GABA<sub>B</sub> HETERODIMERIC RECEPTORS PROMOTE Ca<sup>2+</sup> INFLUX VIA STORE-OPERATED CHANNELS IN RAT CORTICAL NEURONS AND TRANSFECTED CHINESE HAMSTER OVARY CELLS

D. C. NEW, H. AN, N. Y. IP AND Y. H. WONG*  
Department of Biochemistry, the Molecular Neuroscience Center, and the Biotechnology Research Institute, Hong Kong University of Science and Technology, Clearwater Bay, Hong Kong, China

Abstract—The GABA<sub>B</sub> receptors are generally considered to be classical G<sub>i</sub>-coupled receptors that lack the ability to mobilize intracellular Ca<sup>2+</sup> without the aid of promiscuous G proteins. Here, we report the ability of GABA<sub>B</sub> receptors to promote calcium influx into primary cultures of rat cortical neurons and transfected Chinese hamster ovary cells. Chinese hamster ovary cells were transfected with GABA<sub>B1(a)</sub> or GABA<sub>B1(b)</sub> subunits along with GABA<sub>B2</sub> subunits. In experiments using the fluorometric imaging plate reader platform, GABA and selective agonists promoted increases in intracellular Ca<sup>2+</sup> levels in transfected Chinese hamster ovary cells and cortical neurons with the expected order of potency. These effects were fully antagonized by selective GABAB receptor antagonists. To investigate the intracellular pathways responsible for mediating these effects we employed several pharmacological inhibitors. Pertussis toxin abolished GABA<sub>B</sub> mediated Ca<sup>2+</sup> increases, as did the phospholipase C<sub>β</sub> inhibitor U73122. Inhibitor 2-aminethoxydiphenyl borane acts as an antagonist at inositol 1,4,5-trisphosphate receptors and at store-operated channels. In all cell types, 2-aminethoxydiphenyl borane prevented Ca<sup>2+</sup> mobilization. The selective store-operated channel inhibitor 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1H-imidazole hydrochloride prevented increases in intracellular Ca<sup>2+</sup> levels as did performing the assays in Ca<sup>2+</sup>-free buffers. In conclusion, GABA<sub>B</sub> receptors expressed in Chinese hamster ovary cells and endogenously expressed in rat cortical neurons promote Ca<sup>2+</sup> entry into the cell via the activation of store-operated channels, using a mechanism that is dependent on G<sub>i/o</sub> heterotrimeric proteins and phospholipase C<sub>β</sub>. These findings suggest that the neuronal effects mediated by GABA<sub>B</sub> receptors may, in part, rely on the receptor’s ability to promote Ca<sup>2+</sup> influx. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: GPCR, FLIPR, calcium, influx, SOC, GABA.

GABA is the main inhibitory neurotransmitter in the mammalian CNS. Its effects are exerted through cell surface receptors, which are distributed throughout the nervous system and are either ionotropic (termed GABA<sub>A</sub> and GABA<sub>ρ</sub>) or metabotropic (GABA<sub>B</sub>; Bowery et al., 1987). Attempts to clone GABA<sub>B</sub> receptors identified three proteins with the topology of G protein-coupled receptors (GPCRs), GABA<sub>B1(a)</sub> and GABA<sub>B1(b)</sub> as well as a GABA<sub>B2</sub> subunit (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). Expression of any of these subunits alone did not generate a functional receptor. However, co-expression of a GABA<sub>B1</sub> subunit and GABA<sub>B2</sub> resulted in the production of a functional GABA<sub>B</sub> receptor with affinities and effector coupling efficiencies equivalent to endogenous GABA<sub>B</sub> receptors (Jones et al., 1998; Restituito et al., 2005). A great deal of subsequent research has revealed that the heterodimeric GABA<sub>B</sub> receptor is bound together by a coiled-coil interaction between the carboxy terminal tails of a GABA<sub>B1</sub> and a GABA<sub>B2</sub> subunit (Calver et al., 2002). Only the GABA<sub>B1</sub> subunit is able to bind extracellular ligands and agonist activation transmits conformational changes to the GABA<sub>B2</sub> subunit, which is then able to functionally interact with G proteins (Pin et al., 2004).

Functional GABA<sub>B</sub> receptors predominantly interact with G proteins of the G<sub>q/11</sub> family but not with G<sub>α</sub> or G<sub>q</sub> proteins (Odagaki and Koyama, 2001) and, indeed, pertussis toxin (PTX) is effective at inhibiting many GABA<sub>B</sub> receptor-mediated effects (Bowery et al., 2002). GABA<sub>B</sub> receptors have been described to couple to several intracellular pathways in neuronal cells endogenously expressing these GPCRs. For example GABA<sub>B</sub> receptor agonists inhibit the forskolin-stimulated accumulation of cAMP in rat cortical brain slices (Knight and Bowery, 1996), while an increase in CAMP production has also been reported in the rat frontal cortex upon the application of GABA<sub>B</sub> receptor agonists, which is presumably mediated by G<sub>q/11</sub> proteins (Onali and Olianas, 2001). GABA<sub>B</sub> receptors are also known to modulate changes in the membrane K<sup>+</sup> flux of neuronal cells as well as regulating Ca<sup>2+</sup> flux through voltage-gated calcium channels (VGCCs; Parramon et al., 1995; Bowery et al., 2002). There are scattered reports of GABA<sub>B</sub> receptor-mediated effects that are PTX insensitive (Bowery et al., 2002) but it is not yet clear whether GABA<sub>B</sub> receptors are able to couple to G<sub>q/11</sub> a PTX insensitive member of the G<sub>q/11</sub> family that is extensively expressed in neuronal cells (Ho and Wong, 2001), or to other unidentified G proteins and adaptor molecules (Calver et al., 2002). Furthermore, there is an isolated report that GABA<sub>B</sub> receptors promote the accumulation of inositol 1,4,5-trisphosphate (IP<sub>3</sub>) in bovine adrenal chromaffin cells (Parramon et al., 1995).
In an effort to broaden our understanding of the intracellular events activated by GABAB<sub>1</sub> receptors, we have examined their signal transduction mechanisms using both recombinant and native cells. Unlike many G<sub>GTP</sub>-coupled receptors, GABAB<sub>1</sub> receptors appear to be efficiently linked to Ca<sup>2+</sup> mobilization. Intracellular Ca<sup>2+</sup> levels can be regulated by a variety of mechanisms (Verkhratsky, 2005), many of which are known to be activated by GPCRs, and the mobilization of Ca<sup>2+</sup> is a key regulator of numerous physiological and pathophysiological events (Verkhratsky, 2005). Here, we report the ability of GABAB heterodimers in primary cultures of rat cortical neurons and in transfected Chinese hamster ovary (CHO) cells to promote Ca<sup>2+</sup> entry into the cell via store-operated channels (SOCs). GABAB<sub>1</sub> receptors activate SOCs via a pathway that utilizes a PTX-sensitive and phospholipase C (PLC) dependent mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**

CHO-K1 cells were obtained from the American Type Culture Collection (ATCC CCL-61; Rockville, MD, USA). Cell and neuronal culture reagents were purchased from Life Technologies (Gaithersburg, MD, USA). Ninety-six-well plates were obtained from Corning, Inc. (Corning, NY, USA). The bicistronic cloning vector pBudCE4.1 was purchased from Invitrogen (Carlsbad, CA, USA). The GABA<sub>B1</sub><sup>a</sup> and GABA<sub>B2</sub><sup>a</sup> cDNAs were kindly provided by Dr. Fiona Marshall (GlaxoSmithKline, Stevenage, UK). GABA<sub>B1</sub><sup>a</sup> and GABA<sub>B2</sub><sup>a</sup> subunits were subcloned into pBudCE4.1 using NotI/KpnI and HindIII/XbaI restriction enzyme sites, respectively. Receptor ligands and inhibitors were obtained from Tocris (Bristol, UK) or Sigma-RBI (St. Louis, MO, USA). Fluo-4 AM was from Molecular Probes, Inc. (Eugene, OR, USA). Antibodies directed against GABA<sub>B1</sub> and GABA<sub>B2</sub> subunits were obtained from Chemicon International (Temecula, CA, USA). Other chemicals were of analytical grade and purchased from commercial suppliers.

**Generation of stable cell lines**

CHO cells were seeded into 25 cm<sup>2</sup> culture flasks at a density of 3×10<sup>5</sup> cells per flask. On the following day, 2 h before transfection, fresh F12 medium with 10% (v/v) fetal bovine serum (FBS), 50 U/ml penicillin and 50 µg/ml streptomycin was added to the cells. To construct the GABA<sub>B1</sub><sup>a</sup>/CHO cell line, 10 µg of GABA<sub>B1</sub><sup>a</sup> and GABA<sub>B2</sub><sup>a</sup> cDNAs subcloned into the bicistronic pBudCE4.1 vector was introduced into the cells using the calcium phosphate precipitation method (Sambrook et al., 1989). For the GABA<sub>B1</sub><sup>a</sup>/CHO cell line, 10 µg of each of the GABA<sub>B1</sub><sup>a</sup> cDNA subcloned into pcDNA3.1 (+) and the GABA<sub>B2</sub> cDNA subcloned into pCDNA3.1/Zeo (+) were used. Transfected cells were cultured for 16 h at 37 °C in a water-saturated atmosphere of 95% air and 5% CO<sub>2</sub>. Cells were washed with phosphate-buffered saline and cultured in normal growth medium for a further 24 h before selection by the addition of 400 µg/ml zeocin (GABA<sub>B1</sub><sup>a</sup>/CHO) or 800 µg/ml G418 and 400 µg/ml zeocin (GABA<sub>B2</sub><sup>a</sup>/CHO). Following the death of all mock-transfected cells, zeocin resistant cells were re-plated at a low density. Individual colonies were isolated and maintained in growth medium containing 200 µg/ml G418 and/or 100 µg/ml zeocin, as appropriate.

**Preparation of cortical neuronal cultures**

Primary cultures of cortical neurons were initiated from the cortices of 17–18 day old rat (Sprague–Dawley) embryos. Cortical tissue was washed with HEPES buffer, salt solution and incubated with 0.25% trypsin at 37 °C for 15 min. The neurons were supplemented with 5% (v/v) horse serum followed by two rounds of centrifugation (1250 r.p.m., 5 min) and resuspension in DMEM medium. Cells were seeded in 96-well, black-walled microtiter plates at a density of 20,000 cells per well in DMEM medium supplemented with 10% (v/v) horse serum and 1 mM L-glutamine. Four hours after seeding the medium was replaced with neurobasal medium supplemented with 2% (v/v) B27, 50 U/ml penicillin and 50 µg/ml streptomycin. The medium was replaced after 3 days and cultured neuronal cells were assayed by fluorometric imaging plate reader (FLIPR) assay 9–11 days following preparation.

**Western blotting analysis**

Crude membrane proteins from stably transfected cell lines were extracted as previously described (Liu and Wong, 2005). Fifty micrograms of membrane protein was resolved on a 12.5% (v/v) sodium dodecyl sulfate–polyacrylamide gel and transferred to polyvinylidene fluoride membrane. Protein expression was detected using antisera recognizing the C-terminal tail regions of GABA<sub>B1</sub> or GABA<sub>B2</sub> subunits and an enhanced chemiluminescence (ECL) kit.

**Measurement of cAMP levels**

Transfected cells were labeled with [3H]adenosine (1 µCi/ml) in F12 medium containing 1% (v/v) FBS for 20–24 h. Labeled cells were challenged with appropriate drugs for 30 min at 37 °C, in the presence of 50 µM forskolin, and assayed for cAMP levels, as described previously (Ho et al., 2002). When required, cells were incubated in the presence of 100 ng/ml of PTX for approximately 16 h before forskolin and agonist challenge. EC<sub>50</sub> values are given as the mean±S.D. from four independent experiments.

**Measurement of intracellular Ca<sup>2+</sup> using a FLIPR device**

CHO cells were seeded into 96-well, black-walled microtiter plates at a density of 4×10<sup>4</sup> cells per well in F12 medium containing 10% (v/v) FBS. Where indicated, cells were incubated with PTX (100 ng/ml) for 16 h prior to the assay. The following day, the medium was removed and replaced with 200 µl of labeling medium consisting of 1:1 (v/v) Opti-MEM:Hanks’ balanced salt solution (HBSS), 2.5% (v/v) FBS, 20 mM HEPES, pH 7.4, 2.5 mM probenecid and 2 µM Fluo-4 AM and assayed using an optimized FLIPR protocol (New and Wong, 2004). Pharmacological inhibitors were added at appropriate time points before the assay where indicated. Agonists and antagonists were prepared as a 5× solution in HBSS, 20 mM HEPES, pH 7.4 and 2.5 mM probenecid and aliquoted into polypropylene 96-well plates. Following a 60 min incubation of the cells in the labeling medium, cell and drug plates were placed in a FLIPR (Molecular Devices, Sunnyvale, CA, USA). Changes in fluorescence were measured over a period of 120 s following excitation at a wavelength of 488 nm and detection at 510–560 nm. Fifty microliters of drug solution were added to the cell medium at time = 10 s. When required, inhibitors were added to the labeling medium at the indicated times before the drug solutions. Data were collected as relative fluorescence units (RFU), which denotes the fluorescent signal obtained over an arbitrarily set baseline. For each treatment, the response to the vehicle was subtracted from the drug-induced response. Generally, agonist-induced responses were several thousand RFUs over basal. Data were analyzed using Excel and GraphPad Prism, version 3.02. EC<sub>50</sub> and IC<sub>50</sub> values are given as the mean±S.D. from no less than three independent determinations.
**RESULTS**

**Generation and characterization of CHO cells stably expressing GABA<sub>B1(b)/2</sub> subunits**

In order to examine the signaling properties of the GABA<sub>B</sub> receptor, we have established a CHO cell line stably co-expressing the GABA<sub>B1(b)</sub> and GABA<sub>B2</sub> receptors using the bicistronic expression vector pBudCE4.1. Zeocin resistant clonal cells were isolated and tested for their ability to respond to GABA<sub>B1(b)/2</sub> receptors using FLIPR assays. The ability of these GABA<sub>B1(b)/2</sub>/CHO cells to activate intracellular Gi/o-coupled pathways was assessed by the ability of the GABA<sub>B</sub> agonist baclofen to inhibit the forskolin-stimulated production of cAMP. When challenged with baclofen, the cAMP levels in forskolin-stimulated GABA<sub>B1(b)/2</sub>/CHO cells were reduced by approximately 65% with an EC<sub>50</sub> value of 3.7 ± 2.3 μM (n=4; Fig. 1B). This is in good agreement with an EC<sub>50</sub> value of 3.7 μM reported for GABA<sub>B</sub> receptor-mediated cAMP inhibition in HEK-293 cells and an EC<sub>50</sub> value of 59 μM for incorporation of GTPγS into rat brain membranes stimulated by GABA (White et al., 1998). Furthermore, the inhibition of cAMP production was entirely abolished when cells were preincubated with PTX prior to the addition of 1 mM baclofen (Fig. 1B), suggesting the involvement of G<sub>i/o</sub> proteins.

GABA<sub>B</sub> receptors mediate the mobilization of intracellular Ca<sup>2+</sup>

Previous reports have demonstrated that GABA<sub>B</sub> receptors coexpressed with chimeric G proteins allow these Gi/o-coupled receptors to promote intracellular increases in Ca<sup>2+</sup> levels (Wood et al., 2000; Liu et al., 2003). As we have previously observed that several Gi/o-coupled receptors can promote Ca<sup>2+</sup> mobilization in the absence of artificial chimeras (New and Wong, 2004), we examined the ability of GABA<sub>B</sub> receptors expressed in CHO cells to activate pathways that trigger increases in intracellular Ca<sup>2+</sup> levels. GABA<sub>B1(b)/2</sub>/CHO cells were challenged with...
baclofen and the Ca\(^{2+}\) levels monitored in FLIPR assays. 
GABA\(_{B1b(2)}/\)CHO cells, but not parental CHO cells, responded to baclofen with large increases in intracellular Ca\(^{2+}\) with EC\(_{50}\) values in the micromolar range (Fig. 2). Ca\(^{2+}\) mobilization was apparent within 2–3 s of drug addition, reaching a maximum value approximately 20 s after drug addition before returning to basal levels within 50 s. To confirm that the Ca\(^{2+}\) mobilization was mediated by GABA\(_B\) receptors, we tested the response of the GABA\(_B/\)CHO cells to different GABA\(_B\) receptor agonists and antagonists. Dose-response curves constructed using FLIPR assays showed that the agonist GABA had a similar potency and efficacy to baclofen (Fig. 2), with EC\(_{50}\) values of 3.5±3.72 μM (n=3) versus 4.43±1.8 μM (n=3), respectively. However, agonist SKF97541 was approximately 10-fold more potent with an EC\(_{50}\) value of 0.37±0.05 μM (n=3; Fig. 2). The potency order of these three agonists replicates that seen in previous studies (Seabrook et al., 1990; Wood et al., 2000). The baclofen-induced Ca\(^{2+}\) mobilization was completely abolished by co-administration with the antagonists CGP55845 or CGP46381 with IC\(_{50}\) values of 1.5±2.1 μM (n=3) and 22.2±34 μM (n=3), respectively (Fig. 3A). To determine the K\(_B\) value of CGP46381, a Schild analysis was performed. Increasing concentrations of CGP46381 shifted the baclofen dose-response to the right (Fig. 3B) with a K\(_B\) value of 0.82 μM and a slope of 0.94, suggesting potent, competitive inhibition (Fig. 3C) as previously reported (Olpe et al., 1993; Wood et al., 2000).

Identification of intracellular pathways mediating Ca\(^{2+}\) release

Having determined that GABA\(_B\) receptors are able to mediate Ca\(^{2+}\) mobilization in CHO cells, we proceeded to examine the intracellular pathways mediating this effect. PTX treatment of GABA\(_{B1b(2)}/\)CHO cells for 16 h prior to baclofen stimulation completely abolished the agonist-stimulated increases in Ca\(^{2+}\) levels (Fig. 4). This indicates that the responses determined in FLIPR assays are entirely mediated by PTX-sensitive G\(_i/o\) family heterotrimeric proteins.

G\(_{i\gamma}\) subunits released from G\(_i/o\) proteins are able to activate PLC\(_\beta\) (Rebecchi and Penttyala, 2000), potentially modulating Ca\(^{2+}\) release and we reasoned that such a pathway may be operational in GABA\(_{B1b(2)}/\)CHO cells. Indeed, the PLC\(_\beta\) inhibitor U73122 at a concentration of 4 μM reduced Ca\(^{2+}\) mobilization induced by a saturating concentration of baclofen by 86.3±7.8% (n=3; data not shown). Increasing the concentration of U73122-10 μM inhibited responses to agonists by 99.1±4.0% (n=3; Fig. 5A).
In contrast, 4 μM or 10 μM of the inactive homologue of U73122, U73343, was unable to suppress the baclofen-induced Ca\(^{2+}\) mobilization. U73343 (10 μM) inhibited responses by $-3.6\pm9.2\%$ ($n=3$; Fig. 5A). These results demonstrate that GABA\(_B\) receptors induce increases in intracellular Ca\(^{2+}\) levels via a PLC\(\beta\)-mediated pathway.

To identify the downstream effectors and the source of Ca\(^{2+}\), GABA\(_B_{102}\)/CHO cells were challenged with baclofen following treatment with various pharmacological inhibitors. The IP\(_3\) receptor antagonist 2-aminophosphonic acid (2-APB) dose-dependently inhibited Ca\(^{2+}\) mobilization with 10 μM inhibiting the response by at least 75% and 100 μM completely abolishing baclofen-induced responses ($102.1\pm4.1\%$, $n=3$; Fig. 5A). The inhibition of responses by 2-APB implicates intracellular Ca\(^{2+}\) stores as the likely source of the Ca\(^{2+}\) measured in FLIPR assays, but 2-APB is also known to inhibit store-operated Ca\(^{2+}\) entry over the same concentration range that antag-
onizes IP3 receptors (Bootman et al., 2002). Therefore, we preincubated the cells with the selective SOC inhibitor 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl]-1H-imidazole hydrochloride (SKF96365) for 10 min before challenge with baclofen. SKF96365 (10 μM) significantly inhibited FLIPR responses to baclofen while 100 μM SKF96365 completely abolished the response (95±14.3%, n=3; Fig. 5A), suggesting that the majority of Ca2+ measured within the cell was the result of influx from the extracellular medium. This was confirmed when the assays were repeated using Ca2+ free buffers. Under these assay conditions, baclofen was ineffective in promoting increases in intracellular Ca2+ levels even at a concentration of 1 mM (Fig. 6).

As the baclofen-induced Ca2+ fluxes were PLCβ-dependent, we tested the ability of the selective protein kinase C (PKC) inhibitor calphostin C to inhibit increases in intracellular Ca2+ levels. At a concentration of 200 nM, calphostin C was unable to significantly affect Ca2+ mobilization, indicating that this kinase and its downstream effectors do not play a role in GABAB receptor-induced Ca2+ fluxes. As GABAB receptors have been shown to mediate cAMP production in some cell types (10), we also examined the role of protein kinase A (PKA) in GABAβ receptor-mediated Ca2+ flux. The selective inhibitor H89 was completely ineffective in inhibiting the baclofen-induced responses (Fig. 5A).

Examining the role of ryanodine receptors and Ca2+ channels

We also examined the contribution of ryanodine receptors and VGCC-mediated Ca2+ fluxes to the GABAβ receptor-induced intracellular Ca2+ levels. Ryanodine receptor inhibitors ruthenium red and dantrolene at concentrations of 20 μM and 10 μM, respectively, were not able to significantly decrease the response of GABAβ1(b)/2/CHO cells to baclofen (Fig. 5B). Similarly, the VGCC blockers nifedipine (L-type channel blocker; 10 μM), ω-conotoxin GVIA (N-type channel blocker; 1 μM) or SNX-482 (R-type channel blocker; 30 nM) were unable to attenuate the GABAβ receptor-mediated responses (Fig. 5B).

GABAβ1(a)/2 receptors also promote store-operated Ca2+ entry

To determine the ability of GABAβ1(a) containing heterodimeric GABAβ receptors to activate Ca2+ influx via SOCs, we constructed and examined the signaling properties of GABAβ1(a)/2/CHO cells. As observed with GABAβ1(b)/2/CHO cells, baclofen promoted increases in intracellular Ca2+ levels, which were antagonized by CGP55845 (IC50 = 9.8±10.5 μM (n=3); Fig. 7A). Furthermore, PTX, U73122, 2-APB and SKF96365 were all able to completely inhibit baclofen-induced responses over similar concentration ranges as were effective in GABAβ1(b)/2 expressing cells (Fig. 7B). This indicates that GABAβ1(a)+ and GABAβ1(b)+ containing receptors are both able to promote Ca2+ influx via SOCs using a PTX-sensitive, PLCβ-dependent pathway.

Ca2+ influx through SOCs in primary cultures of rat cortical neurons

To determine whether GABAβ receptors are able to promote Ca2+ influx in neuronal cells endogenously expressing GABAβ receptors, a similar set of experiments was performed using primary cultures of cortical neurons isolated from rats and cultured in 96-well plates. Baclofen was able to dose-dependently increase intracellular Ca2+ levels with an EC50 value of 3.7±4.4 μM (n=4; Fig. 8A).
response induced by 1 mM baclofen was antagonized by CGP55845 with an IC\textsubscript{50} value of 9.95±6.5 μM (n=3; Fig. 8B), confirming that GABA\textsubscript{B} receptors were mediating the response. Pretreatment of cells for 16 h with PTX rendered 1 mM baclofen completely ineffective (Fig. 9), indicating that, as in CHO cells, G\textsubscript{i/o} proteins in cortical neurons also transduce the Ca\textsuperscript{2+} mobilizing effects of GABA\textsubscript{B} receptors. The PLC\textsubscript{β} inhibitor U73122 (10 μM) completely inhibited baclofen-induced responses, whereas U73343 was ineffective (Fig. 9). As observed in GABA\textsubscript{B1(a)/2/CHO} cells, 2-APB dose-dependently inhibited agonist-induced responses with 100 μM completely blocking increases in Ca\textsuperscript{2+} levels (Fig. 9). SOC inhibitor SKF96365 completely abolished agonist-induced responses at 10 and 50 μM (Fig. 9). The ryanodine receptor inhibitors ruthenium red and dantrolene, as well as the VGCC blockers nifedipene, α-conotoxin GVIA and SNX-482 were ineffective (data not shown). The accumulated data confirm that the PLC\textsubscript{β}-mediated opening of SOCs by the stimulation of GABA\textsubscript{B} receptors endogenously expressed in cortical neurons operates in a manner similar to GABA\textsubscript{B} receptors exogenously expressed in CHO cells.

**DISCUSSION**

GABA\textsubscript{B} receptors are typical G\textsubscript{i}-coupled receptors, which can efficiently inhibit adenylyl cyclase.  

---

**Fig. 7.** Effect of GABA\textsubscript{B} antagonists and pharmacological inhibitors on the response of GABA\textsubscript{B1(a)/2/CHO} cells to 100 μM baclofen. (A) A CHO cell line stably expressing GABA\textsubscript{B1(a) and GABA\textsubscript{B2} receptor subunits was established. The effect of the selective GABA\textsubscript{B} receptor antagonist CGP55845 on 100 μM baclofen-induced Ca\textsuperscript{2+} levels was characterized using FLIPR. The normalized data are presented as the means±S.D.s of data averaged across three independent experiments performed in duplicate or triplicate. (B) The effects of PTX (100 ng/ml), U73122, U73343, 2-APB and SKF96365 on baclofen-induced Ca\textsuperscript{2+} levels were also examined. For each treatment the data were measured as an increase over the basal value obtained in the presence of the inhibitor. None of the inhibitors used had a significant effect on basal values. The normalized data are presented as the means±S.D. of data averaged across three independent experiments performed in triplicate.
study suggests that activating GABAB receptors containing CHO and GABAB1(b)/2/CHO cells (Figs. 3 and 7A). Sections of evidence. Firstly, the pharmacological profiles for activation of SOCs. This notion is supported by several agonist-induced Ca\(^{2+}\) elevation of intracellular Ca\(^{2+}\) linked receptors appear to possess an inherent ability to increase in intracellular Ca\(^{2+}\) levels. Muscarinic M\(_2\) and M\(_4\), formyl peptide-receptor-like-1 (New and Wong, 2004), somatostatin SST\(_2\) (Nunn et al., 2004) and \(\mu\)-opioid receptors (Smart et al., 1997) are all able to mediate PTX-sensitive elevation of intracellular Ca\(^{2+}\) levels. The present study suggests that activating GABA\(_B\) receptors containing either the B1(a) or B1(b) subunit can also lead to an increase in intracellular Ca\(^{2+}\) levels, primarily through the activation of SOCs. This notion is supported by several lines of evidence. Firstly, the pharmacological profiles for agonist-induced Ca\(^{2+}\) responses in GABA\(_B1(b)/2\)/CHO cells are in agreement with known selectivity of the GABA\(_B\) receptor (Fig. 2); GABA\(_B\) receptor antagonists inhibited the agonist-induced responses in GABA\(_B1(b)/2\)/CHO and GABA\(_B1(b)/2\)/CHO cells (Figs. 3 and 7A). Secondly, a selective inhibitor of SOCs, SKF96365 (Merritt et al., 1990), abolished the GABA\(_B\) receptor-mediated changes in intracellular Ca\(^{2+}\) (Figs. 5A and 7B). The absence of any response when Ca\(^{2+}\) free buffers were used ruled out the possibility that SKF96365 was exerting effects other than on SOCs (Fig. 6). Lastly, the baclofen-induced Ca\(^{2+}\) fluxes and their sensitivity to antagonists and pharmacological inhibitors were also observed in primary cultures of rat cortical neurons (Figs. 8 and 9), where GABA\(_B1(a)\) and GABA\(_B1(b)\) subunits are endogenously co-expressed (Kaupmann et al., 1998). These results strongly suggest that activation of GABA\(_B\) receptors in the CHO stable cell lines and rat cortical neurons can lead to the stimulation of SOCs.

The mechanism by which GABA\(_B\) receptors activate SOCs appears to involve multiple signaling intermediaries. Inhibition of Ca\(^{2+}\) influx by PTX treatment of cells identified G\(_{i/o}\) heterotrimeric proteins as mediators of GABA\(_B\) receptor-generated signals (Figs. 4, 7B and 9). Complete inhibition of Ca\(^{2+}\) influx by U73122 (but not by its inactive analog) implicates the involvement of PLC\(\beta\) (Figs. 5A, 7B and 9). To date there are no known instances of G\(_{i/o}\) proteins directly activating PLC\(\beta\) and it has been demonstrated that constitutively active mutants of G\(_{i/o}\) subunits do not promote PLC\(\beta\) activity (Tsu et al., 1995). However, activation of PLC\(\beta\) by G\(\beta\)\(\gamma\) dimers is an increasingly common observation (Rebecchi and Pentyala, 2000). It therefore seems likely that GABA\(_B\) receptor activation of G\(_{i/o}\) proteins leads to the release of G\(\beta\)\(\gamma\) dimers that activate PLC\(\beta\), which in turn triggers downstream events leading to an increase in intracellular levels of Ca\(^{2+}\). GABA\(_B\) receptor-mediated release of G\(\beta\)\(\gamma\) subunits from G\(_{i/o}\) proteins has previously been shown to result in increased adenyl cyclase activity in the rat frontal cortex (Onali and Olianas, 2001). Furthermore, Xenopus spinal growth cones are repelled by a gradient of baclofen, which is apparently mediated by G\(_i\) protein activation of PLC\(\beta\) (Xiang et al., 2002).

Activation of PLC\(\beta\) leads to the generation of IP\(_3\) and the subsequent release of Ca\(^{2+}\) from intracellular stores, as well as the generation of diacylglycerol and the activation of PKC (Berridge, 1993). 2-APB is an IP\(_3\) receptor antagonist that has been widely used as a pharmacological tool to characterize IP\(_3\)-mediated Ca\(^{2+}\) release from intracellular stores (Boothman et al., 2002). However, 2-APB has also been shown to be equally effective at inhibiting the Ca\(^{2+}\) influx from the extracellular medium (Boothman et al., 2002) and, therefore, its inhibition of baclofen-mediated Ca\(^{2+}\) flux in our experiments (Figs. 5A, 7B and 9) does not allow us to conclude a role for IP\(_3\) receptors in the activation of SOCs. However, the lack of an effect on baclofen-induced Ca\(^{2+}\) influx by calphostin C does allow us to conclude that PKC and its effectors do not significantly contribute to the GABA\(_B\) receptor-mediated activation of SOCs (Fig. 5A). This observation is consistent with previous findings that thapsigargin- and orexin receptor-induced SOC activation are also unaffected by PKC (Venkatachalam et al., 2003; Larsson et al., 2005). A summary of the proposed mechanism of Ca\(^{2+}\) entry is presented schematically in Fig. 10.

These accumulated data suggested to us that GABA\(_B\) receptor activation leads to a G\(_{i/o}\)-dependent, PLC\(\beta\)-mediated...
ated rapid influx of Ca$$^{2+}$$ into the cell through SOCs. Extensive studies on the process of Ca$$^{2+}$$ entry have indicated that several mechanisms may mediate PLC-$$\beta$$- dependent Ca$$^{2+}$$ influx (Putney et al., 2001; Venkatachalam et al., 2002), with contradictory data reported on the requirement of IP$$^3$$ generation and IP$$^3$$ receptor activation. Several studies have shown that IP$$^3$$ is required for store-operated Ca$$^{2+}$$ entry (Zubov et al., 1999) but others have demonstrated that in lacrimal and rat basophilic leukemia cells treated with the PLC-$$\beta$$ inhibitor U73122, Ca$$^{2+}$$ influx is not restored by the application of exogenous IP$$^3$$ and that SOC activating pathways are operational in IP$$^3$$ receptor knockout DT40 B-lymphocytes (Broad et al., 2001). Therefore, there appear to be IP$$^3$$ dependent and independent

![Figure 9](image-url) **Fig. 9.** Effect of PTX and pharmacological inhibitors on the response of primary rat cortical neurons to 1 mM baclofen. For PTX (100 ng/ml), cells were treated for 16 h before assay using the FLIPR platform. The other inhibitors were applied at the indicated concentrations in advance of the assays (the incubation times are as specified in the legend to Fig. 6). For each treatment the data were measured as an increase over the basal value obtained in the presence of the inhibitor. None of the inhibitors used had a significant effect on basal values. The normalized data are presented as the means±S.D. of data averaged across three independent experiments performed in duplicate or triplicate.

![Figure 10](image-url) **Fig. 10.** Proposed mechanism for the activation of Ca$$^{2+}$$ entry mediated by GABA$$\beta$$ receptors. The data presented are illustrated schematically and indicate that agonist activation of GABA$$\beta$$ receptors leads to an influx of Ca$$^{2+}$$ from the extracellular medium. The inhibition of this phenomenon by PTX, U73122, 2-APB and SKF96365 suggests that a mechanism of capacitative calcium entry operates to allow Ca$$^{2+}$$ influx through SOCs. The nature and role of intermediaries between PLC$$\beta$$, IP$$^3$$, intracellular stores and SOCs are not yet fully elucidated (the reader is directed to Putney et al., 2001 and Parekh and Putney, 2005 for full discussions). Our experimental evidence shows that GABA$$\beta$$ receptors do not require the activation of PKA, PKC or VGCCs for SOC entry.
mechanisms of SOC activation. Our data confirm the PLCβ dependence of GABAβ receptors mediated capacitative calcium entry, although the lack of selective, membrane permeable IP3 receptor antagonists has prevented us from demonstrating IP3 receptor involvement in this mechanism.

Several other GPCRs have been demonstrated to activate SOCs, including metabotropic glutamate subtype 1 receptors in dopamine neurons in rat brain slices (Tozzi et al., 2003), muscarinic receptors expressed in lymphatic cell lines (Broad et al., 2001) and endothelin receptors in vascular smooth muscle cells (Kawanabe et al., 2002). To date, no systematic study on the ability of GPCRs to activate SOCs in neuronal and non-neuronal cells has been undertaken. It is, therefore, unclear whether SOC activation by GPCRs is a property common to all Gq/coupled receptors as well as those Gq/o-coupled GPCRs that are able to activate PLCβ, or whether there is an extra degree of control of SOC opening that can only be overcome by a subset of GPCRs.

It has recently been reported that the orexin GPCRs can promote Ca2+ influx into CHO cells via the activation of nonstore-operated Ca2+ entry (Larsson et al., 2005). As with SOCs, these channels are believed to be composed of subtypes of the transient receptor potential channel (TRPC) family (Minke and Cook, 2002; Parekh and Putney, 2005). A distinguishing characteristic of nonstore-operated entry, compared with Ca2+ entry via SOCs, is its lack of sensitivity to 2-APB and SKF96365 (Larsson et al., 2005). The sensitivity to these two blockers of the responses that we observed in transfected CHO cells and cortical neurons indicates that GABAβ receptors do not activate nonstore-operated Ca2+ channels.

GABAβ receptors have previously been shown to suppress (Bowery et al., 2002) or facilitate Ca2+ influx via several sub-types of VGCCs, notably L-type channels (Parramon et al., 1995; Carter and Mynlieff, 2004). We observed no signs of GABAβ receptor-mediated VGCC influx in transfected CHO cells or rat cortical neurons (Fig. 5B). Ryanodine receptors are activated by some Gq/o-coupled GPCRs (Maghzachi, 2000) in a PLCβ-independent manner (Putney et al., 2001), but we conclude that GABAβ receptors do not activate this system when expressed in CHO cells or rat cortical neurons (Fig. 5B). However, our investigation measured Ca2+ levels on a whole cell scale, and we do not rule out the possibility of localized Ca2+ gradients controlled by VGCCs or ryanodine-sensitive stores.

It was apparent from the experiments in which Ca2+ influx was inhibited by SKF96365 or by the use of Ca2+ free buffers that we could not detect Ca2+ release from intracellular stores, even though depletion of Ca2+ from the ER is thought by some to be required before SOCs open (Putney et al., 2001). This would seem to suggest that in our experimental system the IP3 receptor-induced release of Ca2+ from intracellular stores following activation of GABAβ receptors is on a small scale. Alternatively, it is possible that Ca2+ release from intracellular stores is not a prerequisite for SOC activation. Consistent with these findings are observations that in skeletal muscle cells, IP3 receptors mediate SOC opening without necessarily triggering release from internal Ca2+ stores (Launikonis et al., 2003). We are currently investigating the relative contributions of extracellular Ca2+ influx and intracellular Ca2+ mobilization upon activation of PLCβ-dependent pathways by other Gq/o- and Gq/coupled receptors.

GABAβ receptor involvement has been demonstrated to regulate a variety of neuronal functions both directly and indirectly, by modulating the activity of other neurotransmitter systems. GABAβ receptors generate inhibitory postsynaptic potentials that are associated with large hyperpolarizations, which have an inhibitory effect on many neurons (Mott and Lewis, 1994). However, postsynaptic GABAβ receptors in the thalamus are excitatory, with such activity contributing to absence epilepsy (Calver et al., 2002). Postsynaptically, GABAβ receptor activity can directly affect neuronal function by, for example, promoting the phosphorylation and inhibition of GABAβ receptors in a PLCβ-dependent manner (Hahner et al., 1991). Postsynaptic activity is especially effective at inhibiting NMDA receptor-mediated responses using a mechanism that requires G proteins (Morrisett et al., 1991) and, therefore, GABAβ receptors are likely to play an inhibitory role in synaptic plasticity and long-term potentiation. Presynaptically, GABAβ receptors have a depressant effect on excitatory responses in numerous brain regions (Mott and Lewis, 1994), leading to the modulation of the release of numerous neurotransmitters (Calver et al., 2002). Many of these post- and presynaptic effects are, in part, regulated by the GABAβ receptor regulation of K+ channels and VGCCs. However, in light of our findings that GABAβ receptor activation in neuronal cells leads to increases in intracellular Ca2+ levels, we suggest that the role of SOCs in neurophysiology be considered.

It is currently unclear as to whether Ca2+ influx through SOCs is simply required for the replenishment of ER stores or whether it serves some independent function. Nevertheless, SOC activation has been implicated in neuroplasticity (Baba et al., 2003), DNA synthesis and ordered cell cycle progression in retinal neuroepithelial cells (Sugioka et al., 1999), while reduced SOC activity is associated with the generation of the Aβ42 protein, a key component in the generation of plaques associated with the development of Alzheimer’s disease (Yoo et al., 2000). We anticipate that GABAβ receptor-mediated activation of SOCs contributes to a complex interplay of Ca2+ releasing and sequestering pathways that regulate the intracellular levels of Ca2+. This may be required for the regulation of electric charge and the effective control of neuronal functions, including neurotransmitter release, excitability, synaptic plasticity and gene expression (Mott and Lewis, 1994; Vergher, 2005).

The broad expression profile of GABAβ receptors at both pre- and post-synaptic sites in the CNS (Mott and Lewis, 1994) has led to their use as therapeutic targets, and treatments with anti-convulsant, anti-ulcer, anti-amnesic and many other efficacies are being actively pursued (Bolser et al., 1995; Kerr and Ong, 1995; Nava et al., 2001;
Brown et al., 2003). We have previously examined the responses of GABA<sub>B</sub> receptors in FLIPR assays using a transient transfection system (Liu et al., 2003). We found weak but consistent Ca<sup>2+</sup> increases in response to high doses of baclofen that were potentiated by the chimeric G<sub>α</sub> subunits 16z25 and 16z44. Our generation of CHO cells stably expressing GABA<sub>B</sub> receptors provides a much more robust screening platform that replicates the signal transduction pathways seen in neuronal cells. We believe that this will enable more efficient drug screening for agonists, antagonists and allosteric modulators of GABA<sub>B</sub> receptors. In conclusion, we have identified a GABA<sub>B</sub> receptor-activated signal transduction pathway in cortical neurons that is mediated by G<sub>i/o</sub> proteins and leads to the activation of SOCs.

Acknowledgments—The authors are grateful to Agnes Chan, Estella Tong, Fred Luk and Fanny Ip for assistance with the preparation of the cortical neurons and subcloning. This work was supported in part by the Hong Kong Jockey Club and grants from the Innovation and Technology Commission of Hong Kong (ITSI 226/01 and ITS/113/03), the Research Grants Council of Hong Kong (HKUST 3/03C), and the University Grants Committee (AoE/B-15/01). N.Y.I. and Y.H.W. were recipients of the Croucher Senior Research Fellowship.

REFERENCES


