Br. J. Pharmacol. (1992), **105**, 893–898

Effectiveness of $GABA_B$ antagonists in inhibiting baclofen-induced reductions in cytosolic free Ca concentration in isolated melanotrophs of rat

I. Shibuya, [2]S. Kongsamut & [1]W.W. Douglas

Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut, 06510, U.S.A.

1 The purpose of the present experiments was to assess the activities of $GABA_B$ receptor antagonists in mammalian isolated melanotrophs.

2 Cytosolic free Ca concentration ($[Ca^{2+}]_i$) in rat melanotrophs in primary culture was monitored with the fluorescent probe, fura-2.

3 (−)-Baclofen lowered $[Ca^{2+}]_i$ in a concentration-dependent manner with an EC_{50} of 0.96 μM. The reduction in $[Ca^{2+}]_i$ produced by (−)-baclofen at a maximally effective concentration (100 μM) was similar to that produced by the classic transmitter inhibitory to melanotroph secretion, dopamine, at a corresponding concentration (100 nM), or by perifusion with a nominally Ca-free solution.

4 The $GABA_B$ receptor antagonists, 3-aminopropyl(diethoxymethyl)phosphinic acid (CGP 35348), 2-hydroxy saclofen, phaclofen and 4-amino-3-(5-methoxybenzo[b]furan-2-yl) butanoic acid (9H), had inhibitory effects on the reduction in $[Ca^{2+}]_i$ produced by (−)-baclofen (3 μM). Of the antagonists tested, CGP 35348 was the most potent with an IC_{50} of 60 μM, compared to 120 to 400 μM for the others. CGP 35348 acted competitively.

5 CGP 35348 alone had no effect on basal $[Ca^{2+}]_i$, or on the changes in $[Ca^{2+}]_i$ produced by dopamine (10 nM) or the specific $GABA_A$ receptor agonist, muscimol (10 μM).

6 The evidence indicates that of the antagonists tested, CGP 35348 offers the greatest promise for pharmacological analysis of the functional significance of the $GABA_B$ receptors in melanotrophs.

Keywords: Melanotroph; $GABA_B$ receptors; $GABA_B$ antagonists; CGP 35348; 2-hydroxy saclofen; phaclofen; baclofen; $GABA_A$ receptors; cytosolic calcium; fura-2

Introduction

Melanotrophs, present as a virtually homogeneous population in the intermediate lobe of the pituitary gland, differ from other adenohypophyseal cells in being directly innervated. The nerves, which course from the hypothalamus through the infundibular stalk to form synaptic-like contacts with the melanotrophs, exert a tonic inhibitory influence on melanotroph secretion which otherwise occurs at a high spontaneous rate. The classic inhibitory transmitter is dopamine (see Holzbauer & Racké, 1985), but γ-aminobutyric acid (GABA) has also been detected in the nerves by immunohistochemical methods (Oertel *et al.*, 1982; Vincent *et al.*, 1982; Sakaue *et al.*, 1988), seemingly as a co-stored transmitter (Stoeckel *et al.*, 1985; Vuillez *et al.*, 1987; Schimchowitsch *et al.*, 1991).

The physiological function of this GABA is not clear (see Kongsamut *et al.*, 1991) but *in vitro* experiments have shown that application of GABA to melanotrophs directly affects electrical activity (Taraskevich & Douglas, 1982; 1985), output of hormones (Tomiko *et al.*, 1983; Demeneix *et al.*, 1984) and the concentration of cytosolic free Ca ($[Ca^{2+}]_i$) (Nemeth *et al.*, 1988; Taraskevich & Douglas, 1990). Moreover, release of endogeneous GABA has been demonstrated, albeit indirectly, by a 'postsynaptic' effect recorded in melanotrophs upon stimulation of the pituitary stalk (MacVicar & Pittman, 1986; Williams *et al.*, 1989).

One factor that has hampered progress in understanding the physiological relevance of GABA in the control of melanotroph function is that receptors of both $GABA_A$ and $GABA_B$ type are present on the cells (see discussion), and although the former are readily blocked by bicuculline, the classic $GABA_A$ antagonist, it is only recently that a $GABA_B$ antagonist effective in melanotrophs has been found; this is CGP 34348, a novel compound previously shown to possess effective $GABA_B$ antagonist activity on a variety of central nervous system preparations (Olpe *et al.*, 1990). The effectiveness of this compound in melanotrophs was detected in experiments conducted on isolated neurointermediate lobes of the toad, *Xenopus laevis* (Shibuya *et al.*, 1991), a species with remarkably powerful, bicuculline-resistant inhibitory effects of GABA on melanotroph secretion (Verburg van Kemenade *et al.*, 1987). In this preparation, CGP 35348 possessed useful antagonist activity, which contrasted with the lack of activity of other $GABA_B$ antagonists, namely, phaclofen, 2-hydroxy saclofen (Kerr *et al.*, 1987; 1988) and 9H (Beattie *et al.*, 1989). Thus CGP 35348, but not the other compounds, blocked the intense secreto-inhibitory effects of the $GABA_B$ agonist, baclofen, and of GABA given in the presence of bicuculline (to eliminate any $GABA_A$ component). Moreover, this block was selective: responses to dopamine or to $GABA_A$-receptor activation were unimpaired (Shibuya *et al.*, 1991). Observations on rat neurointermediate lobes indicated that CGP 35348 was also effective in countering baclofen-induced inhibition of melanotroph secretion in a mammalian species (Shibuya *et al.*, 1991).

The purpose of the present experiments was to obtain evidence on the direct effects of various $GABA_B$ antagonists in mammalian melanotrophs to define more fully the actions of such drugs on these endocrine cells and thus their possible usefulness as pharmacological agents with which to analyse physiological function. We have used isolated melanotrophs of rat in primary culture, and have assessed the activity of CGP 35348 and several other $GABA_B$ antagonists in counter-

[1] Author for correspondence.
[2] Present address: Department of Biological Research, Hoechst Roussel Pharmaceuticals Inc., Somerville, NJ 08876, U.S.A.

ing $GABA_B$-induced reductions in $[Ca^{2+}]_i$ using the fluorescent Ca probe, fura-2. There are several indications that the inhibitory effects on melanotroph secretion of both dopamine (acting through D_2 receptors) and GABA (acting through $GABA_B$ receptors) are the consequence of such reductions, which have been interpreted as likely to arise from actions of the transmitters impeding Ca influx through voltage-dependent Ca channels of low threshold (Nemeth *et al.*, 1988; 1990; Taraskevich & Douglas, 1990). Furthermore, by studying the quenching effect of the Ca surrogate, Mn, on the fura-2 signal, we have obtained evidence for a brisk influx of Ca through channels of this type in isolated melanotrophs of rat in basal (unstimulated) conditions, and moreover, have shown that dopamine acting through D_2 receptors or GABA acting through $GABA_B$ receptors closes these channels as witnessed by arrest of quenching (Shibuya *et al.*, 1992). Our decision to assess the effects of the $GABA_B$ antagonists by measurements of $[Ca^{2+}]_i$ was influenced by two factors: firstly, this parameter is an early event following receptor occupancy; and secondly, it is more easily obtained and quantified than secretory responses.

Methods

Dissociation and culture of melanotrophs

Male Sprague-Dawley rats (weighing 200–350 g) were decapitated under ether anesthesia and neurointermediate lobes of the pituitary gland were isolated and incubated in standard solution containing (mM): NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 15 and glucose 11 with 0.1% bovine serum albumin, and pH adjusted to 7.4 with NaOH. Melanotrophs were dissociated and cultured for 1 to 4 days according to the procedures previously described (Tomiko *et al.*, 1984).

Fura-2 loading and cell perifusion

Culture medium was removed and the cells were washed with standard solution and then loaded, in the same solution, with acetoxymethyl esters of fura-2 (fura-2/AM, 1 μM) at 35°C for 1 h. The cells were then washed once with dye-free standard solution and kept at room temperature until used. A portion of the cell suspension amounting to about 1/30 of the cells obtained from one rat was transferred to a perifusion chamber, the bottom of which consisted of a glass coverslip. This was placed on the stage of an inverted microscope (Nikon, Diaphot) and perifusion with standard solution was begun 5 to 10 min later when the cells had settled and attached to the cover glass. The volume of the perifusion solution in the chamber was kept constant (about 130 μl) by adding and withdrawing the solution at the same rate (1.5 ml min^{-1}) with a roller pump (Rainin, Rabbit) and the temperature was maintained at 37°C by circulating water around the chamber. Different solutions were selected by tap and the half time for a complete change of the solution in the chamber was about 7 s.

Measurement of fluorescence

This was carried out on clumps of 5 to 30 cells by epi-illumination using optical filters and a dichroic mirror (Omega Optical, VT, U.S.A.). Light emitted from a 150 W Xenon lamp (Oriel, CT, U.S.A.) was introduced into the inverted microscope after passing through excitation filters (380 and 340 nm, with 10 nm band width) held in a filter wheel (Oriel, CT, U.S.A.). The excitation filters were alternated every 1.5 s electro-mechanically and a shutter (Vincent Assoc., NY, U.S.A.) placed just before the excitation filters was opened for about 50 ms after one or other of the filters came to a stop in the light path. The beam then was passed through an appropriate neutral density filter, and reflected by a dichroic mirror (reflecting wavelengths <400 nm) onto the melanotrophs in the perifusion chamber via an objective lens (CF Fluor 20×, Nikon). The fluorescence emitted by the cells traversed the dichroic mirror, an emission filter (540 nm with 40 nm band pass) and an iris diaphragm before it was introduced into a photomultiplier tube (Hamamatsu photonics, 1P21). The output voltage from the photomultiplier was digitized with a Labmaster DMA (Scientific Solutions, OH, U.S.A.). Control of the various components and aquisition of digitized data were accomplished by a computer programme developed by one of us (I.S.). The fluorescence intensities after background subtraction at 340 and at 380 nm (F340 and F380, respectively) were recorded together with the ratio of F340 over F380 (R), displayed on a monitor screen, stored on a hard disk, and plotted on an xy-plotter. $[Ca^{2+}]_i$ was calculated from R by use of the following equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times \beta,$$

where K_d is the dissociation constant for fura-2 (224 nM); R_{max} and R_{min} are the ratios for unbound and bound forms of the fura-2/Ca^{2+} complex, respectively; and β is the ratio between maximum and minimum fluorescence intensities of fura-2 at 380 nm excitation. R_{max} and R_{min} were estimated with the fluorescence intensities of fura-2 solution (5 μM) containing 10 mM $CaCl_2$ and 1 mM EDTA, respectively. Autofluorescence in melanotrophs was negligible ($<0.1\%$) compared with the fluorescence in the fura-2 loaded cells.

Drugs

The sources of the drugs used were as follows: 3-aminopropyl (diethoxymethyl)phosphinic acid (CGP 35348), phaclofen, (−)-baclofen, and (+)-baclofen (Dr H. Bittiger, Ciba-Geigy, Basel, Switzerland); 2-hydroxy saclofen (2OH saclofen; Research Biochemicals Inc., Natick, MA, U.S.A.), and 4-amino-3-(5-methoxybenzo[b]furan-2-yl) butanoic acid (9H; Dr P. Berthelot, Laboratoire de Pharmacie Chimique, Lille, France).

Results

(−)-Baclofen lowers $[Ca^{2+}]_i$ in a concentration-dependent manner

Repetitive exposure to the same concentration of (−)-baclofen given at 5 min intervals had similar effects on $[Ca^{2+}]_i$ (examples are seen in Figure 3). The fall in $[Ca^{2+}]_i$ induced by (−)-baclofen was concentration-dependent (Figure 1a) with threshold effects appearing between 100 nM and 1 μM, maximum effects between 10 and 100 μM, and EC_{50} around 1 μM. The fall in $[Ca^{2+}]_i$ began within a few seconds of introducing (−)-baclofen to the chamber and reached the maximum within 30 s. Upon removing (−)-baclofen, $[Ca^{2+}]_i$ rebounded rapidly and, with the exception of the lowest concentrations of the drug, consistently overshot the pre-existing levels. These spike-like overshoots in $[Ca^{2+}]_i$ subsided, in turn, to around the control levels in 3–5 min. The changes in $[Ca^{2+}]_i$ obtained with dopamine (Figure 1b) closely resembled those obtained with (−)-baclofen and, moreover, the amplitude of the reductions in $[Ca^{2+}]_i$ in response to maximally effective concentrations of the two drugs were also similar. Furthermore, the reductions in $[Ca^{2+}]_i$ obtained with maximally effective concentrations of the two drugs were comparable with those obtained during perifusion with a nominally Ca-free solution ($CaCl_2$ replaced with $MgCl_2$). The reintroduction of Ca caused an overshooting transient increase in $[Ca^{2+}]_i$ much like that seen after exposure to (−)-baclofen or dopamine (Figure 1). The (+)-isomer of baclofen, which lacks the $GABA_B$ agonistic activity (its relative potency to (−)-baclofen is 0.001, Hill & Bowery, 1981), was without effect on $[Ca^{2+}]_i$ at concentrations below 100 μM, although it caused a small fall in $[Ca^{2+}]_i$ at 1 mM (Figure 2). The con-

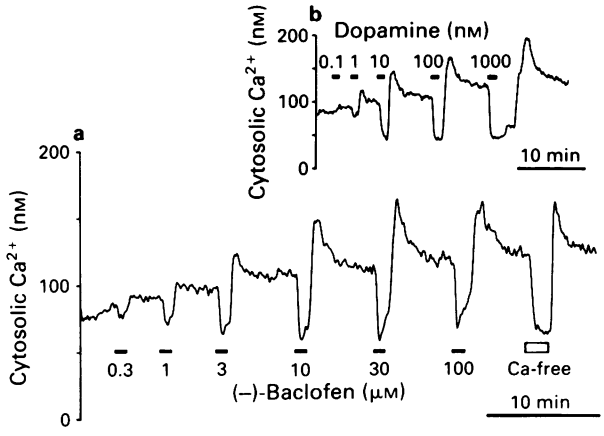

Figure 1 Representative traces of cytosolic free Ca concentration ($[Ca^{2+}]_i$) in isolated and perifused rat melanotrophs showing responses to increasing concentrations of (−)-baclofen (a) and dopamine (b), and for comparison (in a), exposure to a (nominally) Ca-free solution ($CaCl_2$ replaced with $MgCl_2$). The periods of exposure to the various solutions are indicated by the bars. Note that the concentration-dependence of the responses to (−)-baclofen and to dopamine and that maximally effective concentrations lower $[Ca^{2+}]_i$ to the same extent as is obtained with the Ca-free solution, and also the overshooting elevation in $[Ca^{2+}]_i$ on withdrawing the drugs or restoring Ca.

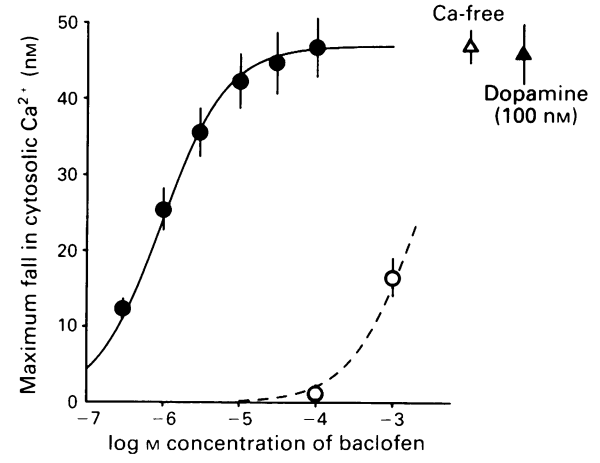

Figure 2 Concentration-response curve for (−)-baclofen (●), vertical lines show s.e.mean. Each value is the mean of the maximum reduction in $[Ca^{2+}]_i$ obtained in 5 to 8 tests; vertical lines show s.e.mean. The sigmoid curve shown for (−)-baclofen was obtained by the method of least squares. The EC_{50} of (−)-baclofen was 0.96 μM. For comparison are shown the values obtained with dopamine in a maximally effective concentration ($n = 7$) and with Ca-free solution ($n = 12$). Note that (+)-baclofen (○, $n = 5$) had little activity.

centration-response curve for (−)-baclofen yielded an EC_{50} of 0.94 μM.

Effects of CGP 35348 and other GABA$_B$ antagonists on the fall in $[Ca^{2+}]_i$ induced by (−)-baclofen

To assess the effects of the $GABA_B$ antagonists against responses to (−)-baclofen, the latter was used at a concentration of 3 μM which consistently produced a substantial yet submaximal fall in $[Ca^{2+}]_i$. Prior addition of CGP 35348 inhibited, in a concentration-dependent manner, the effects of (−)-baclofen, both the decrease and the overshoot in $[Ca^{2+}]_i$. The inhibitory effect, which was reversible, was evident at 30 μM CGP 35348 and complete at 300 μM (Figure 3a–c). By itself, CGP 35348 did not affect $[Ca^{2+}]_i$ at any concentration tested, the highest being 1 mM (data not shown). For comparison with CGP 35348, similar experiments were performed with several other $GABA_B$ antagonists. Among these, 2OH saclofen (300 μM), albeit less potent than CGP 35348, also substantially reduced the fall in $[Ca^{2+}]_i$ caused by (−)-baclofen (3 μM, Figure 3d). Both phaclofen (300 μM) and 9H (300 μM) were weaker antagonists and only partially inhibited the effect of (−)-baclofen (3 μM) (Figure 3e and f). We did not attempt to assess the baclofen-antagonist activity of 2OH saclofen or phaclofen at concentrations higher than 300 μM, since each of these drugs at higher concentrations (0.5 to 1 mM) itself affected basal levels of $[Ca^{2+}]_i$. The concentration-response curves for the several $GABA_B$ antagonists at different concentrations are presented in Figure 4. The IC_{50}

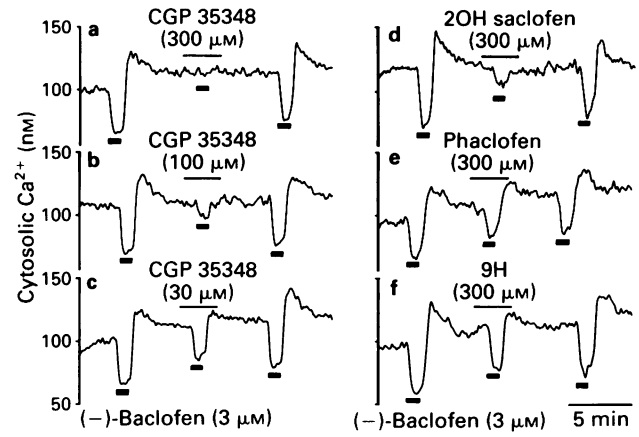

Figure 3 The effects, on baclofen-induced lowering of $[Ca^{2+}]_i$, of various $GABA_B$ antagonists (CGP 35348, a-c; 2OH saclofen, d; phaclofen, e; and 9H, f). All the substances tested had some inhibitory effect (which was in each instance reversible) but at the highest concentration tested (300 μM) only CGP 35348 blocked completely. The period of exposure to (−)-baclofen is indicated by the thick bars under each response and that to the antagonists by the thin bars above the response.

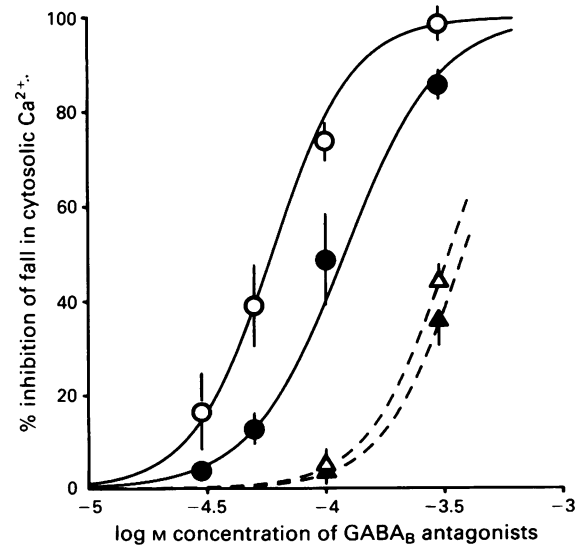

Figure 4 Concentration-response curves for the inhibitory effects of several $GABA_B$ antagonists on the fall in $[Ca^{2+}]_i$ induced by (−)-baclofen (3 μM). The %inhibition achieved with the antagonists was expressed as the value during exposure to baclofen plus antagonists as a % of the mean values obtained before and after with (−)-baclofen alone. Each value is the mean of 3 to 7 tests, vertical lines show s.e.mean. The curves were obtained by the method of least squares. IC_{50} for CGP 35348 (○), 60 μM; for 2OH saclofen (●), 120 μM; for phaclofen (△), 330 μM; and for 9H (▲), 380 μM.

for CGP 35348 was about 60 μM and that of 2OH saclofen about 120 μM. A Schild plot (Arunlakshana & Schild, 1959) of the results obtained with CGP 35348 at three different concentrations (50, 100 and 300 μM) against (−)-baclofen yields a pA_2 of 4.8 and a slope of 1.08 (Figure 5), the latter indicating that CGP 35348 is a competitive antagonist.

Selectivity of CGP 35348: lack of effect on changes in $[Ca^{2+}]_i$ elicited by muscimol or dopamine

CGP 35348 at a concentration (300 μM) which, as above noted, completely suppresses the effect of the $GABA_B$ agonist (−)-baclofen (3 μM) on $[Ca^{2+}]_i$, did not alter the corresponding effects of submaximal concentrations of the $GABA_A$ agonist muscimol (10 μM) or of dopamine (10 nM) (Figure 6). By contrast, the changes in $[Ca^{2+}]_i$ elicited by muscimol were abolished by the specific $GABA_A$ antagonist, bicuculline (bicuculline methiodide; BMI, 30 μM) and those elicited by dopamine were abolished by the specific D_2 antagonist, sulpiride (1 μM).

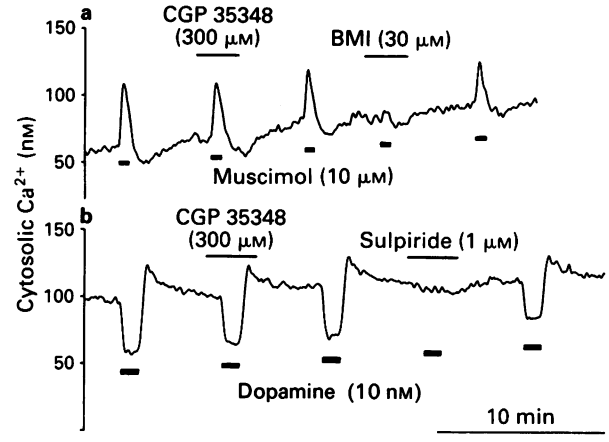

Figure 6 Indications of selectivity: CGP 35348 in a concentration sufficient to block the response to (−)-baclofen (3 μM) does not affect responses to (a) muscimol (10 μM) or (b) dopamine (10 nM). These responses are, however, blocked by the specific $GABA_A$ antagonsist, bicuculline methiodide (BMI; 30 μM) and by the selective D_2 receptor antagonist, sulpiride (1 μM), respectively. Similar results were obtained in three other experiments.

Discussion

GABA antagonists

The initial studies on the direct effects of GABA on secretion in isolated melanotrophs, performed on rat, revealed that GABA has a biphasic effect; eliciting first a sharp transient increase in secretion followed by a more persistent reduction (Tomiko *et al.*, 1983). The first phase was susceptible to block by the specific $GABA_A$ receptor antagonist, bicuculline, and was attributed to the $GABA_A$-mediated depolarization observed in similar cell preparations (Taraskevich & Douglas, 1982). The second phase was resistant to block by bicuculline (Tomiko, 1983; Demeneix *et al.*, 1984). The latter authors found that the $GABA_B$ agonist, baclofen could reduce melanotroph secretion and suggested that the bicuculline-resistant inhibitory effect of GABA was probably mediated by $GABA_B$ receptors. Support for this interpretation has been provided by the recent demonstration, on isolated neurointermediate lobes of toads and rats, that the inhibitory effects on melanotroph secretion of baclofen and of GABA given in the presence of bicuculline were both suppressed by the specific $GABA_B$ antagonist, CGP 35348 (Shibuya *et al.*, 1991). The present results show that CGP 35348 acts directly on the melanotrophs to inhibit the effect of baclofen to lower $[Ca^{2+}]_i$. Our evidence indicates that the antagonism is competitive, as reported for the effects of CGP 35348 in brain slices (Seabrook *et al.*, 1990). In the isolated melanotrophs of the rat, inhibitory activity, albeit weaker than that of CGP 35348, was also noted in 2OH saclofen, phaclofen and 9H. By contrast, no antagonist activity was noted with these three drugs in the neurointermediate lobes of the toad, the preparation on which these $GABA_B$ antagonists were previously compared (Shibuya *et al.*, 1991). This disparity may result from species differences. Alternatively, the lack of activity of the $GABA_B$ antagonists other than CGP 35348 in the toad lobes may reflect a poor ability to penetrate the tissue (cf. results on brain obtained by Olpe *et al.*, 1990 and Malcangio *et al.*, 1991).

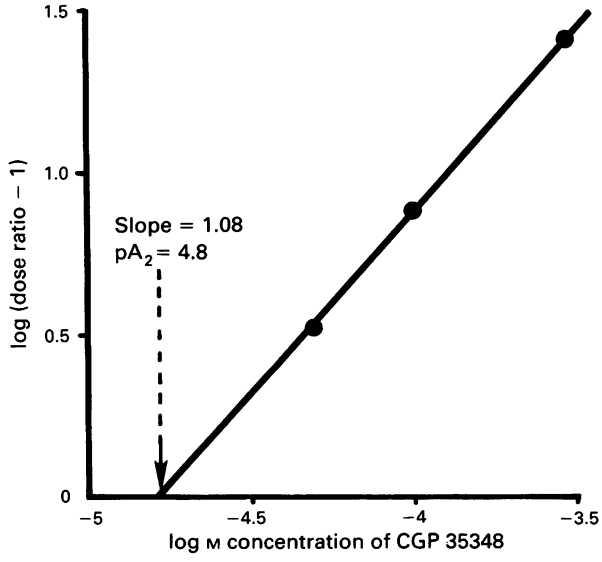

Figure 5 A Schild plot for CGP 35348 against (−)-baclofen. Dose-ratios were calculated from the EC_{50} values estimated from concentration-response curves for (−)-baclofen obtained in the absence of CGP 35348 (shown in Figure 2), or in the presence of CGP 35348 (50, 100 and 300 μM). pA_2 was estimated to be 4.8 from the x-intercept. The slope of 1.08 indicates that CGP 35348 is a competitive antagonist.

Efficacy of $GABA_B$ antagonists

The molar ratio of $GABA_B$ antagonist to agonist (baclofen) required for adequate block in the isolated melanotrophs was high: approximately 100: 1 for CGP 35348. This reinforces our previously stated view (Shibuya *et al.*, 1991a), based also on the need for a great excess of CGP 35348 to inhibit the responses to baclofen in the toad lobes, that the $GABA_B$ receptors in melanotrophs may differ from those in the central or peripheral nervous systems in which effective antagonism against baclofen has been obtained with much lower molar ratios; for example, 3: 1 for CGP 35348 in hippocampal CA1 pyramidal neurones (Olpe *et al.*, 1990); and 5: 1 for 2OH saclofen in cat spinal cord (Curtis *et al.*, 1988). Bowery (1989) has mentioned the possible existence of subtypes of the $GABA_B$ receptors. In any event, CGP 35348 is effective in melanotrophs and is the most potent of the $GABA_B$ antagonists we tested. This, along with its selectivity, rapid reversibility, and effectiveness on neurointermediate lobes, indicates that CGP 35348 is the most promising of the pharmacological antagonists with which to assess involvement of $GABA_B$ receptor activation in the physiological control of melanotrophs. Effectiveness in tissue preparations is an essential attribute of a pharmacological tool, and in this regard, it may be noted that Olpe *et al.* (1990) have empha-

sized that CGP 35348 readily penetrates the brain following systemic administration. Furthermore, Malcangio *et al.* (1991) found that CGP 35348 administered systemically antagonized baclofen-induced antinociception, whereas 2OH saclofen and phaclofen given by the same route were inactive.

In summary, the present experiments support the view that bicuculline-insensitive GABA receptors on melanotrophs belong to the class of $GABA_B$ receptors, although they are perhaps somewhat different from those described in neuronal tissues. They also show that of the $GABA_B$ antagonists tested, CGP 35348, which has by itself no effect on basal $[Ca^{2+}]_i$, is the most potent and is without effect on responses mediated through dopamine D_2 receptors or $GABA_A$ receptors. These properties, taken in conjunction with the previously demonstrated effectiveness of CGP 35348 as a $GABA_B$ antagonist in whole neurointermediate lobes, indicate that CGP 35348 may be a useful pharmacological tool with which to study the participation of $GABA_B$-mediated events in the physiological control of melanotroph function.

We thank Dr H. Bittiger (Ciba-Geigy, Basel) for generously providing CGP 35348, (−)-baclofen, (+)-baclofen and phaclofen; and Dr P. Berthelot (Laboratoire de Pharmacie Chimique, Lille) for 9H. This work was supported by a Javits Investigator Award (NS 09137) to W.W.D. and an NRSA postdoctoral fellowship (NS 07136) to S.K.

References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BEATTIE, D.T., CURTIS, D.R., DEBAERT, M., VACCHER, C. & BERTHELOT, P. (1989). Baclofen antagonism by 4-amino-3-(5-methoxybenzo[b]furan-2-yl)butanoic acid in the cat spinal cord. *Neurosci. Lett.*, **100**, 292–294.

BOWERY, N. (1989). $GABA_B$ receptors and their significance in mammalian pharmacology. *Trends Pharmacol. Sci.*, **10**, 401–407.

CURTIS, D.R., GYNTHER, B.D., BEATTIE, D.T., KERR, D.I.B. & PRAGER, R.H. (1988). Baclofen antagonism by 2-hydroxy saclofen in the cat spinal cord. *Neurosci. Lett.*, **92**, 97–101.

DEMENEIX, B.A., DESAULLES, E., FELTZ, P. & LOEFFLER, J.P. (1984). Dual population of $GABA_A$ and $GABA_B$ receptors in rat pars intermedia demonstrated by release of α-MSH caused by barium ions. *Br. J. Pharmacol.*, **82**, 183–190.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

HILL, D.R. & BOWERY, N.G. (1981). ^{3}H-baclofen and ^{3}H-GABA bind to bicuculline-insensitive $GABA_B$ sites in rat brain. *Nature*, **290**, 149–152.

HOLZBAUER, M. & RACKÉ, K. (1985). The dopaminergic innervation of the intermediate lobe and of the neural lobe of the pituitary gland. *Med. Biol.*, **63**, 97–116.

KERR, D.I.B., ONG, J., PRAGER, R.H., GYNTHER, B.D. & CURTIS, D.R. (1987). Phaclofen: a peripheral and central baclofen antagonist. *Brain Res.*, **405**, 150–154.

KERR, D.I.B., ONG, J., JOHNSTON, G.A.R., ABBENANTE, J. & PRAGER, R.H. (1988). 2-Hydroxy saclofen: an improved antagonist at central and peripheral $GABA_B$ receptors. *Neurosci. Lett.*, **92**, 92–96.

KONGSAMUT, S., SHIBUYA, I. & DOUGLAS, W.W. (1991). Why are several inhibitory transmitters present in the innervation of pituitary melanotrophs? Actions and interactions of dopamine, GABA and neuropeptide Y on secretion from neurointermediate lobes of *Xenopus laevis*. *Neuroendocrinol.*, **54**, 599–606.

MACVICAR, B.A. & PITTMAN, Q.J. (1986). Novel synaptic responses mediated by dopamine and γ-aminobutyric acid in neuroendocrine cells of the intermediate pituitary. *Neurosci. Lett.*, **64**, 35–40.

MALCANGIO, M., GHELARDINI, C., GIOTTI, A., MALMBERG-AIELLO, P. & BARTOLINI, A. (1991). CGP 35348, a new $GABA_B$ antagonist, prevents antinociception and muscle-relaxant effect induced by baclofen. *Br. J. Pharmacol.*, **103**, 1303–1308.

NEMETH, E.F., TARASKEVICH, P.S. & DOUGLAS, W.W. (1988). Intracellular free calcium in melanotrophs of rat pituitary pars intermedia and responses to diverse secretagogues and inhibitors. *J. Gen. Physiol.*, **92**, 9a.

NEMETH, E.F., TARASKEVICH, P.S. & DOUGLAS, W.W. (1990). Cytosolic Ca^{2+} in melanotrophs: Pharmacological insights into regulatory influences of electrical activity and ion channels. *Endocrinol.*, **126**, 754–758.

OERTEL, W.H., MUGNAINI, E., TAPPAZ, M.L., WEISE, V.K., DAHL, A.-L., SCHMECHEL, D.E. & KOPIN, I.J. (1982). Central GABAergic innervation of neurointermediate pituitary lobe: biochemical and immunocytochemical study in the rat. *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 675–679.

OLPE, H.-R., KARLSSON, G., POZZA, N.F., BRUGGER, F., STEINMANN, N., VAN RIESEN, H., FAGG, G., HALL, R.G., FRÖSTL, W. & BITTIGER, H. (1990). CGP 35348: a centrally active blocker of $GABA_B$ receptors. *Eur. J. Pharmacol.*, **187**, 27–38.

SAKAUE, M., SAITO, N., TANIGUCHI, H., BABA, S. & TANAKA, C. (1988). Immunohistochemical localization of γ-aminobutyric acid in the rat pituitary gland and related hypothalamic regions. *Brain Res.*, **446**, 343–353.

SCHIMCHOWITSCH, S., VUILLEZ, P., TAPPAZ, M.L., KLEIN, M.J. & STOECKEL, M.E. (1991). Systematic presence of GABA-immunoreactivity in the tubero-infundibular and tubero-hypophyseal dopaminergic axonal systems: an ultrastructural immunogold study on several mammals. *Exp. Brain Res.*, **83**, 575–586.

SEABROOK, G.R., HOWSON, W. & LACEY, M.G. (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.

SHIBUYA, I., KONGSAMUT, S. & DOUGLAS, W.W. (1991). Studies on pituitary melanotrophs reveal the novel $GABA_B$ antagonists CGP 35-348 to be the first such compound effective on endocrine cells. *Proc. R. Soc. B*, **243**, 129–137.

SHIBUYA, I., KONGSAMUT, S. & DOUGLAS, W.W. (1992). Importance of voltage-dependent Ca channels of low threshold in maintenance of basal cytosolic free Ca and its reduction by dopamine and baclofen: evidence from Mn quenching of fura-2 signals in isolated melanotrophs of rats. *J. Physiol. (Lond.)*, **446**, 310P.

STOECKEL, M.E., TAPPAZ, M.L., HINDELANG, C., SEWERYN, C. & PORTE, A. (1985). Opposite effects of monosodium glutamate on the dopaminergic and GABAergic innervations of the median eminence and the intermediate lobe in the mouse. *Neurosci. Lett.*, **56**, 249–255.

TARASKEVICH, P.S. & DOUGLAS, W.W. (1982). GABA directly affects electrophysiological properties of pituitary pars intermedia cells. *Nature*, **299**, 733–734.

TARASKEVICH, P.A. & DOUGLAS, W.W. (1985). Pharmacological and ionic features of γ-aminobutyric acid receptors influencing electrical properties of melanotrophs isolated from the rat pars intermedia. *Neurosci.*, **14**, 301–308.

TARASKEVICH, P.S. & DOUGLAS, W.W. (1990). Dopamine (D_2) or γ-aminobutyric acid ($GABA_B$) receptor activation hyperpolarizes rat melanotrophs and pertussis toxin blocks these responses and the accompanying fall in $[Ca]_i$. *Neurosci. Lett.*, **112**, 205–209.

TOMIKO, S.A. (1983). Studies on cellular events controlling secretion in melanotrophs of pituitary pars intermedia. *Ph.D. Thesis, Yale University*.

TOMIKO, S.A., TARASKEVICH, P.S. & DOUGLAS, W.W. (1983). GABA acts directly on cells of pituitary pars intermedia to alter hormone output. *Nature*, **301**, 706–77.

TOMIKO, S.A., TARASKEVICH, P.S. & DOUGLAS, W.W. (1984). Effects of veratridine, tetrodotoxin and other drugs that alter electrical behaviour on secretion of melanocyte-stimulating hormone from melanotrophs of the pituitary pars intermedia. *Neurosci.*, **12**, 1223–1228.

VERBURG VAN KEMENADE, B.M.L., JENKS, B.G., LENSSEN, F.J.A. & VAUDRY, H. (1987). Characterization of GABA receptors in the neurointermediate lobe of the amphibian *Xenopus laevis*. *Endocrinol.*, **120**, 622–628.

VINCENT, S.R., HÖKFELT, T. & WU, J.-Y. (1982). GABA neuron systems in hypothalamus and the pituitary gland: immunohistochemical demonstration using antibodies against glutamate decarboxylase. *Neuroendocrinol.*, **34**, 117–125.

VUILLEZ, P., PÉREZ, S.C. & STOECKEL, M.E. (1987). Colocalization of GABA and tyrosine hydroxylase immunoreactivities in the axons innervating the neurointermediate lobe of the rat pituitary: an ultrastructural immunogold study. *Neurosci. Lett.*, **79**, 53–58.

WILLIAMS, P.J., MACVICAR, B.A. & PITTMAN, Q.J. (1989). Identification of a GABA-activated chloride-mediated synaptic potential in rat pars intermedia. *Brain Res.*, **483**, 130–134.

(Received November 12, 1991
Revised December 10, 1991
Accepted December 18, 1991)