Neuroscience 137 (2006) 1347–1358

$GABA_B$ HETERODIMERIC RECEPTORS PROMOTE Ca^{2+} 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 $GABA_B$ receptors are generally considered to be classical G_i-coupled receptors that lack the ability to mobilize intracellular Ca^{2+} without the aid of promiscuous G proteins. Here, we report the ability of $GABA_B$ 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 $GABA_{B1(a)}$ or $GABA_{B1(b)}$ subunits along with $GABA_{B2}$ subunits. In experiments using the fluorometric imaging plate reader platform, GABA and selective agonists promoted increases in intracellular Ca^{2+} levels in transfected Chinese hamster ovary cells and cortical neurons with the expected order of potency. These effects were fully antagonized by selective $GABA_B$ receptor antagonists. To investigate the intracellular pathways responsible for mediating these effects we employed several pharmacological inhibitors. *Pertussis* toxin abolished $GABA_B$ mediated Ca^{2+} increases, as did the phospholipase Cβ 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^{2+} mobilization. The selective store-operated channel inhibitor 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1*H*-imidazole hydrochloride prevented increases in intracellular Ca^{2+} levels as did performing the assays in Ca^{2+} free buffers. In conclusion, $GABA_B$ receptors expressed in Chinese hamster ovary cells and endogenously expressed in rat cortical neurons promote Ca^{2+} entry into the cell via the activation of store-operated channels, using a mechanism that is dependent on $G_{i/o}$ heterotrimeric proteins and phospholipase Cβ. These findings suggest that the neuronal effects mediated by $GABA_B$ receptors may, in part, rely on the receptor's ability to promote Ca^{2+} 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 $GABA_A$ and $GABA_C$) or metabotropic ($GABA_B$; Bowery et al., 1987). Attempts to clone $GABA_B$ receptors identified three proteins with the topology of G protein-coupled receptors (GPCRs), $GABA_{B1(a)}$ and $GABA_{B1(b)}$ as well as a $GABA_{B2}$ 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 $GABA_{B1}$ subunit and $GABA_{B2}$ resulted in the production of a functional $GABA_B$ receptor with affinities and effector coupling efficiencies equivalent to endogenous $GABA_B$ receptors (Jones et al., 1998; Restituito et al., 2005). A great deal of subsequent research has revealed that the heterodimeric $GABA_B$ receptor is bound together by a coiled-coil interaction between the carboxy terminal tails of a $GABA_{B1}$ and a $GABA_{B2}$ subunit (Calver et al., 2002). Only the $GABA_{B1}$ subunit is able to bind extracellular ligands and agonist activation transmits conformational changes to the $GABA_{B2}$ subunit, which is then able to functionally interact with G proteins (Pin et al., 2004).

Functional $GABA_B$ receptors predominantly interact with G proteins of the $G_{i/o}$ family but not with G_s or G_q proteins (Odagaki and Koyama, 2001) and, indeed, *pertussis* toxin (PTX) is effective at inhibiting many $GABA_B$ receptor-mediated effects (Bowery et al., 2002). $GABA_B$ receptors have been described to couple to several intracellular pathways in neuronal cells endogenously expressing these GPCRs. For example $GABA_B$ 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 $GABA_B$ receptor agonists, which is presumably mediated by Gβγ subunit released from $G_{i/o}$ proteins (Onali and Olianas, 2001). $GABA_B$ receptors are also known to modulate changes in the membrane K^+ flux of neuronal cells as well as regulating Ca^{2+} flux through voltage-gated calcium channels (VGCCs; Parramon et al., 1995; Bowery et al., 2002). There are scattered reports of $GABA_B$ receptor-mediated effects that are PTX insensitive (Bowery et al., 2002) but it is not yet clear whether $GABA_B$ receptors are able to couple to G_z, a PTX insensitive member of the $G_{i/o}$ 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 $GABA_B$ receptors promote the accumulation of inositol 1,4,5-trisphosphate (IP_3) in bovine adrenal chromaffin cells (Parramon et al., 1995).

*Corresponding author. Tel: +2358-7328; fax: +2358-1552.
E-mail address: boyung@ust.hk (Y. H. Wong).

Abbreviations: CHO, Chinese hamster ovary; FBS, fetal bovine serum; FLIPR, fluorometric imaging plate reader; GPCR, G protein-coupled receptor; HBSS, Hanks' balanced salt solution; IP_3, inositol 1,4,5-trisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLCβ, phospholipase Cβ; PTX, *pertussis* toxin; RFU, relative fluorescence units; SKF96365, 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1*H*-imidazole hydrochloride; SOC, store-operated channels; VGCC, voltage-gated calcium channels; 2-APB, 2-aminethoxydiphenyl borane.

0306-4522/06$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2005.10.033

In an effort to broaden our understanding of the intracellular events activated by $GABA_B$ receptors, we have examined their signal transduction mechanisms using both recombinant and native cells. Unlike many $G_{i/o}$-coupled receptors, $GABA_B$ receptors appear to be efficiently linked to Ca^{2+} mobilization. Intracellular Ca^{2+} 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^{2+} is a key regulator of numerous physiological and pathophysiological events (Verkhratsky, 2005). Here, we report the ability of $GABA_B$ heterodimers in primary cultures of rat cortical neurons and in transfected Chinese hamster ovary (CHO) cells to promote Ca^{2+} entry into the cell via store-operated channels (SOCs). $GABA_B$ receptors activate SOCs via a pathway that utilizes a PTX-sensitive and phospholipase Cβ (PLCβ)-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 $GABA_{B1}$ and $GABA_{B2}$ cDNAs were kindly provided by Dr. Fiona Marshall (GlaxoSmithKline, Stevenage, UK). $GABA_{B1(b)}$ and $GABA_{B2}$ subunits were subcloned into pBudCE4.1 using *Not*I/*Kpn*I and *Hind*III/*Xba*I 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 $GABA_{B1}$ and $GABA_{B2}$ 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^2 culture flasks at a density of 3×10^5 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 $GABA_{B1(b)/2}$/CHO cell line, 10 μg of $GABA_{B1(b)}$ and $GABA_{B2}$ 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 $GABA_{B1(a)/2}$/CHO cell line, 10 μg of each of the $GABA_{B1(a)}$ cDNA subcloned into pcDNA3.1 (+) and the $GABA_{B2}$ 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_2. 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 ($GABA_{B1(b)/2}$/CHO) or 800 μg/ml G418 and 400 μg/ml zeocin ($GABA_{B1(a)/2}$/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 buffered 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 antiserum recognizing the C-terminal tail regions of $GABA_{B1}$ or $GABA_{B2}$ subunits and an enhanced chemiluminescence (ECL) kit.

Measurement of cAMP levels

Transfected cells were labeled with [^{3}H]adenine (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_{50} values are given as the mean±S.D. from four independent experiments.

Measurement of intracellular Ca^{2+} using a FLIPR device

CHO cells were seeded into 96-well, black-walled microtiter plates at a density of 4×10^4 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 aliquotted 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 monitored 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_{50} and IC_{50} values are given as the mean±S.D. from no less than three independent determinations.

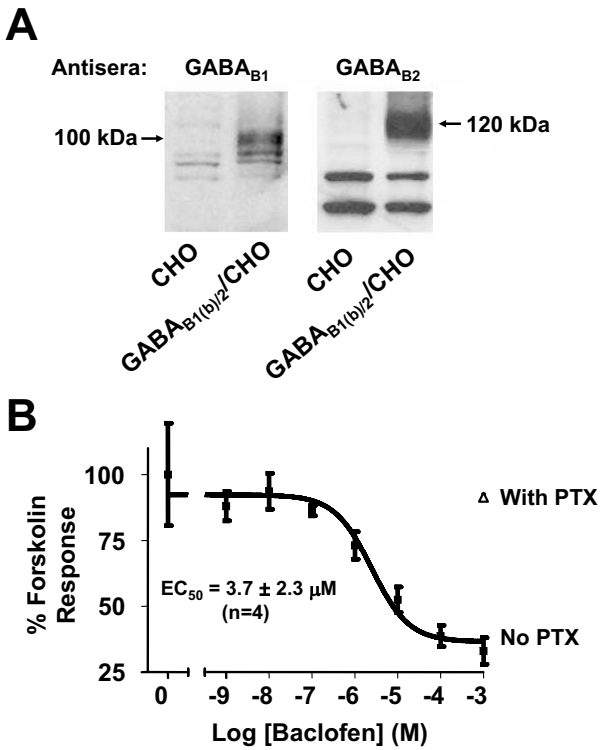

Fig. 1. Characterization of $GABA_B$ subunits stably expressed in CHO cells. (A) $GABA_{B1(b)/2}$/CHO cells were harvested and membrane proteins were prepared. Fifty micrograms of the membrane proteins were separated on a 12% SDS-PAGE gel and transferred to polyvinylidene fluoride membrane by electroblotting. Membranes were probed with antibodies raised against the C-terminal region of either $GABA_{B1}$ or $GABA_{B2}$ subunits. Fluorographs were visualized with an enhanced chemiluminescence detection kit. (B) $GABA_{B1(b)/2}$/CHO cells were labeled with [^{3}H]adenine in the absence (■) or presence (△) of PTX and assayed for cAMP accumulation in the presence of 50 μM forskolin and increasing concentrations of baclofen. [^{3}H]cAMP was measured as a fraction of the total tritiated adenine nucleotides per assay well. The normalized data are presented as the means±S.D. of data averaged across four independent experiments performed in triplicate.

RESULTS

Generation and characterization of CHO cells stably expressing $GABA_{B1(b)/2}$ subunits

In order to examine the signaling properties of the $GABA_B$ receptor, we have established a CHO cell line stably co-expressing the $GABA_{B1(b)}$ and $GABA_{B2}$ receptors using the bicistronic expression vector pBudCE4.1. Zeocin resistant clonal cells were isolated and tested for their ability to respond to $GABA_B$ receptor agonists by inhibiting the forskolin-stimulated intracellular cAMP levels. Several clones were identified that responded to 500 μM of the selective $GABA_B$ agonist (*RS*)-baclofen (to be referred to as baclofen) by inhibiting cAMP accumulation up to 50–60%, whereas parental CHO cells did not respond to baclofen (data not shown). Membrane preparations of the best responding clone were subjected to Western blotting analysis using antisera directed against C-terminal regions of $GABA_{B1}$ or $GABA_{B2}$ subunits. Antisera against $GABA_{B1}$ proteins identified a band of approximately 100 kDa that was not expressed in parental CHO cells (Fig. 1A). Antisera against $GABA_{B2}$ subunits labeled a diffuse band at approximately 128 kDa, whose expression was not detected in CHO cells (Fig. 1A). This is in agreement with previous data identifying $GABA_B$ subunit expression in rat brain and transfected HEK-293 cells (4).

The ability of these $GABA_{B1(b)/2}$/CHO cells to activate intracellular $G_{i/o}$-coupled pathways was assessed by the ability of the $GABA_B$ agonist baclofen to inhibit the forskolin-stimulated production of cAMP. When challenged with baclofen, the cAMP levels in forskolin-stimulated $GABA_{B1(b)/2}$/CHO cells were reduced by approximately 65% with an EC_{50} value of 3.7±2.3 μM (n=4; Fig. 1B). This is in good agreement with an EC_{50} value of 3.7 μM reported for $GABA_B$ receptor-mediated cAMP inhibition in HEK-293 cells and an EC_{50} 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 $G_{i/o}$ proteins.

$GABA_B$ receptors mediate the mobilization of intracellular Ca^{2+}

Previous reports have demonstrated that $GABA_B$ receptors coexpressed with chimeric G proteins allows these $G_{i/o}$-coupled receptors to promote intracellular increases in Ca^{2+} levels (Wood et al., 2000; Liu et al., 2003). As we have previously observed that several $G_{i/o}$-coupled receptors can promote Ca^{2+} mobilization in the absence of artificial chimeras (New and Wong, 2004), we examined the ability of $GABA_B$ receptors expressed in CHO cells to activate pathways that trigger increases in intracellular Ca^{2+} levels. $GABA_{B1(b)/2}$/CHO cells were challenged with

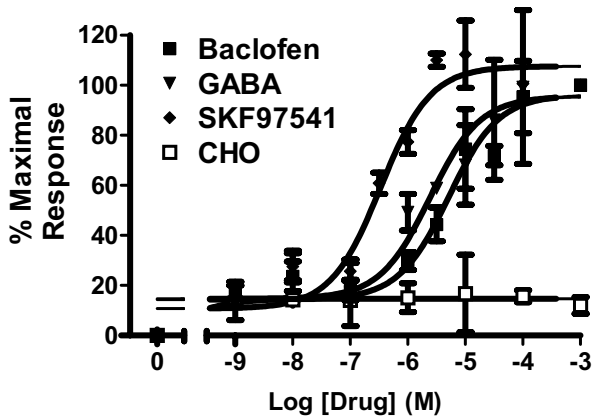

Fig. 2. Ca^{2+} mobilization in $GABA_{B1(b)/2}$/CHO cells challenged with $GABA_B$ receptor agonists. FLIPR assays were used to construct dose-response curves of $GABA_{B1(b)/2}$/CHO cell activation by three agonists, baclofen, GABA and SKF97541. The normalized data are presented as the means±S.D. of data averaged across three independent experiments performed in triplicate. The response of the parental CHO cell line to baclofen was also examined and responses normalized to the maximal baclofen-induced response in the stable cell line.

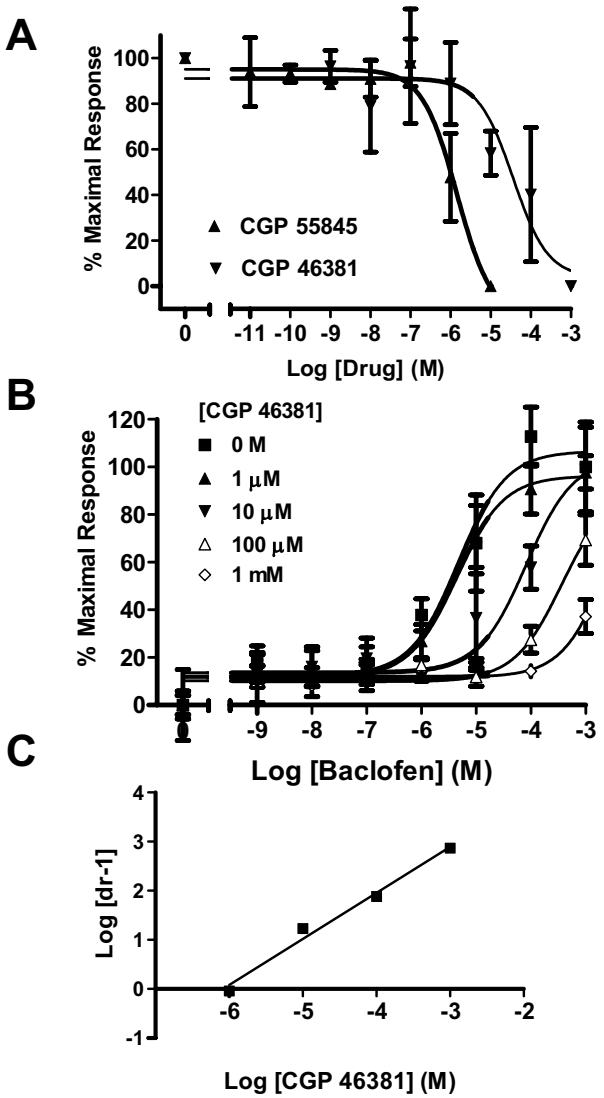

Fig. 3. Selective $GABA_B$ receptor antagonists inhibit agonist induction of Ca^{2+} mobilization in $GABA_{B1(b)/2}$/CHO cells. (A) The ability of 10 μM of baclofen to mobilize Ca^{2+} in $GABA_{B1(b)/2}$/CHO cells using FLIPR assays was determined in the presence of increasing concentrations of antagonists CGP55845 and CGP46381. 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 ability of increasing concentrations of CGP46381 to shift the dose-response curve of $GABA_{B1(b)/2}$/CHO cells to baclofen (1 μM to 1 mM) was determined (in triplicate and normalized to the response of the cells to 1 mM baclofen in the absence of antagonist) and C, was transformed into a Schild plot to determine the K_B of CGP46381.

baclofen and the Ca^{2+} levels monitored in FLIPR assays. $GABA_{B1(b)/2}$/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 $GABA_{B(1b)/2}$/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 $GABA_{B1(b)/2}$/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.

Gβγ subunits released from $G_{i/o}$ proteins are able to activate PLCβ (Rebecchi and Pentyala, 2000), potentially modulating Ca^{2+} release and we reasoned that such a pathway may be operational in $GABA_{B1(b)/2}$/CHO cells. Indeed, the PLCβ 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).

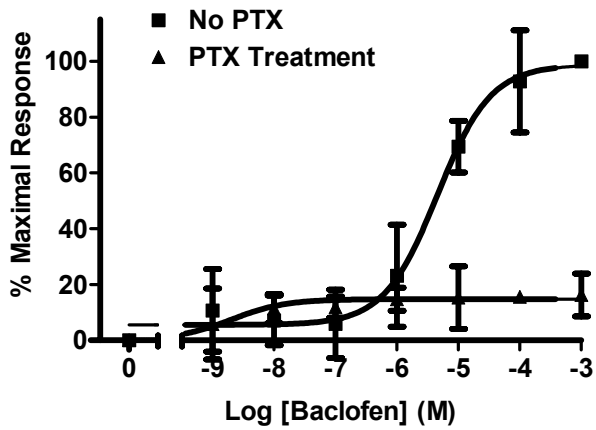

Fig. 4. Effect of PTX on the response of $GABA_{B1(b)/2}$/CHO cells to baclofen. $GABA_{B1(b)/2}$/CHO cells were seeded in 96-well plates 6 h before the addition of PTX (100 ng/ml) or vehicle to the cells. Dose-response curves were constructed using baclofen in the FLIPR platform. The normalized data are presented as the means±S.D. of data averaged across three independent experiments performed in triplicate.

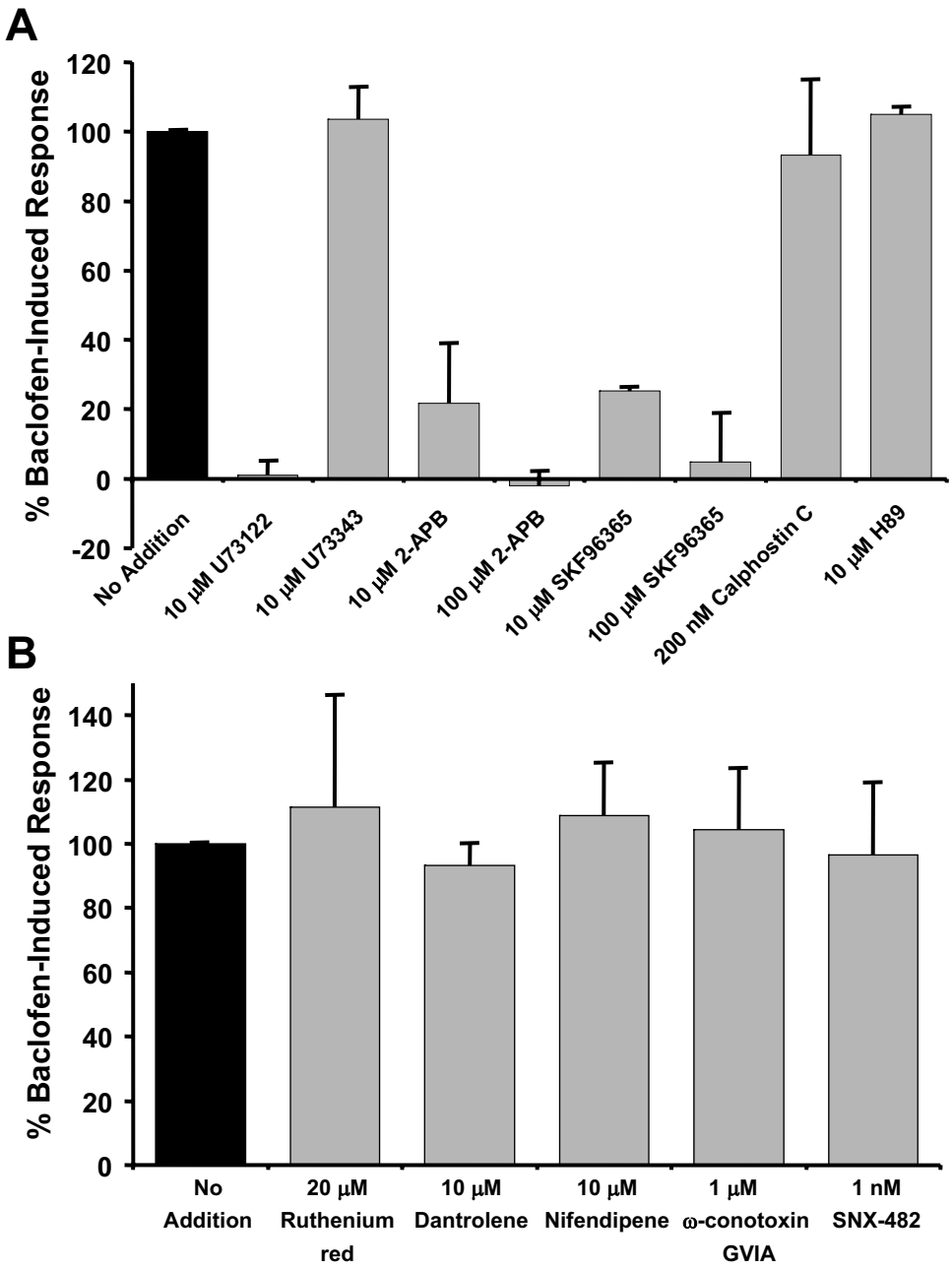

Fig. 5. Effect of pharmacological inhibitors on the response of $GABA_{B1(b)/2}$/CHO cells to 100 μM baclofen. FLIPR assays were used to examine the effect of the indicated inhibitors. U73122, U73343, 2-APB, calphostin C and H89 were added to the cell labeling medium 20 min before assay. SKF96365 and all inhibitors indicated in panel B were added 10 min in advance. 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.

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±9.2% (n=3; Fig. 5A). These results demonstrate that $GABA_B$ receptors induce increases in intracellular Ca^{2+} levels via a PLCβ-mediated pathway.

To identify the downstream effectors and the source of Ca^{2+}, $GABA_{B1(b)/2}$/CHO cells were challenged with baclofen following treatment with various pharmacological inhibitors. The IP_3 receptor antagonist 2-aminethoxydiphenyl borane (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±4.1%, 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-

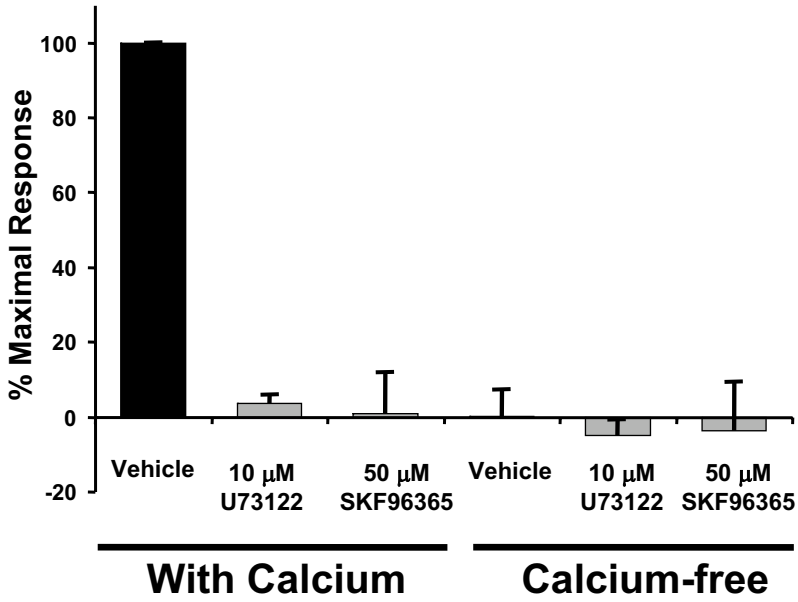

Fig. 6. Effect of extracellular Ca^{2+} on the $GABA_B$ receptor-induced intracellular Ca^{2+} levels. The response of $GABA_{B1(b)/2}$/CHO cells to baclofen (100 μM) in FLIPR assays was determined in the presence (with calcium) or absence (calcium-free) of Ca^{2+} in the extracellular labeling medium. The effect of U73122 and SKF96365 on baclofen-induced basal Ca^{2+} levels in both types of labeling medium was also determined. The normalized data are presented as the means±S.D. of data averaged across three independent experiments performed in duplicate or triplicate.

onizes IP_3 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-1*H*-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 Ca^{2+} measured within the cell was the result of influx from the extracellular medium. This was confirmed when the assays were repeated using Ca^{2+} free buffers. Under these assay conditions, baclofen was ineffective in promoting increases in intracellular Ca^{2+} levels even at a concentration of 1 mM (Fig. 6).

As the baclofen-induced Ca^{2+} fluxes were PLCβ dependent, we tested the ability of the selective protein kinase C (PKC) inhibitor calphostin C to inhibit increases in intracellular Ca^{2+} levels. At a concentration of 200 nM, calphostin C was unable to significantly affect Ca^{2+} mobilization, indicating that this kinase and its downstream effectors do not play a role in $GABA_B$ receptor-induced Ca^{2+} fluxes. As $GABA_B$ receptors have been shown to mediate cAMP production in some cell types (10), we also examined the role of protein kinase A (PKA) in $GABA_B$ receptor-mediated Ca^{2+} flux. The selective inhibitor H89 was completely ineffective in inhibiting the baclofen-induced responses (Fig. 5A).

Examining the role of ryanodine receptors and Ca^{2+} channels

We also examined the contribution of ryanodine receptors and VGCC-mediated Ca^{2+} fluxes to the $GABA_B$ receptor-induced intracellular Ca^{2+} 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 $GABA_{B1(b)/2}$/CHO cells to baclofen (Fig. 5B). Similarly, the VGCC blockers nifendipene (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 $GABA_B$ receptor-mediated responses (Fig. 5B).

$GABA_{B1(a)/2}$ receptors also promote store-operated Ca^{2+} entry

To determine the ability of $GABA_{B1(a)}$ containing heterodimeric $GABA_B$ receptors to activate Ca^{2+} influx via SOCs, we constructed and examined the signaling properties of $GABA_{B1(a)/2}$/CHO cells. As observed with $GABA_{B1(b)/2}$/CHO cells, baclofen promoted increases in intracellular Ca^{2+} levels, which were antagonized by CGP55845 (IC_{50}= 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 $GABA_{B1(b)/2}$ expressing cells (Fig. 7B). This indicates that $GABA_{B1(a)}$- and $GABA_{B1(b)}$-containing receptors are both able to promote Ca^{2+} influx via SOCs using a PTX-sensitive, PLCβ-dependent pathway.

Ca^{2+} influx through SOCs in primary cultures of rat cortical neurons

To determine whether $GABA_B$ receptors are able to promote Ca^{2+} influx in neuronal cells endogenously expressing $GABA_B$ 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 Ca^{2+} levels with an EC_{50} value of 3.7±4.4 μM ($n=4$; Fig. 8A). The

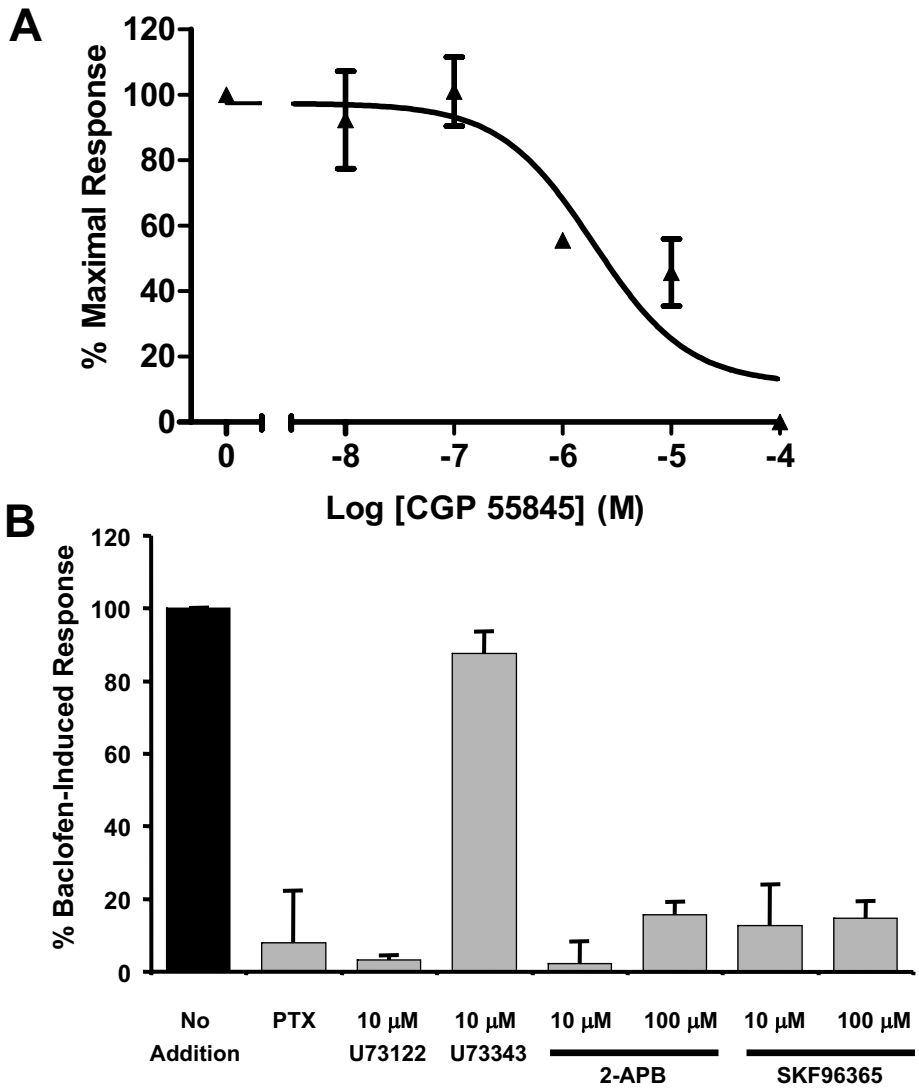

Fig. 7. Effect of $GABA_B$ antagonists and pharmacological inhibitors on the response of $GABA_{B1(a)/2}$/CHO cells to 100 μM baclofen. (A) A CHO cell line stably expressing $GABA_{B1(a)}$ and $GABA_{B2}$ receptor subunits was established. The effect of the selective $GABA_B$ receptor antagonist CGP55845 on 100 μM baclofen-induced Ca^{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^{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.

response induced by 1 mM baclofen was antagonized by CGP55845 with an IC_{50} value of 9.95±6.5 μM (n=3; Fig. 8B), confirming that $GABA_B$ 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, $G_{i/o}$ proteins in cortical neurons also transduce the Ca^{2+} mobilizing effects of $GABA_B$ receptors. The PLCβ inhibitor U73122 (10 μM) completely inhibited baclofen-induced responses, whereas U73343 was ineffective (Fig. 9). As observed in $GABA_{B1(b)/2}$/CHO cells, 2-APB dose-dependently inhibited agonist-induced responses with 100 μM completely blocking increases in Ca^{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 nifendipene, ω-conotoxin GVIA and SNX-482 were ineffective (data not shown). The accumulated data confirm that the PLCβ-mediated opening of SOCs by the stimulation of $GABA_B$ receptors endogenously expressed in cortical neurons operates in a manner similar to $GABA_B$ receptors exogenously expressed in CHO cells.

DISCUSSION

$GABA_B$ receptors are typical G_i-coupled receptors, which can efficiently inhibit adenylyl cyclase (Bowery et al.,

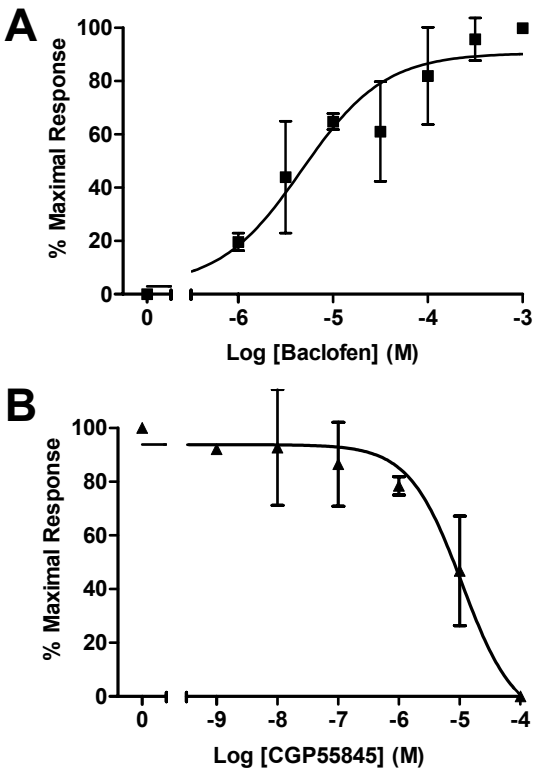

Fig. 8. Ca^{2+} mobilization in primary cultures of rat cortical neurons challenged with a $GABA_B$ receptor agonist and antagonized by CGP55845. (A) FLIPR assays were used to determine the response of primary rat cortical neurons challenged with increasing concentrations of baclofen. (B) Primary cultures of cortical neurons were simultaneously challenged with 1 mM baclofen and increasing concentrations of CGP55845. For both panels, the normalized data are presented as the means±S.D. of data averaged across three independent experiments performed in duplicate or triplicate.

2002), but generally thought to require the expression of $G\alpha_{q/i}$ chimeras to activate PLCβ and mobilize intracellular Ca^{2+} (Wood et al., 2000; Liu et al., 2003). Although most G_i-coupled receptors are unable to mobilize intracellular Ca^{2+} in the absence of $G\alpha_{q/i}$ chimeras, a number of G_i-linked receptors appear to possess an inherent ability to elevate 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 μ-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 $GABA_{B1(a)/2}$/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 $GABA_{B1}$ and $GABA_{B2}$ 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 PLCβ (Figs. 5A, 7B and 9). To date there are no known instances of $G\alpha_{i/o}$ proteins directly activating PLCβ and it has been demonstrated that constitutively active mutants of $G\alpha_{i/o}$ subunits do not promote PLCβ activity (Tsu et al., 1995). However, activation of PLCβ by Gβγ 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 Gβγ dimers that activate PLCβ, which in turn triggers downstream events leading to an increase in intracellular levels of Ca^{2+}. $GABA_B$ receptor-mediated release of Gβγ subunits from $G_{i/o}$ proteins has previously been shown to result in increased adenylyl 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 PLCβ (Xiang et al., 2002).

Activation of PLCβ 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 (Bootman et al., 2002). However, 2-APB has also been shown to be equally effective at inhibiting the Ca^{2+} influx from the extracellular medium (Bootman 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, PLCβ-medi-

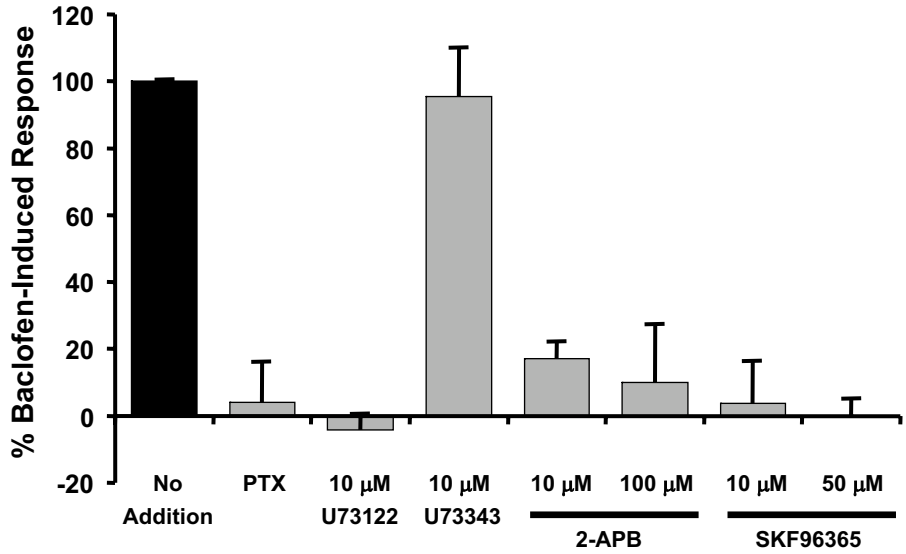

Fig. 9. Effect of PTX and pharmacological inhibitors on the response of primary rat cortical neurons to 1 mM baclofen. For PTX (100 ng/ml), cells were treated for 16 h before assay using the FLIPR platform. The other inhibitors were applied at the indicated concentrations in advance of the assays (the 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.

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 PLCβ-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 PLCβ 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

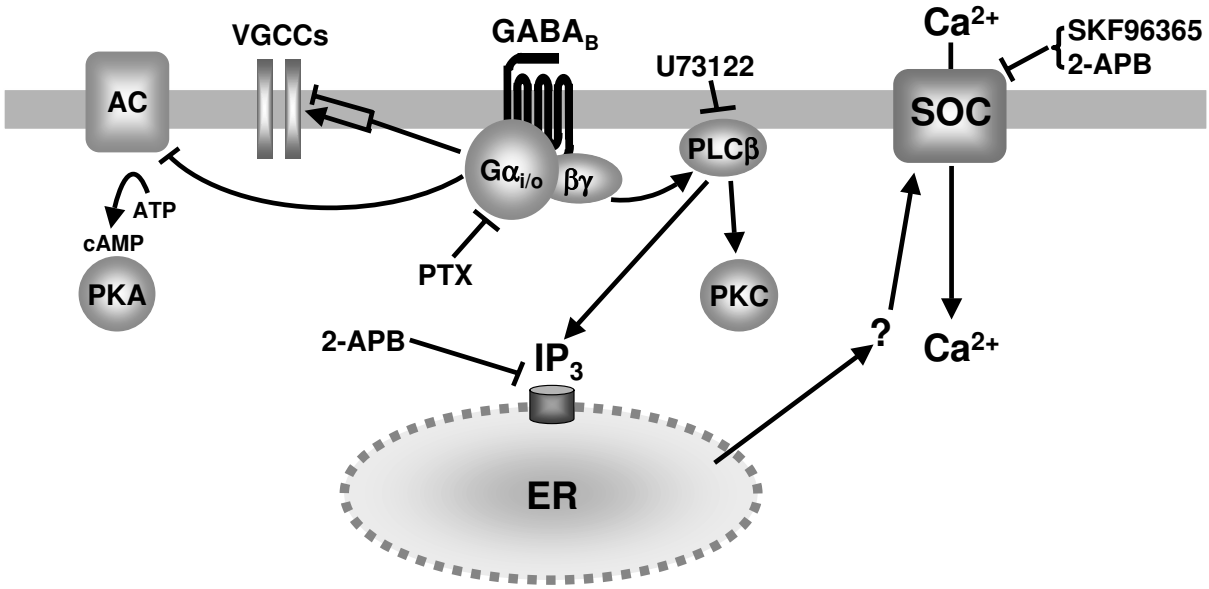

Fig. 10. Proposed mechanism for the activation of Ca^{2+} entry mediated by $GABA_B$ receptors. The data presented are illustrated schematically and indicate that agonist activation of $GABA_B$ receptors leads to an influx of Ca^{2+} from the extracellular medium. The inhibition of this phenomenon by 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 PLCβ, 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 $GABA_B$ 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 $GABA_B$ receptor-mediated capacitative calcium entry, although the lack of selective, membrane permeable IP_3 receptor antagonists has prevented us from demonstrating IP_3 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 is a property common to all G_q-coupled receptors as well as those $G_{i/o}$-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 Ca^{2+} influx into CHO cells via the activation of nonstore-operated Ca^{2+} 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 Ca^{2+} 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 $GABA_B$ receptors do not activate nonstore-operated Ca^{2+} channels.

$GABA_B$ receptors have previously been shown to suppress (Bowery et al., 2002) or facilitate Ca^{2+} influx via several sub-types of VGCCs, notably L-type channels (Parramon et al., 1995; Carter and Mynlieff, 2004). We observed no signs of $GABA_B$ receptor-mediated VGCC influx in transfected CHO cells or rat cortical neurons (Fig. 5B). Ryanodine receptors are activated by some $G_{i/o}$-coupled GPCRs (Maghazachi, 2000) in a PLCβ-independent manner (Putney et al., 2001), but we conclude that $GABA_B$ receptors do not activate this system when expressed in CHO cells or rat cortical neurons (Fig. 5B). However, our investigation measured Ca^{2+} levels on a whole cell scale, and we do not rule out the possibility of localized Ca^{2+} gradients controlled by VGCCs or ryanodine-sensitive stores.

It was apparent from the experiments in which Ca^{2+} influx was inhibited by SKF96365 or by the use of Ca^{2+} free buffers that we could not detect Ca^{2+} release from intracellular stores, even though depletion of Ca^{2+} 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 IP_3 receptor-induced release of Ca^{2+} from intracellular stores following activation of $GABA_B$ receptors is on a small scale. Alternatively, it is possible that Ca^{2+} release from intracellular stores is not a prerequisite for SOC activation. Consistent with these findings are observations that in skeletal muscle cells, IP_3 receptors mediate SOC opening without necessarily triggering release from internal Ca^{2+} stores (Launikonis et al., 2003). We are currently investigating the relative contributions of extracellular Ca^{2+} influx and intracellular Ca^{2+} mobilization upon activation of PLCβ-dependent pathways by other $G_{i/o}$- and G_q-coupled receptors.

$GABA_B$ receptor involvement has been demonstrated to regulate a variety of neuronal functions both directly and indirectly, by modulating the activity of other neurotransmitter systems. $GABA_B$ 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 $GABA_B$ receptors in the thalamus are excitatory, with such activity contributing to absence epilepsy (Calver et al., 2002). Postsynaptically, $GABA_B$ receptor activity can indirectly affect neuronal function by, for example, promoting the phosphorylation and inhibition of $GABA_A$ 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, $GABA_B$ receptors are likely to play an inhibitory role in synaptic plasticity and long-term potentiation. Presynaptically, $GABA_B$ 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 $GABA_B$ receptor regulation of K^+ channels and VGCCs. However, in light of our findings that $GABA_B$ receptor activation in neuronal cells leads to increases in intracellular Ca^{2+} levels, we suggest that the role of SOCs in neurophysiology be considered.

It is currently unclear as to whether Ca^{2+} 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 $A\beta_{42}$ protein, a key component in the generation of plaques associated with the development of Alzheimer's disease (Yoo et al., 2000). We anticipate that $GABA_B$ receptor-mediated activation of SOCs contributes to a complex interplay of Ca^{2+} releasing and sequestering pathways that regulate the intracellular levels of Ca^{2+}. 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; Verkhratsky, 2005).

The broad expression profile of $GABA_B$ 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 $GABA_B$ receptors in FLIPR assays using a transient transfection system (Liu et al., 2003). We found weak but consistent Ca^{2+} increases in response to high doses of baclofen that were potentiated by the chimeric $G\alpha$ subunits 16z25 and 16z44. Our generation of CHO cells stably expressing $GABA_B$ 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 $GABA_B$ receptors. In conclusion, we have identified a $GABA_B$ receptor-activated signal transduction pathway in cortical neurons that is mediated by $G_{i/o}$ 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 the subcloning. This work was supported in part by the Hong Kong Jockey Club and grants from the Innovation and Technology Commission of Hong Kong (ITS/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

Baba A, Yasui T, Fujisawa S, Yamada RX, Yamada MK, Nishiyama N, Matsuki N, Ikegaya Y (2003) Activity-evoked capacitative Ca^{2+} entry: implications in synaptic plasticity. J Neurosci 23:7737–7741.

Berridge MJ (1993) Inositol trisphosphate and calcium signalling. Nature 361:315–325.

Bolser DC, Blythin DJ, Chapman RW, Egan RW, Hey JA, Rizzo C, Kuo SC, Kreutner W (1995) The pharmacology of SCH 50911: a novel, orally-active GABA-B receptor antagonist. J Pharmacol Exp Ther 274:1393–1398.

Bootman MD, Collins TJ, Mackenzie L, Roderick HL, Berridge MJ, Peppiatt CM (2002) 2-Aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca^{2+} entry but an inconsistent inhibitor of $InsP_3$-induced Ca^{2+} release. FASEB J 16:1145–1150.

Bowery NG, Hudson AL, Price GW (1987) $GABA_A$ and $GABA_B$ receptor site distribution in the rat central nervous system. Neuroscience 20:365–383.

Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, Raiteri M, Bonner TI, Enna SJ (2002) International Union of Pharmacology. XXXIII. Mammalian gamma-aminobutyric $acid_B$ receptors: structure and function. Pharmacol Rev 54:247–264.

Broad LM, Braun FJ, Lievremont JP, Bird GS, Kurosaki T, Putney JW Jr (2001) Role of the phospholipase C-inositol 1,4,5-trisphosphate pathway in calcium release-activated calcium current and capacitative calcium entry. J Biol Chem 276:15945–15952.

Brown JT, Gill CH, Farmer CE, Lanneau C, Randall AD, Pangalos MN, Collingridge GL, Davies CH (2003) Mechanisms contributing to the exacerbated epileptiform activity in hippocampal slices of $GABA_{B1}$ receptor subunit knockout mice. Epilepsy Res 57:121–136.

Calver AR, Davies CH, Pangalos M (2002) $GABA_B$ receptors: from monogamy to promiscuity. Neurosignals 11:299–314.

Carter TJ, Mynlieff M (2004) γ-Aminobutyric acid type B receptors facilitate L-type and attenuate N-type Ca^{2+} currents in isolated hippocampal neurons. J Neurosci Res 76:323–333.

Hahner L, McQuilkin S, Harris RA (1991) Cerebellar $GABA_B$ receptors modulate function of $GABA_A$ receptors. FASEB J 5:2466–2472.

Ho MK, Wong YH (2001) G_z signaling: emerging divergence from G_i signaling. Oncogene 20:1615–1625.

Ho MKC, New DC, Wong YH (2002) Co-expressions of different opioid receptor types differentially modulate their signaling via G_{16}. Neurosignals 11:115–122.

Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao WJ, Johnson M, Gunwaldsen C, Huang LY, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA, Gerald C (1998) $GABA_B$ receptors function as a heteromeric assembly of the subunits $GABA_B$R1 and $GABA_B$R2. Nature 396:674–679.

Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A, Bettler B (1998) $GABA_B$-receptor subtypes assemble into functional heteromeric complexes. Nature 396:683–687.

Kawanabe Y, Hashimoto N, Masaki T (2002) Ca^{2+} channels involved in endothelin-induced mitogenic response in carotid artery vascular smooth muscle cells. Am J Physiol 282:C330–C337.

Kerr DI, Ong J (1995) $GABA_B$ receptors. Pharmacol Ther 67:187–246.

Knight AR, Bowery NG (1996) The pharmacology of adenylyl cyclase modulation by $GABA_B$ receptors in rat brain slices. Neuropharmacology 35:703–712.

Larsson KP, Peltonen HM, Bart G, Louhiuori LM, Penttonen A, Antikainen M, Kukkonen JP, Akerman KEO (2005) Orexin-A-induced Ca^{2+} entry: evidence for involvement of TRPC channels and protein kinase C regulation. J Biol Chem 280:1771–1781.

Launikonis BS, Barnes M, Stephenson DG (2003) Identification of the coupling between skeletal muscle store-operated Ca^{2+} entry and the inositol trisphosphate receptor. Proc Natl Acad Sci USA 100: 2941–2944.

Liu AMF, Ho MKC, Wong CSS, Chan JHP, Pau AHM, Wong YH (2003) $G\alpha_{16/z}$ chimeras efficiently link a wide range of G protein-coupled receptors to calcium mobilization. J Biomol Screen 8:39–49.

Liu AMF, Wong YH (2005) μ-Opioid receptor-mediated phosphorylation of IκB kinase in human neuroblastoma SH-SY5Y cells. Neurosignals 14:136–142.

Maghazachi AA (2000) Intracellular signalling events at the leading edge of migrating cells. Int J Biochem Cell Biol 32:931–943.

Merritt JE, Armstrong WP, Benham CD, Hallam TJ, Jacob R, Jaxa-Chamiec A, Leigh BK, McCarthy SA, Moores KE, Rink TJ (1990) SKandF 96365, a novel inhibitor of receptor-mediated calcium entry. Biochem J 271:515–522.

Minke B, Cook B (2002) TRP channel proteins and signal transduction. Physiol Rev 82:429–472.

Morrisett RA, Mott DD, Lewis DV, Swartzwelder HS, Wilson WA (1991) $GABA_B$-receptor-mediated inhibition of the N-methyl-D-aspartate component of synaptic transmission in the rat hippocampus. J Neurosci 11:203–209.

Mott DD, Lewis DV (1994) The pharmacology and function of central $GABA_B$ receptors. Int Rev Neurobiol 36:97–223.

Nava F, Carta G, Bortolato M, Gessa GL (2001) γ-Hydroxybutyric acid and baclofen decrease extracellular acetylcholine levels in the hippocampus via $GABA_B$ receptors. Eur J Pharmacol 430:261–263.

New DC, Wong YH (2004) Characterization of CHO cell lines stably expressing a $G\alpha_{16/z}$ chimera for high throughput screening of GPCRs. Assay Drug Dev Tech 2:269–280.

Nunn C, Cervia D, Langenegger D, Tenaillon L, Bouhelal R, Hoyer D (2004) Comparison of functional profiles at human recombinant somatostatin sst2 receptor: simultaneous determination of intracellular Ca^{2+} and luciferase expression in CHO-K1 cells. Br J Pharmacol 142:150–160.

Odagaki Y, Koyama T (2001) Identification of Gα subtype(s) involved in γ-aminobutyric $acid_B$ receptor-mediated high-affinity guanosine triphosphatase activity in rat cerebral cortical membranes. Neurosci Lett 297:137–141.

Olpe HR, Steinmann MW, Ferrat T, Pozza MF, Greiner K, Brugger F, Froestl W, Mickel SJ, Bittiger H (1993) The actions of orally active $GABA_B$ receptor antagonists on GABAergic transmission in vivo and in vitro. Eur J Pharmacol 233:179–186.

Onali P, Olianas MC (2001) βγ-Mediated enhancement of corticotropin-releasing hormone-stimulated adenylyl cyclase activity by activation of gamma-aminobutyric acid$_B$ receptors in membranes of rat frontal cortex. Biochem Pharmacol 62:183–190.

Parekh AB, Putney JW Jr (2005) Store-operated calcium channels. Physiol Rev 85:757–810.

Parramon M, Gonzalez MP, Herrero MT, Oset-Gasque MJ (1995) GABA$_B$ receptors increase intracellular calcium concentrations in chromaffin cells through two different pathways: their role in catecholamine secretion. J Neurosci Res 41:65–72.

Pin JP, Kniazeff J, Binet V, Liu J, Maurel D, Galvez T, Duthey B, Havlickova M, Blahos J, Prezeau L, Rondard P (2004) Activation mechanism of the heterodimeric GABA$_B$ receptor. Biochem Pharmacol 68:1565–1572.

Putney JW Jr, Broad LM, Braun FJ, Lievremont JP, Bird GS (2001) Mechanisms of capacitative calcium entry. J Cell Sci 114:2223–2229.

Rebecchi MJ, Pentyala SN (2000) Structure, function, and control of phosphoinositide-specific phospholipase C. Physiol Rev 80:1291–1335.

Restituito S, Couve A, Bawagan H, Jourdain S, Pangalos MN, Calver AR, Freeman KB, Moss SJ (2005) Multiple motifs regulate the trafficking of GABA$_B$ receptors at distinct checkpoints within the secretory pathway. Mol Cell Neurosci 28:747–756.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Seabrook GR, Howson W, Lacey MG (1990) Electrophysiological characterization of potent agonists and antagonists at pre- and postsynaptic GABA$_B$ receptors on neurones in rat brain slices. Br J Pharmacol 101:949–957.

Smart D, Hirst RA, Hirota K, Grandy DK, Lambert DG (1997) The effects of recombinant rat μ-opioid receptor activation in CHO cells on phospholipase C, $[Ca^{2+}]_i$ and adenylyl cyclase. Br J Pharmacol 120:1165–1171.

Sugioka M, Zhou WL, Hofmann HD, Yamashita M (1999) Ca^{2+} mobilization and capacitative Ca^{2+} entry regulate DNA synthesis in cultured chick retinal neuroepithelial cells. Int J Dev Neurosci 17:163–172.

Tozzi A, Bengtson CP, Longone P, Carignani C, Fusco FR, Bernardi G, Mercuri NB (2003) Involvement of transient receptor potential-like channels in responses to mGluR-I activation in midbrain dopamine neurons. Eur J Neurosci 18:2133–2145.

Tsu RC, Lai HW, Allen RA, Wong YH (1995) Differential coupling of the formyl peptide receptor to adenylate cyclase and phospholipase C by the pertussis toxin-insensitive G_z protein. Biochem J 309: 331–339.

Venkatachalam K, van Rossum DB, Patterson RL, Ma HT, Gill DL (2002) The cellular and molecular basis of store-operated calcium entry. Nat Cell Biol 4:E263–E272.

Venkatachalam K, Zheng F, Gill DL (2003) Regulation of canonical transient receptor potential (TRPC) channel function by diacylglycerol and protein kinase C. J Biol Chem 278:29031–29040.

Verkhratsky A (2005) Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons. Physiol Rev 85:201–279.

White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM, Marshall FH (1998) Heterodimerization is required for the formation of a functional GABA$_B$ receptor. Nature 396:679–682.

Wood MD, Murkitt KL, Rice SQ, Testa T, Punia PK, Stammers M, Jenkins O, Elshourbagy NA, Shabon U, Taylor SJ, Gager TL, Minton J, Hirst WD, Price GW, Pangalos M (2000) The human GABA$_{B1b}$ and GABA$_{B2}$ heterodimeric recombinant receptor shows low sensitivity to phaclofen and saclofen. Br J Pharmacol 131: 1050–1054.

Xiang Y, Li Y, Zhang Z, Cui K, Wang S, Yuan XB, Wu CP, Poo MM, Duan S (2002) Nerve growth cone guidance mediated by G protein-coupled receptors. Nat Neurosci 5:843–848.

Yoo AS, Cheng I, Chung S, Grenfell TZ, Lee H, Pack-Chung E, Handler M, Shen J, Xia W, Tesco G, Saunders AJ, Ding K, Frosch MP, Tanzi RE, Kim TW (2000) Presenilin-mediated modulation of capacitative calcium entry. Neuron 27:561–572.

Zubov AI, Kaznacheeva EV, Nikolaev AV, Alexeenko VA, Kiselyov K, Muallem S, Mozhayeva GN (1999) Regulation of the miniature plasma membrane Ca^{2+} channel I_{min} by inositol 1,4,5-trisphosphate receptors. J Biol Chem 274:25983–25985.

(Accepted 18 October 2005)
(Available online 15 December 2005)