recordings, coverslips containing cultured neurons or HEK 293 cells were transferred into a glass-bottomed chamber and visualized under differential interference contrast and epifluorescent video microscopy. Cells were bathed in an extracellular recording solution composed of (in mM): NaCl 140, KCl 5.4, HEPES 25, CaCl$_2$ 1.3, glucose 33, and tetrodotoxin 0.001, pH 7.35; osmolarity, 310–320 mOsm. Recordings were made with pipettes (resistance 2–5 MΩ) filled with intracellular solution that contained (in mM): CsCl 140, HEPES 10, 1,2-bis(2-aminophenyl)ethane-N,N,N',N'-tetraacetic acid 10, pH 7.25; osmolarity, 300–315 mOsm. Na$_2$ATP (4 mM) and MgCl$_2$ (1 mM) were included in the intracellular recording solution to support the process of protein phosphorylation, thereby preventing current rundown during a prolonged period of whole-cell recording (Chen et al., 1990). Currents were recorded under standard whole-cell voltage-clamp configuration using an Axopatch 1D amplifier.

**Preparation of neuronal cultures.** Methods for preparing cultures from the medulla containing the solitary complex were dissection (Yu, 1989). The tissue was treated with trypsin and triturated using a Pasteur pipette. The cells were then plated onto collagen-coated 25 mm glass coverslips and set into a standard 35 mm culture dish. The cultures were maintained in minimum essential medium supplemented with 10% fetal bovine serum, 10% heat-inactivated horse serum, and 1 U/ml insulin. Cells were used for recording 30–48 hr after transfection.

**Results**

**Modulation of GABA$_A$ receptor-mediated currents by endogenous PTK activity**

To investigate the role of protein tyrosine phosphorylation in regulating the function of GABA$_A$ receptors in the CNS, our initial experiments focused on endogenous PTKs and were performed using cultured spinal dorsal horn neurons. As shown in Figure 1, pressure ejection of GABA (100 µM) produced an inward current response at a holding membrane potential of −60 mV. The currents had a reversal potential of −80 mV, with a slightly inward rectified current–voltage ($I–V$) relationship within the range of holding membrane potential from −100 to +60 mV (Fig. 1) and were blocked by bath application of GABA$_A$ receptor antagonist bicuculline (20 µM; data not shown), consistent with GABA$_A$ receptor mediation of these currents (Macdonald and Olsen, 1994). Extracellular application of genistein (50 µM), a membrane-permeable inhibitor of PTKs (Akiyama et al., 1987; O’Dell et al., 1991), produced a reversible reduction in the amplitude of the GABA$_A$ currents without altering the $I–V$ curve or the reversal potential, suggesting that the reduction of the current by genistein is attributable to a change in channel conductance rather than an alteration of driving force. On average, currents were reduced to 0.44 ± 0.05 times control within 5 min after the drug application ($n = 30$). We next investigated the specificity of genistein as a PTK inhibitor by examining the effect of daitzein, an inactive analog of genistein (Akiyama et al., 1987; Wang and Saltar, 1994), on the GABA$_A$ currents. As shown in Figure 2, in contrast to genistein, bath perfusion of the same cells with daitzein (50 µM) produced no change in the amplitude of the currents (1.09 ± 0.11; $n = 4$). To determine whether the modulation is unique to GABA$_A$ receptors in dorsal horn neurons, we also...
investigated effects of genistein on GABA currents in cultured dorsal medulla neurons. Bath application of genistein (50 μM) reduced the amplitude of currents by 0.45 and 0.51% of control, respectively, in two neurons tested.

Modulation of GABA<sub>A</sub> receptor-mediated currents by activity of an exogenous PTK

To examine the effect of exogenous PTK on the function of GABA<sub>A</sub> receptors, we applied the cytosolic PTK pp60<sup>c-src</sup> (30 U/ml) directly into cultured spinal dorsal horn neurons via the recording pipette (Wang and Salter, 1994). This resulted in a progressive increase in the current amplitude, which reached a steady level within 5–10 min. On average, the current amplitude increased to 1.86 ± 0.23 times the initial level after 10 min (n = 4) (Fig. 3A,B). As with genistein, pp60<sup>c-src</sup> did not affect the I–V relationship or the reversal potential (Fig. 3C). To confirm that the effect of pp60<sup>c-src</sup> is caused by its tyrosine kinase activity, we then applied this enzyme to neurons that had been incubated with genistein (50 μM) for 10 min. In all cases, pretreatment with genistein prevented the potentiation of GABA<sub>A</sub> current by pp60<sup>c-src</sup> (Fig. 3B) (1.01 ± 0.06; n = 4).

GABA-activated currents were also found to be potentiated by pp60<sup>c-src</sup> in two cultured dorsal medulla neurons tested.

Figure 1. Genistein, an inhibitor of PTKs, suppresses GABA<sub>A</sub> receptor-mediated currents in a cultured spinal dorsal horn neuron. A, Genistein (100 μM) applied in the bath medium inhibited currents evoked by pressure application of GABA (100 μM) from a micropipette whose tip was positioned within 50 μM of the neuron. Currents were recorded under whole-cell configuration at a holding membrane potential of −60 mV in all figures, unless specifically indicated otherwise. B, Genistein reduced the slope conductance but not the reversal potential of the GABA currents in the same neuron. On the left are individual current traces evoked at membrane potentials from −80 to +60 with a step of 20 mV in the absence (○) and presence (●) of genistein (100 μM) in the bath medium. On the right are the I–V curves constructed from recordings shown on the left.

Figure 2. Genistein produced a reduction in the amplitude of GABA currents by inhibiting activity of PTKs. Daidzein, an inactive analog of genistein, does not mimic the effect of genistein on GABA<sub>A</sub> currents. The left panel shows representative GABA<sub>A</sub> current traces obtained from the same neuron in the presence or absence of genistein (100 μM) and daidzein (100 μM), respectively. The graph on the right shows averaged currents from four spinal dorsal horn neurons for each treatment. Currents were normalized by taking currents in the presence of the respective drug over control.

Figure 3. Recombinant pp60<sup>c-src</sup> potentiated GABA currents in cultured neurons. A, Intracellular perfusion of the PTK pp60<sup>c-src</sup> (30 U/ml, ●) potentiated GABA currents in control neurons but not in neurons that had been treated with genistein (100 μM, ○) 10 min before the start of whole-cell recordings. B, Graph of normalized currents recorded in the presence of pp60<sup>c-src</sup> alone (n = 4) and pp60<sup>c-src</sup> plus genistein (n = 4). Currents were normalized by taking currents recorded at 10 min (I<sub>10</sub>) over those recorded at 1 min (I<sub>1</sub>) after the start of whole-cell recordings. C, I–V relationship constructed from individual currents evoked at holding membrane potentials from −80 to +60 after the potentiation by pp60<sup>c-src</sup> has been established.
receptor subunits. To test this hypothesis, whole homogenates of the cultured spinal dorsal horn neurons were immunoprecipitated with monoclonal antibody recognizing β2/β3 subunits of the rat GABA_α receptor, the most common subunits of native CNS GABA_α receptors (Benke et al., 1991; Fritschy et al., 1992). Proteins were resolved on SDS-PAGE and probed with a polyclonal antiphosphotyrosine antibody. As shown in Figure 4A, the whole homogenate contains many tyrosine-phosphorylated proteins, consistent with the presence of endogenously active PTKs in these cells. The GABA_α receptor antibody isolated a tyrosine-phosphorylated protein band that migrates at ~58 kDa, a predicted molecular weight for β subunits of GABA_α receptors (Benke et al., 1991), indicating that the β2/β3 subunits are tyrosine phosphorylated. In other experiments, we immunoprecipitated the whole homogenate with the antiphosphotyrosine antibody and similarly probed the resulting blot with anti-β2/β3 antibody. This anti-β2/β3 blot revealed an immunoreactive band migrating at the predicted molecular weight of β subunits in both the whole homogenate and the antiphosphotyrosine immunoprecipitate (Fig. 4B). Thus, these results demonstrate that β2/β3 subunits of the GABA_α receptor in neuronal cultures are phosphorylated at tyrosine residues by endogenous PTKs. Furthermore, treatment of the neurons with genistein (100 μM; 10 min), before immunoprecipitation with anti-β2/β3 antibody, caused a reproducible decrease in antiphosphotyrosine immunoreactivity of the GABA_α receptor subunits (n = 2) (Fig. 4C). These results suggest that the level of tyrosine phosphorylation of the β2/β3 subunits of the GABA_α receptor is modulated by genistein treatment.

Modulation of recombinant GABA_α receptors by endogenous PTKs

To investigate the contribution of the β subunit to tyrosine phosphorylation modulation of the receptor function, we next examined effects of genistein on GABA_α currents in HEK 293 cells transiently expressing recombinant GABA_α receptors consisting of various combinations of rat α1, β2, and γ2 subunits. To identify the transfected cells for electrophysiological studies, cDNA encoding GFP (Marshall et al., 1995) was used as a gene marker and cotransfected with GABA_α receptor subunit cDNAs. We first studied the modulation in cells expressing the α1β2γ2 subunit combination.

Figure 5A is an example of transfected cells visualized under epifluorescent illumination with a standard FITC filter. Under standard whole-cell recording configuration, all fluorescent cells tested expressed functional GABA_α channels, as evidenced by their current responses to pressure ejections of GABA (100 μM) (Fig. 5B), confirming the utility of cotransfection of GFP cDNA as a gene expression marker in electrophysiological studies of recombinant GABA_α receptors. The induced GABA currents were potentiated by diazepam (5 μM) but were insensitive to inhibition by Zn²⁺ (100 μM), consistent with the classic pharmacology of recombinant GABA_α receptors containing αβγ subunits (Angelotti et al., 1993a; Macdonald and Olsen, 1994; Connolly et al., 1996a). As illustrated in Figure 5C, application of genistein led to a reversible reduction of the current amplitude, suggesting a tonic modulation of the receptor function by endogenous PTKs.

To determine whether the presence of β2 subunit in the
GABA<sub>A</sub> receptor complex is sufficient for the receptor modulation, we next attempted to examine the modulation in cells transfected with either α<sub>1</sub>β<sub>2</sub> or α<sub>1</sub>γ<sub>2</sub> subunits. To our surprise, although functional channels were detected in all fluorescent cells transfected with the α<sub>1</sub>β<sub>2</sub> subunit combination, no current response to GABA was recorded in any fluorescent cells transfected with α<sub>1</sub>γ<sub>2</sub> subunits. GABA currents induced in cells expressing the combination of α<sub>1</sub>β<sub>2</sub> subunits were considerably smaller than those recorded in cells expressing α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> subunits (448 ± 105 pA, n = 7, vs 1173 ± 317 pA, n = 8). These currents were substantially inhibited by a low concentration of Zn<sup>2+</sup> (10 μM) but not notably affected by extracellular Zn<sup>2+</sup> at a concentration of 100 μM. Genistein (50 μM) reversibly reduced the peak GABA currents in these cells. On the left are individual current traces recorded before (Control), 5 min after application of drug (Genistein), and 10 min after change of the bathing medium (Wash). The graph on the right summarizes data from eight individual cells.

DISCUSSION

In the present work, we have observed that the GABA<sub>A</sub> receptor-mediated currents in cultured spinal and brainstem neurons were inhibited by bath application of genistein. This effect is likely attributable to specific inhibition of PTK activity because daidzein, which is structurally similar to genistein but has no effect on PTK activity, did not affect the GABA current (Fig. 1). These results suggest that the endogenous PTKs may play an important role in maintaining the function of native GABA<sub>A</sub> receptors in these neurons. This hypothesis is further supported by the demonstrated effects of intracellular...
application of the exogenous PTK pp60c-src. Application of pp60c-src potentiated the GABA_A currents, and the effect is mediated through its kinase activity: it was prevented by pretreatment of the neurons with genistein. Thus, the present work strongly suggests that native GABA_A receptors in the CNS are potentiated by endogenous PTKs. This is in contrast, in most cases, to the modulation of the receptor by serine/threonine-specific phosphorylation. Several GABA_A receptor subunits have been shown to be phosphorylated and modulated by cAMP-dependent protein kinase A, protein kinase C, or the type II calcium/calmodulin-dependent protein kinase (Browning et al., 1993; Raymond et al., 1993; Levitan, 1994; Macdonald and Olsen, 1994). Generally serine/threonine phosphorylation of GABA_A receptors has been found to reduce GABA_A receptor activity, and conversely, dephosphorylation of the receptor is often associated with the enhanced receptor function (Raymond et al., 1993; Levitan, 1994; Macdonald and Olsen, 1994) (but see Angelotti et al., 1993b; Leidenheimer et al., 1993; Lin et al., 1996).

One simple explanation to account for the effect of PTKs on the receptor function is the direct phosphorylation of the receptor subunits at their tyrosine residues: most of the GABA_A receptor subunits contain tyrosine residues (Macdonald and Olsen, 1994; McKernan and Whiting, 1996). By using immunoprecipitation with a subunit-specific antibody, we identified a major antiphosphotyrosine-reactive band to be the β subunit(s), providing the first evidence for in situ phosphorylation of native GABA_A receptor subunits at the tyrosine residues by endogenous PTKs. Given that this antibody reacts with both β and β subunits (Ewert et al., 1990) and that the two proteins are similar in size they are recognized as a single band (Benke et al., 1991), it is not possible using this protocol to determine which of these two β subunits is responsible for the observed tyrosine phosphorylation. It should be noted, however, that to date there has been no evidence for any population of native CNS GABA_A receptors containing more than one type of β subunit and that the β subunit is by far the most abundant β subunit of native GABA_A receptors in the mammalian CNS (McKernan and Whiting, 1996). Moreover, the β subunit of purified native GABA_A receptors has been reported to be tyrosine phosphorylated in vitro by the recombinant PTK v-src (Valenzuela et al., 1995). In addition to the β subunits, Valenzuela et al. (1995) found that the γ subunit of the purified GABA_A receptors can be phosphorylated by v-src. In the present study, we did not observe any detectable tyrosine-phosphorylated protein band that corresponds to the predicted molecular weight of γ subunits (between 41–43 kDa) (Benke et al., 1991; Moss et al., 1995; McKernan and Whiting, 1996). This result argues against in situ tyrosine-specific phosphorylation of γ subunits of the native GABA_A receptor by endogenous PTKs in our preparation. Alternatively, because the γ subunits have been suggested to be sensitive to protease activity (Moss et al., 1992), the failure to detect the phosphorylated band corresponding to γ subunits could simply be attributable to the low level of the intact γ subunit proteins on the Western blots.

The role of β2 and/or β3 subunit phosphorylation in functional modulation of the GABA_A receptors by protein tyrosine phosphorylation has not been studied previously. Valenzuela et al. (1995) found that inhibiting PTK activity reduces GABA currents in Xenopus oocytes expressing either α1β1 or α1β1γ2 subunit combinations. Because the α subunit thus far has not been shown to be tyrosine phosphorylated, these results would argue for a contribution of β1 subunit phosphorylation to the functional modulation of the receptor. In contrast, Moss et al. (1995) have reported that it is the γ2, but not the β1, subunit that is fully accountable for the functional modulation of the receptor function by protein tyrosine phosphorylation. They used transient transfection of A293 cells with cDNAs encoding subunit α1/β1γ2 along with site-directed mutagenesis and found that both β1 and γ2 subunits are tyrosine phosphorylated in cells cotransfected with the v-src cDNA; however, phosphorylation of the γ2 subunit alone affects receptor function (Moss et al., 1995). With respect to the effect of β1 phosphorylation on GABA_A receptor function, the different conclusions of Valenzuela and Moss may stem from differences in the expression systems (oocytes versus mammalian cells). Neither of these studies examined a contribution of β2 and/or β3 subunits to the modulation.

In the present work we have demonstrated that inhibition of PTK activity with genistein reduces both the amplitude of GABA_A currents and the level of tyrosine phosphorylation of β2/β3 subunits of the GABA_A receptor in cultured neurons. Moreover, we also observed a reduction of GABA currents in cells expressing either α1β2 or α1β2γ2 subunits. Together, these results suggest that GABA_A receptors are functionally regulated by the state of protein tyrosine phosphorylation in these cells. As mentioned previously, the α1 subunit has not been found to be tyrosine phosphorylated in either the present work or any previous work (Moss et al., 1995; Valenzuela et al., 1995). Thus, an involvement of phosphorylation of this subunit in the observed modulation by endogenous PTK is unlikely. The ability of genistein to inhibit GABA currents in cells expressing only α1 and β2 subunits and lacking the γ2 subunit suggests that the presence of the γ2 subunit is also not required for the modulation. One may still argue for a contribution from an endogenously expressed γ subunit; potential expression of some endogenous GABA_A receptor subunits in HEK cells has recently been proposed (Ueno et al., 1996). This possibility, however, can be ruled out because GABA_A currents recorded from cells transfected with the α1β2 combination have pharmacological characteristics of currents gated through GABA_A receptors lacking a γ subunit; the currents are highly sensitive to Zn2+ inhibition but resistant to modulation by benzodiazepines (Macdonald and Olsen, 1994a; Connolly et al.,
1996a). Thus, the present work strongly supports the importance of the role of β subunits in the modulation of the GABA<sub>b</sub> receptor function by endogenous PTKs. We should point out, however, that the sufficient role of the β2 subunit in the functional modulation of the GABA<sub>b</sub> receptors does not exclude a possible contribution from a γ subunit to the functional modulation in γ subunit-containing receptors. The failure to produce a functional channel in cells transfected with the α1γ2 combination, lacking the β subunit, precludes a clarification of this issue in the present study.

Another pertinent point that we believe warrants a special comment is the apparent requirement for a β subunit to produce a functional GABA<sub>b</sub> receptor. Among the three combinations (α1β2, α1γ2, and α1β2γ2) tested, GABA currents can be recorded only in cells expressing GABA<sub>b</sub> receptors containing β2 subunits (α1β2 and α1β2γ2), suggesting a requirement for the β subunit in combination with the α1 subunit to form a functional GABA<sub>b</sub> channel. These observations are in agreement with those of Angelotti et al. (1993a) and Krishek et al. (1994). These authors found that no functional GABA<sub>b</sub> receptors can be detected by electrophysiological recording in L292 cells or A293 cells expressing α1γ2, indicating that this subunit combination fails to form functional receptors in mammalian expression systems (but see Verdoorn et al., 1990). The failure of subunit combinations lacking a β subunit to produce functional GABA<sub>b</sub> channels may be attributable to the inability of the receptor complexes to access the cell surface (Q. Wan and Y. T. Wang, unpublished observation). This has also been suggested in a recent study, which reported that cell surface expression of GABA<sub>b</sub> receptors could be detected only in cells transfected with α1 and β2 subunit, regardless of the presence or absence of the γ2 subunit (Connolly et al., 1996a). Thus, these results suggest an important role for β subunits in targeting GABA<sub>b</sub> receptor complex to the membrane surface, which is a prerequisite for forming a functional GABA<sub>b</sub> channel. Because β2/β3 subunits are the most abundant subunits of the native GABA<sub>b</sub> receptors in the CNS (Benke et al., 1991) and may play an important role in relocating the receptors between distinct neuronal domains (Connolly et al., 1996b), phosphorylation of the β2/β3 subunits, and hence modulation of the receptor function, by endogenous PTKs may represent a novel mechanism by which plasticity of GABA<sub>b</sub> receptor-mediated synaptic inhibition is mediated in the mammalian CNS.

REFERENCES

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