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A patch clamp study of γ-aminobutryic acid (GABA)-induced macroscopic currents in rat melanotrophs in cell culture

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1 The macroscopic currents induced in cultured rat melanotrophs by exogenous γ-aminobutyric acid (GABA) were analysed using the patch clamp recording technique.

2 Using various concentrations of intra- and extracellular chloride it was demonstrated that the conductance activated by GABA was chloride selective. Since these currents were blocked with bicuculline and enhanced with chlordiazepoxide the involvement of $GABA_A$ receptors similar to those in the CNS is indicated.

3 When chloride was symmetrically distributed across the membrane the voltage/current relationship was linear; pronounced rectification of GABA mediated currents was evident when there was an asymmetrical distribution of chloride.

4 With concentrations of GABA greater than 10 μM a fading of the current was seen during prolonged (5–10 s) applications. This effect appeared to be due to a decline of conductance rather than a shift of the chloride equilibrium potential.

5 Values for the Hill coefficient derived from dose-response curves suggested that the binding of 2 molecules of GABA to the receptor is required for the activation of the chloride channel.

6 There was no indication of a direct, $GABA_B$ receptor-mediated change of conductance.

Introduction

The pars intermedia (PI) of the rat contains a homogeneous population of hormone secreting cells known as melanotrophs whose secretory products include α-melanocyte stimulating hormone (α-MSH), corticotropin-like intermediate lobe polypeptide, β-endorphin and β-lipotropin hormone (Eberle, 1981). The induction of skin darkening in amphibians is a well-known biological effect of α-MSH. However, the physiological role of this and other PI hormones in mammals is not clear (Howe, 1973). Nonetheless these cells provide a useful model for examining many aspects of the control of pituitary function by the central nervous system (CNS). In the case of the PI this regulation by the CNS comprises inhibitory and, to a lesser extent, stimulatory inputs in the form of direct synaptic contacts between melanotrophs and nerve terminals releasing neurotransmitters including acetylcholine, dopamine, 5-hydroxytryptamine and γ-aminobutyric acid (GABA). The focus of this study is on the electrophysiological actions of the inhibitory transmitter GABA in melanotrophs maintained *in vitro*.

The neurochemical evidence for a role of GABA in the regulation of the secretory activity of melanotrophs includes: (1) immunoreactive glutamic acid decarboxylase (GAD), a selective marker for GABAergic neurones, has been detected in synapse-like junctions around secretory cells of the rat PI; (2) mechanical or electrolytic lesioning of the pituitary stalk abolishes GAD immunoreactivity indicating that it arises, not from local GABAergic neurones, but from descending pathways originating, most likely, in the hypothalamus (Oertel *et al.*, 1982; Vincent *et al.*, 1982); (3) Anderson & Mitchell (1986) have demonstrated the presence of GABA binding, uptake, and calcium-dependent release in the rat neurointermediate lobe; and, (4) GABA has been shown briefly to stimulate and then inhibit the release of α-MSH from dispersed melanotrophs (Tomiko *et al.*, 1983). From the observation that the barium-stimulated release of α-MSH is selectively enhanced by isoguvacine, a $GABA_A$ receptor agonist, and reduced

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by baclofen, a $GABA_B$ receptor agonist (Demeneix et al., 1984; 1986) it has been inferred that the stimulatory and inhibitory actions of GABA itself reflect the consequences of $GABA_A$ and $GABA_B$ receptor occupation, respectively.

The changes of electrical activity of melanotrophs caused by exogenous GABA were first described in dissociated cell cultures by Douglas and his co-workers (Taraskevich & Douglas, 1985). Using intracellular current-clamp methods, this group showed that GABA caused a depolarization of cultured melanotrophs, an effect which was associated with a dramatic decline of the membrane input resistance. MacVicar & Pittman (1986) have demonstrated recently that stimulation of the pituitary stalk in the acutely excised gland evokes, in melanotrophs, an inhibitory postsynaptic potential (i.p.s.p.) which by its ionic pharmacological properties appeared to be mediated by GABA.

We describe here the results of experiments in which the currents induced by exogenous GABA in cultured melanotrophs were characterized in terms of some of their biophysical and pharmacological properties. Some of these data have been presented in abstract form (Kehl & McBurney, 1986).

Methods

Dispersion and culture of cells

For a typical cell culture preparation, whole pituitary glands were obtained from 6 adult rats of either sex. The excised glands were rinsed in sterile, nominally calcium-and magnesium-free phosphate buffered saline (CMF-PBS) containing 4% bovine serum albumin (BSA), 150 u ml^{-1} penicillin and 150 $\mu g\ ml^{-1}$ streptomycin. All subsequent work was carried out in a sterile cabinet using an aseptic technique. Under a stereomicroscope the neurointermediate lobe, comprised of the closely-apposed PI and the pars nervosa, was separated from the pars distalis. To facilitate the movement of tissue through narrow-tipped plastic pipettes each of the neurointermediate lobes was then bisected using crossed microknives. The protocol for the subsequent enzymatic treatment of the tissue consisted of, first, a 50 min incubation in 1 ml of CMF-PBS containing 4% BSA, 0.1% hyaluronidase (Sigma, Type II), 5 $\mu g\ ml^{-1}$ deoxyribonuclease (Sigma) and 0.125% collagenase (Sigma, Type IA) and, second, a 20 min incubation in 1 ml of CMF-PBS containing 5 $\mu g\ ml^{-1}$ deoxyribonuclease and 0.1% protease (Sigma, Type IX). Both courses of enzyme treatment were done at 37°C on the stage of an orbital shaker. At the end of the incubation in the proteolytic enzyme a 300 μl aliquot of foetal calf serum was added to the reaction vial and the tissue fragments were then washed in three 1 ml volumes of culture medium (see below for composition). Subsequently, the cells were mechanically dispersed in CMF-PBS containing 5 $\mu g\ ml^{-1}$ deoxyribonuclease by drawing the tissue through needles of a successively smaller diameter (19-21-23-25-26 gauge). The cell suspension was then transferred to a microcentrifuge tube containing 800 μl of CMF-PBS in 4% BSA and spun for 8 min at 50 *g*. After resuspension in the 500 μl of culture medium a cell count was obtained using a hemocytometer. Yields were in the range of 80,000 to 100,000 cells per rat and cell viability was greater than 90% based on the exclusion of the supravital dye erythrocin B. The cell suspension was then further diluted in culture medium sufficient to allow the addition of a 100 μl aliquot containing 20,000–25,000 cells to each of the 24 wells in a multidish.

In preliminary experiments cells were plated on to a collagen-coated plastic (Aclar, Allied Corporation) coverslip (100 mm^2) placed in the bottom of each well. Attachment of melanotrophs to this substrate occurred slowly over a 4–7 day period and seemed to be dependent on the prior adhesion and proliferation of support cells. This meant that the culture medium could not be changed for up to 1 week after plating nor could the cells be used in experiments since unattached cells would drift in the perfusing medium. Consequently in most cases the cells were plated on to an astrocyte monolayer grown on collagen-coated cover slips in the preceding week. The rat cortical astrocytes were prepared using a modified version of the technique described by McCarthy & De Vellis (1980). Attachment to the astrocyte monolayer occurred within 1–2 h of plating and the cultures could therefore be used in experiments as early as the following day.

The culture medium (500 μl per well) which contained no antibiotics was changed every fourth day and consisted of Alpha-Modified Eagle's Medium (MEM): Ham's F-12: Dulbecco's MEM in the ratio of 1:6:3. It was supplemented with 1% chick embryo extract, 2 mM glutamine, 10% foetal calf serum, 1 μM cytosine arabinoside (to inhibit proliferation of support cells), 25 mM hydroxyethylpiperazine ethanesulphonic acid (HEPES) and 0.13 mM phenol red. Cells were maintained for up to 40 days *in vitro* in a 37°C incubator with a humified atmosphere comprising 5% CO_2 in air. Under these conditions the pH of the incubation medium varied between 7.0 and 7.3.

Recording technique

Experiments were done at 18–22°C in a perspex chamber (see Figure 1 of McBurney & Neering, 1985) mounted on the stage on an inverted phase contrast microscope. In the recording well of the chamber a coverslip to which cells had attached was constantly perfused with medium at a rate of 0.5–1.0 ml min^{-1}.

Melanotrophs could be easily distinguished from support cells by their distinctive morphological features which included: brightness under phase contrast microscopy; a rounded, often spherical shape; and a conspicuous nucleus. The typical electrophysiological characteristics of cells having this appearance were: a resting membrane potential between −50 mV and −80 mV; the spontaneous or current-evoked discharge of tetrodotoxin-sensitive overshooting action potentials; a high input resistance (1−10 GΩ); and voltage-dependent currents carried by sodium, calcium or potassium. These features are the same as those reported as occurring in identified melanotrophs (Douglas & Tarashekvich, 1978). Although cells of this type often existed in clusters of up to 10, the data of this study were obtained from isolated cells.

Whole cell recordings were made using conventional procedures (Hamill *et al.*, 1981). Briefly, macroscopic currents were measure on a List EPC-5 or EPC-7 patch clamp amplifier incorporating a 500 MΩ feedback resistor in the current-to-voltage converter. A typical patch electrode pulled from borosilicate glass (Clark Electromedical, GC120F-15) had a tip diameter of approximately 2 μm and a resistance of 5 MΩ. In an experiment, after immersing the Sylgard (Dow)-coated electrode tip in the bath, the zero-current voltage was adjusted and all command potentials were subsequently referenced to this level. There was no series resistance compensation. During off-line analysis, command voltages were corrected for junction potentials arising from the use of intra- and extracellular solutions of different compositions (Fenwick *et al.*, 1982). Following the formation of a tight seal (> 5 GΩ) the membrane in the tip of the electrode was ruptured by the brief application of suction to obtain a whole cell recording. The success of this manoeuvre was indicated by the sudden appearance of capacitive currents at the onset and offset of small hyperpolarising voltage commands. Signals were recorded on magnetic tape (Racal 4DS; d.c. − 2.5 KHz) and analysed off-line either directly from the oscilloscope screen (Gould 4020) or from chart records of low-speed screen dumps from the same oscilloscope. Traces for figures were obtained by plotting digitised (2048 pts/record) current and voltage responses.

The composition of the standard extracellular solution (ES1) was (in mM): NaCl 140, KCl 3.5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 5, HEPES 10, adjusted to pH 7.2, with NaOH. In some experiments designed to assess the ionic dependency of GABA-induced currents a modified extracellular solution (ES2) was made by the equimolar substitution of 89.5 mM sodium isethionate for NaCl. Drug solutions were prepared by the appropriate dilution, in ES1 or ES2, of frozen aliquots of distilled water solutions of GABA (10 mM), (±)-baclofen (Ciba-Geigy; 10 mM), glycine (20 mM), bicuculline methiodide (10 mM), and chlordiazepoxide HCl (Roche; 2 mM). Unless indicated otherwise chemicals were obtained from Sigma. Pipette solutions (see Table 1) were stored at 4°C and passed through a filter (0.2 μm pore size) before use. Since it is widely accepted that in the whole cell configuration the pipette solution rapidly equilibrates with the cell cytoplasm (see, for example, Figure 8 of Fenwick *et al.*, 1982) the term of intracellular solution (IS) shall be assumed to be synonymous with pipette solution.

Table 1 The composition of pipette solutions used for whole cell recordings

	IS1	*IS2*	*IS3*	*IS4*
CsCl	100	—	—	25
KCl	—	100	—	—
Cs_2SO_4	—	—	50	37.5
TEA-Cl	20	20	—	5
TEA-OH	—	—	20	15
$MgCl_2$	5	5	5	5
HEPES	20	20	20	20
EGTA	10	10	10	10
Sucrose	—	—	50	37.5
$[Cl^-]$	130	130	10	40

Concentrations shown in mM; the pH was adjusted to 7.2 with NaOH. The measured osmolarity of the intracellular solution was always 5−10% hypo-osmotic with respect to the extracellular solution; pCa is less than 8 with this concentration of EGTA.

Except where indicated, a U-tube tool of a construction similar to that described by Fenwick *et al.* (1982) was used to apply drugs. A multi-way valve was used to select one of up to 6 test solutions and suction was provided by a perfusion pump positioned on the outlet side of the U-tube. The duration and frequency of solenoid valve closures, which caused the efflux of solutions from the 100−200 μm diameter hole in the bend of the U-tube, were under TTL control (Digitimer D4030). To allow visual monitoring of its operation, the sodium salt of phenol red (Sigma; tissue culture grade; 0.7 mM) was included in all solutions applied with the U-tube; the application of ES1 containing phenol red had no effect on membrane currents.

Where applicable, values are expressed as the mean ± the standard deviation (s.d.) of the mean.

Results

All of the cells tested responded to GABA and, although not studied systematically, the wide variation of reponsivenesss to GABA such as that demon-

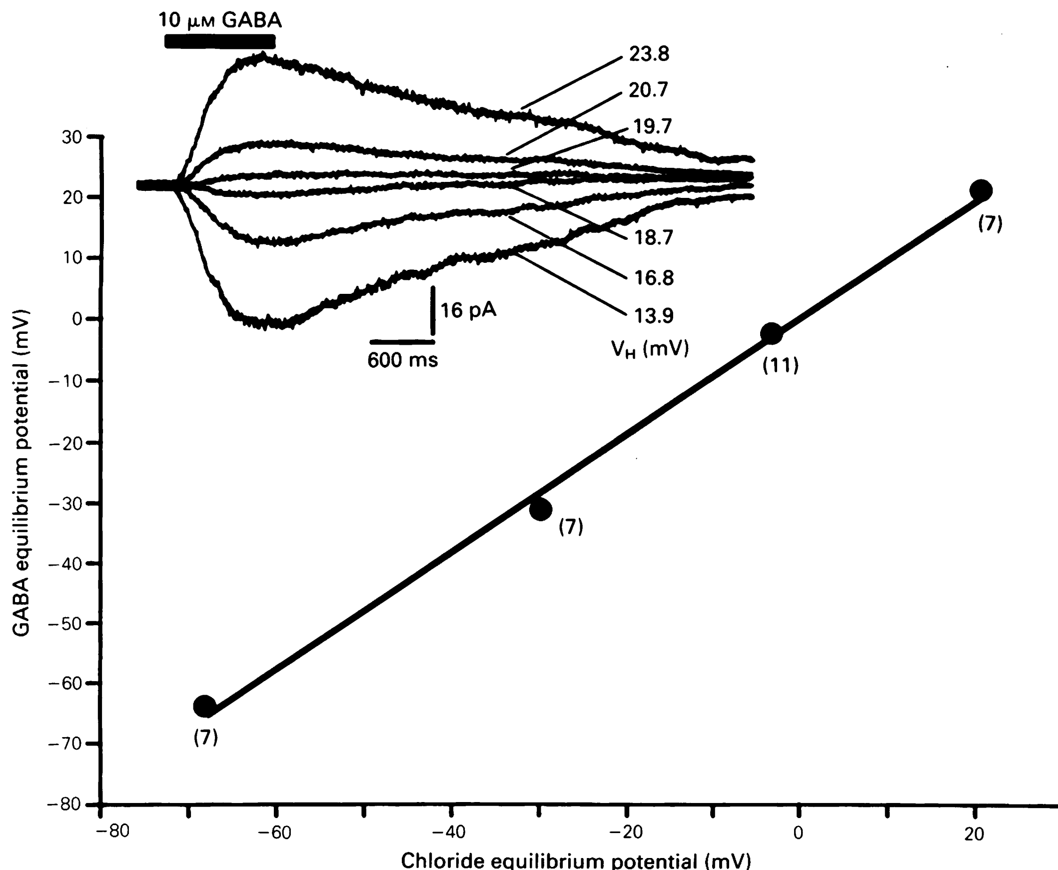

Figure 1 The experimentally determined reversal potentials for GABA (mean ± s.d. (*n*)) are plotted against the corresponding values for E_{Cl}. The slope of 0.97 ($r = 0.99$) for the line fitted by linear regression analysis indicates the strong positive correlation between E_{Cl} and E_{GABA} suggesting that an increased conductance for chloride is primarily involved. The method for generating families of traces such as those shown in the inset is discussed in the text. In the example shown E_{GABA} was near 19 mV. The horizontal bar above the traces in this and subsequent figures indicates the timing of the drug ejection from the U-tube tool. Downward-going responses represent inward positive current and correspond to an outward movement of chloride.

strated to occur in bovine chromaffin cells (Bormann & Clapham, 1985) was not evident in malanotrophs regardless of the age of the culture. Even at concentrations as high as 100 μM, the inhibitory transmitter glycine had no effect on membrane currents in any of the 10 cells to which it was applied. In current-clamp studies GABA was applied by pressure ejection from a blunt-tipped (2–5 μm diameter) pipette positioned near the cell. With 50 μM GABA in the 'puffer pipette' a brief application caused a rapid and reversible shift of the membrane potential to the equilibrium potential for chloride. The input resistance usually declined to the extent that it became impossible to measure accurately the voltage changes elicited by the injection of currents which in control and recovery responses produced 10–20 mV hyperpolarizations (data not shown). These results are virtually identical to those obtained by Taraskevich & Douglas using intracellular electrodes (1982) or patch electrodes (1985).

In voltage-clamp experiments GABA was applied using a U-tube tool (see Methods). With nearly equivalent concentrations of chloride on the extracellular and cytoplasmic faces of the membrane and with the membrane potential clamped at −60 mV, a brief application of 10 μM GABA produced an increase of the membrane current (I_{GABA}) and noise (e.g., Figure 2a). The inward flow of positive

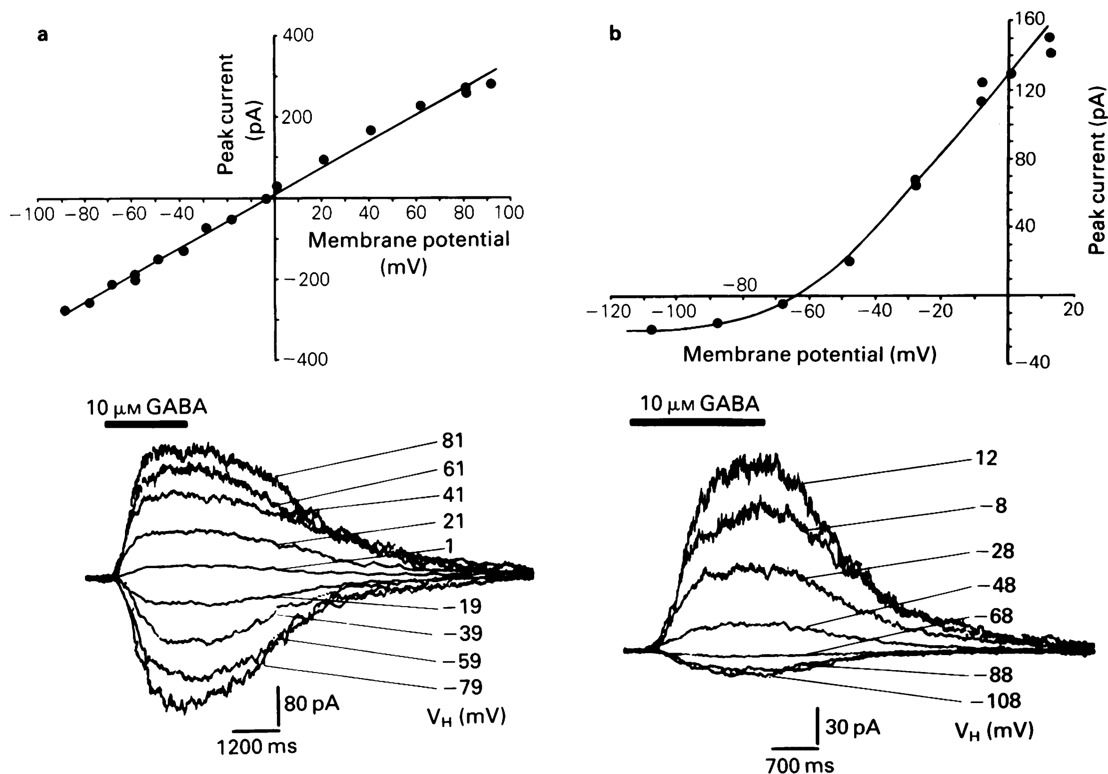

Figure 2 (a) The voltage/current relationship obtained when chloride was very nearly symetrically distributed across the membrane. The peak amplitudes of the GABA-induced currents elicited were linearly related to the membrane potential; there is no clear evidence for either inward or outward retification. The line fitted by linear regression analysis indicated a reversal potential of −2.6 mV for the response to GABA (IS1/ES1; E_{Cl} = −3.5 mV). Shown below are some of the current responses (superimposed with leakage current subtracted) from which the graph was derived. (b) As for (a) but in a cell where the intracellular concentration of chloride was much less than that in the bathing medium (IS3/ES1; E_{Cl} = −68 mV). Note the pronounced outward-going rectification of currents evoked at potentials positive to E_{Cl}.

current recorded under these conditions was consistent with an outward movement of chloride (see below). The increased current noise probably arises from the stochastic variation in the number of open ion channels around their mean level at any time point during the response (Colquhuon & Hawkes, 1977). In each of 4 cells (IS2/ES1) which responded vigorously to 10 μM GABA, a subsequent exposure to 50 μM (±)-baclofen had no effect on the resting membrane conductance.

Ionic dependence of GABA-induced currents

To reduce errors arising from extrapolation of voltage/current (V/I) curves showing inward- or outward-going rectification arising from an asymmetrical distribution of chloride across the membrane (e.g., Figure 2b), the reversal potential of the responses induced by GABA was determined in the following manner. Before an experiment the Nernstian value for the chloride equilibrium potential (E_{Cl}) was calculated for the particular combination of intracellular (patch pipette) and extracellular solutions which was to be used. This was prompted by the fact that GABA activates a chloride conductance in the neurones in the CNS. The procedure in an experiment (e.g., traces of Figure 1) was to hold the membrane near the calculated value for E_{Cl} and 10 or 20 μM GABA was then applied for 1–2 s. After recovery to the pretreatment current level the holding potential was then incremented or decremented, depending on the polarity of the current response, and after a 40–60 s interval GABA was re-applied. After several such cycles it was possible to 'tune' the membrane to a voltage at which an application of GABA would elicit a very small or no membrane current. The latter voltage was defined as

Table 2 The relationship between the equilibrium potential for chloride (E_{Cl}) and GABA (E_{GABA})

Intra-/extracellular solution	E_{Cl}	E_{GABA}
IS1/ES1	−3.5 mV	−2.1 ± 0.9 mV (5)
IS2/ES1	−3.5 mV	−2.1 ± 0.6 mV (6)
IS3/ES1	−68 mV	−64 ± 0.8 mV (7)
IS4/ES1	−30 mV	−31 ± 1.1 mV (7)
IS1/ES2	20 mV	20 ± 0.9 mV (7)

Shown in each row is the value for E_{Cl} and the corresponding value for E_{GABA} measured in whole cell recording using the indicated combination of intra- and extracellular solutions. E_{Cl} was calculated by substituting the appropriate values for $[Cl^-]_{IS}$ and $[Cl^-]_{ES}$ into the Nernst equation: $58 \log [Cl^-]_{IS}/[Cl^-]_{ES}$.
*Data shown represent the mean ± s.d. of number (n) in parentheses.

the equilibrium potential for the response to GABA (E_{GABA}).

In this way it was possible to show in different cells using various combination of IS1-4 or ES1 or ES2 that the conductance activated by GABA was primarily, if not exclusively, one for chloride (Table 2). Thus E_{GABA} shifted by approximately 56 mV per 10 fold change of the ratio of the intra- and extracellular chloride concentrations, in close agreement with the value of 58 mV predicted by the Nernst equation. The positive relationship between E_{GABA} and E_{Cl} is shown in the graph of Figure 1 where the line fitted by linear regression analysis had a slope of 0.97 and a correlation coefficient of 0.99.

In pyramidal neurones of the rat hippocampus GABA activates a potassium-dependent conductance (Gahwiler & Brown, 1985; Newberry & Nicoll, 1984). If this same conductance mechanism is also present in PI cells in these recording conditions it must contribute only slightly to the total membrane current given the close correlation between E_{GABA} and E_{Cl}. Consistent with this interpretation is the fact that blockade of potassium channels with intracellular caesium had no apparent effect on E_{GABA}, the reversal potential for responses with 100 mM internal caesium chloride (−2.1 ± 0.9 mV, $n = 5$) being virtually identical to that obtained with the same concentration of intracellular potassium chloride (−2.2 ± 0.4 mV, $n = 6$). Because there was no significant difference between these two latter groups the data were pooled for the purpose of plotting the graph shown in Figure 1. Although all of the intracellular solutions contained tetraethylammonium (TEA) to block a delayed rectifier current, TEA has been shown by others to have no effect on the baclofen-induced potassium-dependent conductance (Andrade *et al.*, 1986).

Rectification of membrane currents

In spinal cord and hippocampal neurones the relationship between membrane voltage and the magnitude of GABA-induced currents is non-linear; the membrane conductance (G) cannot accurately be predicted by Ohm's law:

$$G = I/V$$

For example, in hippocampal cells the conductance may increase up to 10 fold between −60 mV and 10 mV, with the change being particularly prominent at positive holding potentials (Segal & Barker, 1984). A qualitatively similar effect at the single channel level has also been demonstrated (Gray & Johnston, 1985). Two mechanically distinct processes may contribute to this non-linearity. One of these is the rectification predicted by barrier models such as that represented by the Goldman-Hodgkin-Katz (GHK) equation. Briefly, the GHK equation predicts that with an intracellular concentration of chloride lower than that on the outside of the cell there would be an outward-going rectification of GABA induced currents. That is to say as the membrane potential was shifted in the depolarizing direction from E_{Cl} the currents would become larger than those at hyperpolarized membrane potentials comparably displaced from E_{Cl}. With symmetrical chloride concentrations a linear V/I relationship is predicted by the GHK equation. The fact that the V/I relationship in CNS neurones often shows outward rectification when chloride is symmetrically distributed across the membrane has been attributed to a second mechanism, namely a weak voltage-dependence of GABA-induced responses (Bormann *et al.*, 1987).

In the present study the relationship between membrane voltage and GABA-induced currents was assessed using 1−2 s applications of 10 or 20 μM GABA. To reduce complications arising from desensitization, GABA was applied at intervals of no less than 30 s. When the distribution of chloride across the membrane was very nearly symmetrical (IS1/ES1) the V/I curve indicated no obvious rectification of GABA-induced membrane currents (6 cells). For example, in the cell from which the data of Figure 2a were derived the GABA-induced chord conductance (G_{GABA}) between −90 and 90 mV was relatively constant at 3.3 nS.

Illustrated in Figure 2b is the V/I profile obtained in a cell where the intracellular concentration of chloride was reduced by partial substitution of caesium sulphate for caesium chloride. With this asymmetrical distribution of chloride there was a pronounced outward-going rectification of the current elicited by 10 μM GABA. Using a value of −64 mV for E_{GABA} (Table 2) the conductance at −8 mV (2.0 nS) was approximately 5 fold greater than that measured at −108 mV (0.4 nS).

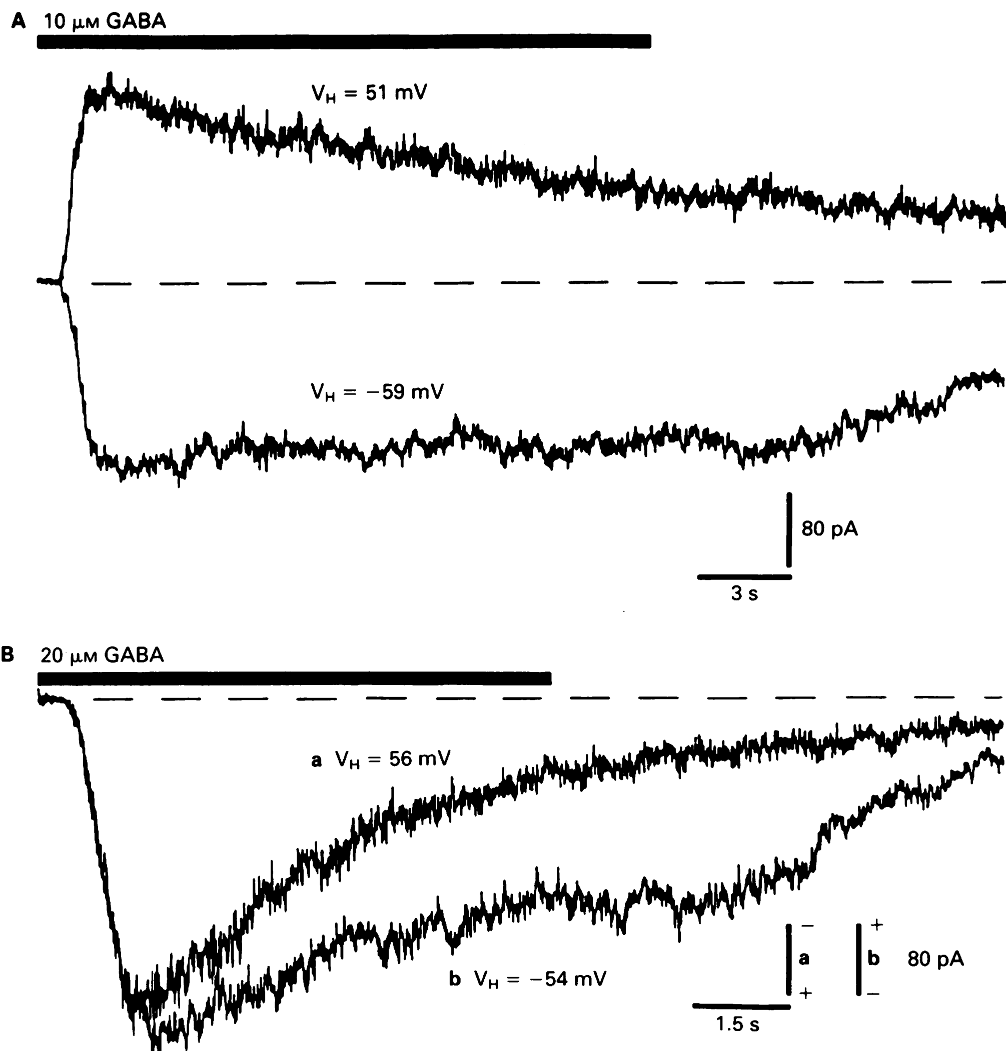

Figure 3 Fading of GABA-induced currents. (A) At a holding potential of −59 mV the inward current rapidly reached and maintained a plateau over the 10 s period during which GABA was applied. In a subsequent application when the membrane was clamped at 51 mV the outward current faded over the course of the drug application (IS1/ES1; E_{Cl} = −3.5 mV). (B) When 20 μM GABA was applied to this cell there was a decline of the current at −54 mV but this was less than observed subsequently at 56 mV. See text for comments (IS1/ES1; E_{Cl} = −3.5 mV). In (A) and (B) leakage currents have been subtracted and the broken line indicates the current level before drug application.

Fading of membrane currents

An important factor in assessing the efficacy of any neurotransmitter is the degree to which the response to the agonist declines with repeated or prolonged exposures. The tendency of GABA-induced currents to decline or fade was assessed in the present study by examining the effects of prolonged applications of the drug.

At a concentration of 10 μM and at negative holding potentials there was very little, if any, fading of the response to prolonged applications of GABA. The traces of Figure 3A show the results of one such experiment in which the cell was clamped at −59 mV and GABA was applied for 10 s. Note that the current rapidly reached and maintained a plateau level for as long as GABA was being ejected from the U-tube. The persistence of the current for a considerable period

after the application was stopped reflects the build-up of a pool of GABA-containing solution around the cell and the time taken for this pool to dissipate. After allowing 60 s for recovery the application was repeated, this time at a holding potential of 51 mV. Although the peak conductance at this potential was approximately the same as that measured at − 59 mV (3.9 nS and 3.6 nS, respectively), there was a progressive decline of the amplitude of the current despite the continued presence of the GABA. By the end of the application I_{GABA} had declined by approximately 50%.

With 20 μM GABA the current faded regardless of the holding potential but here again the same relative effect was observed: the kinetics of the decline of currents evoked at positive holding potentials was faster than at hyperpolarized levels. Shown in Figure 3B is the decline of I_{GABA} which occurred over the course of an 8 s application of 20 μM GABA. To illustrate better the apparent voltage sensitivity of the fading of currents, the response obtained at 56 mV has been inverted and superimposed on the current trace recorded at − 54 mV. Taking a value for E_{GABA} of − 2 mV (IS1/ES1; Table 2) and assuming that E_{Cl} was not significantly altered by the GABA-induced chloride current, the peak conductance changes were approximately 7 nS and 5.8 nS at − 54 mV and 56 mV, respectively.The inward current elicited at − 54 mV slowly declined so that at the end of the application it was reduced to 40% of the initial peak response. The fading of the outward current induced by 20 μM GABA was more pronounced than that which was observed for the inward current. Thus, when the cell was clamped at 56 mV there was a 75% decline of I_{GABA} at the end of the application, a value which was almost two fold greater than that measured at − 54 mV.

Since $I_{GABA} = G\ (V_H - E_{GABA}) = G\ (V_H - E_{Cl})$ a possible explanation for the fading of inward- and outward-going currents induced by 20 μM GABA would be a reduction of the driving force $(V_H - E_{Cl})$ due to the influx (at positive potentials) or efflux (at negative potentials) of chloride (e.g., Huguenard & Alger, 1986). An example of experiments done to address the possible contribution of a shift of E_{Cl} in the fading of GABA-induced currents is shown in Figure 4a. In this cell the membrane potential was stepped at 0.5 Hz between − 54 mV and − 4 mV. The latter potential was very near the predicted value for E_{GABA} (IS1/ES1; E_{Cl} = − 3.5 mV). GABA, at a concentration of 20 μM, was applied to the cell for 10 s. At − 54 mV the amplitude of the current measured at the end of the 10 s application was only 30% of the peak response. If this reduction of I_{GABA} was caused by hyperpolarizing shift of E_{Cl} due to an efflux of chloride, then at − 4 mV the current would have been expected to reflect this shift, first by changing its polarity and thereafter by becoming progressively larger as the application continued. However, although it declined, apparently in parallel with the response measured at − 54 mV, the small current measured at − 4 mV did not change at any time from its inward direction to an outward one. It may be concluded therefore that a change of E_{Cl} did not contribute to the fading of I_{GABA}.

Illustrated in Figure 4b are the currents obtained when the same cell was stepped between − 54 mV and 56 mV. Analysis of the envelopes of the inward and outward currents confirm previously discussed results showing the faster decline of I_{GABA} at positive holding potentials. Taken together with the results in Figure 4a, these data suggest that the fading of membrane currents in these circumstances is due to a reduction of G_{GABA}, and furthermore that this process is accelerated at positive membrane potentials.

Also evident in the traces of Figure 4b are relaxations of the membrane current during the hyperpolarizing and, to a lesser extent, depolarizing commands. The basis for these relaxations, which have also been observed in hippocampal pyramidal neurones (Segal & Barker, 1984), is undetermined. Relaxations of this type would be consistent with a voltage-dependence of the gating mechanism such as demonstrated for spinal cord neurones (Bormann *et al.*, 1987), though the involvement of other unknown processes cannot be ruled out. This does not necessarily conflict with the observation that the V/I relationship, determined as for Figure 2a, was linear since the macroscopic current depends, *inter alia*, on the probability of channel opening and the unitary conductance(s); and, if, for example, the single channel current rectified in the inward direction, as is the case in bovine chromaffin cells (Bormann & Clapham, 1985), then this could mask any voltage-dependence of channel gating. The extent to which either of these variables is affected by the membrane potential remains to be assessed by single channel analysis.

Relationship between GABA concentration and membrane current

Dose-response curves were generated by applying low concentrations of GABA (0.5–5.0 μM) to cells voltage-clamped at − 80 mV to − 60 mV (IS1/ES1; E_{Cl} = − 3.5 mV). In the experiments GABA was applied from the U-tube for 1–3 s at intervals of 30–40 s. In the representative example of Figure 5 there is a slope of 1.5 (r = 0.99) for the line fitted by linear regression analysis to the data points on the double logarithmic plot of GABA concentration versus peak current. Of the seven Hill plots generated in this manner the mean (± s.d.) of the Hill coefficients was 1.5 ± 0.15.

Pharmacological manipulation of GABA-induced currents

The clinical anxiolytic diazepam (Valium) enhances

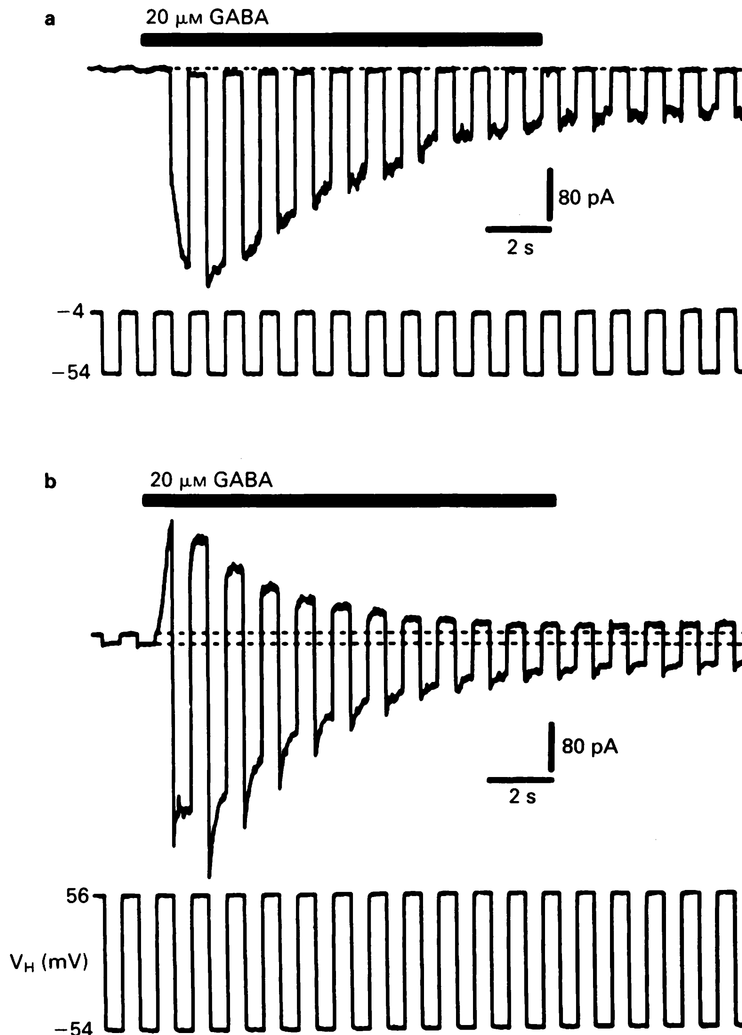

Figure 4 (a) Current (upper) and voltage traces illustrating the action of 20 μM GABA in a cell where the holding potential was stepped at 0.5 Hz between −4 and −54 mV. The small inward current recorded at −4 mV is consistent with the proximity to E_{Cl} (IS1/ES1 = −3.5 mV). The fading of the current at −54 mV cannot be due to a hyperpolarizing shift of E_{Cl} since the current at −4 mV never became positive although it too declined during the application. (b) The response in the same cell when the membrane potential was stepped between −54 mV and 56 mV. The envelopes of the currents induced by GABA indicate a more rapid decline at the positive potential (cf. Figure 3B).

GABA-mediated responses and GABAergic synaptic transmission in the CNS (Choi & Fischbach, 1981). Figure 6a illustrates the typical effect (3 of 3 cells) of chlordiazepoxide, a congener of diazepam, on the GABA-induced membrane currents in a melanotroph held at −60 mV and having a chloride equilibrium potential near −4 mV. For each of two successive applications of a U-tube solution containing 10 μM GABA the peak inward current was approximately 190 pA. However, after switching to a U-tube solution containing 10 μM GABA and 10 μM chlordiazepoxide the peak inward current for each of two successive applications was of the order of 330 pA, nearly twice the amplitude of control responses. The lower traces of Figure 6a indicate the complete reversibility of the effect of chlordiazepoxide.

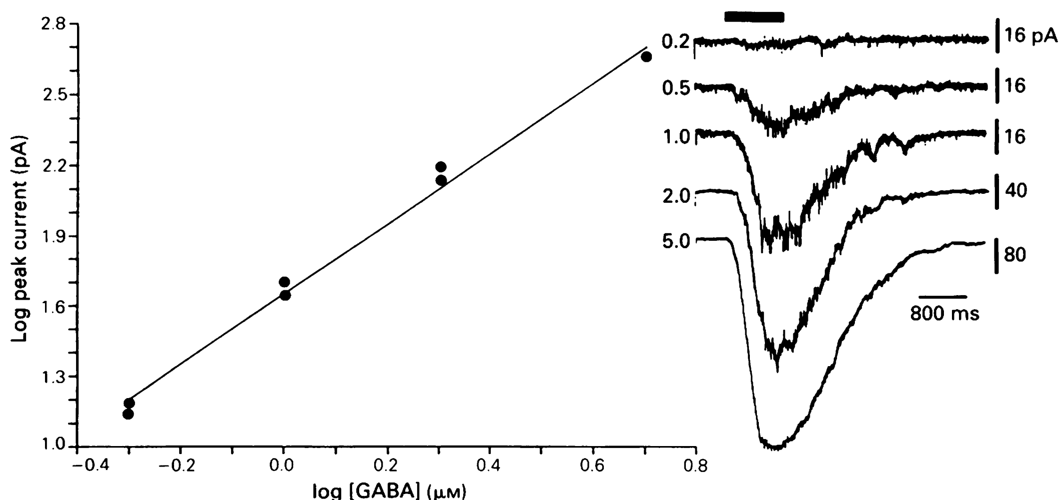

Figure 5 The relationship between the concentration of GABA and the macroscopic current. The slope of the line fitted by linear regression analysis is 1.5 ($r = 0.99$) which suggests that two molecules of GABA must bind to the receptor to open the chloride channel. Traces of current responses on which the graph is partially based are shown to the right. The number at the beginning of each trace indicates the concentration of GABA (μM). Because it was not possible to gauge accurately the small currents evoked with the 0.2 μM solution those points were not graphed. $V_H = -80$ mV.

Consistent with its actions in the CNS, bicuculline blocked GABA-induced currents in melanotrophs (5 of 5 cells). In Figure 6b, for example, the peak currents obtained when 10 μM GABA and 5 μM bicuculline methiodide were simultaneously applied represented only 10–15% of the amplitude of currents recorded in control applications containing 10 μM GABA but no bicuculline. The complete reversibility of the effect of the antagonist was evident in the recovery responses obtained 5 min after returning to a U-tube solution containing only the agonist.

Discussion

We have examined some aspects of whole cell responses to GABA in voltage-clamped PI cells which, by their electrophysiological and gross morphological features, appear to represent melanotrophs. Currents induced by GABA in melanotrophs were primarily, if not exclusively, due to an increased conductance for chloride. We have also found in outside-out membrane patches obtained from these cells that GABA activates chloride channels (Kehl & McBurney, unpublished observations). Unlike spinal neurones which respond equally well to GABA and glycine, PI cells are responsive only to GABA; concentrations of glycine of up to 100 μM did not affect the resting conductance. In this regard melanotrophs are similar to adrenal chromaffin cells (Bormann & Clapham, 1985) but less so to hippocampal neurones in which a detectable chloride current is obtained with 100 μM glycine (Segal & Barker, 1984). GABA-induced currents in melanotrophs were blocked by bicuculline and enhanced by chlordiazepoxide, a benzodiazepine. From these ionic and pharmacological characteristics it may be concluded that $GABA_A$ type receptors are present on the membranes of rat melanotrophs.

The results of studies with cultured spinal cord, hippocampal and chromaffin cells have indicated a weak voltage-dependency of the gating of chloride channels coupled to $GABA_A$ receptors. We could not uncover clear evidence for a such voltage-dependency of GABA-mediated chloride currents in melanotrophs (see also Demeneix *et al.*, 1986). A pronounced outward-going rectification was apparent, however, in cells having an intracellular concentration of chloride less than that in the extracellular medium. This rectification is of physiological significance since in intact cells (i.e., those in which the intracellular recording technique is assumed to have no effect on the internal chloride concentration) E_{Cl} is at a level very near the resting membrane potential. Interestingly, there is an apparent discrepancy in the value for E_{Cl} in melanotrophs in the acutely excised gland (MacVicar & Pittman, 1986) vis-a-vis isolated melanotrophs maintained *in vitro* using cell culture methods (Taraskevich & Douglas, 1985). In the latter prepara-

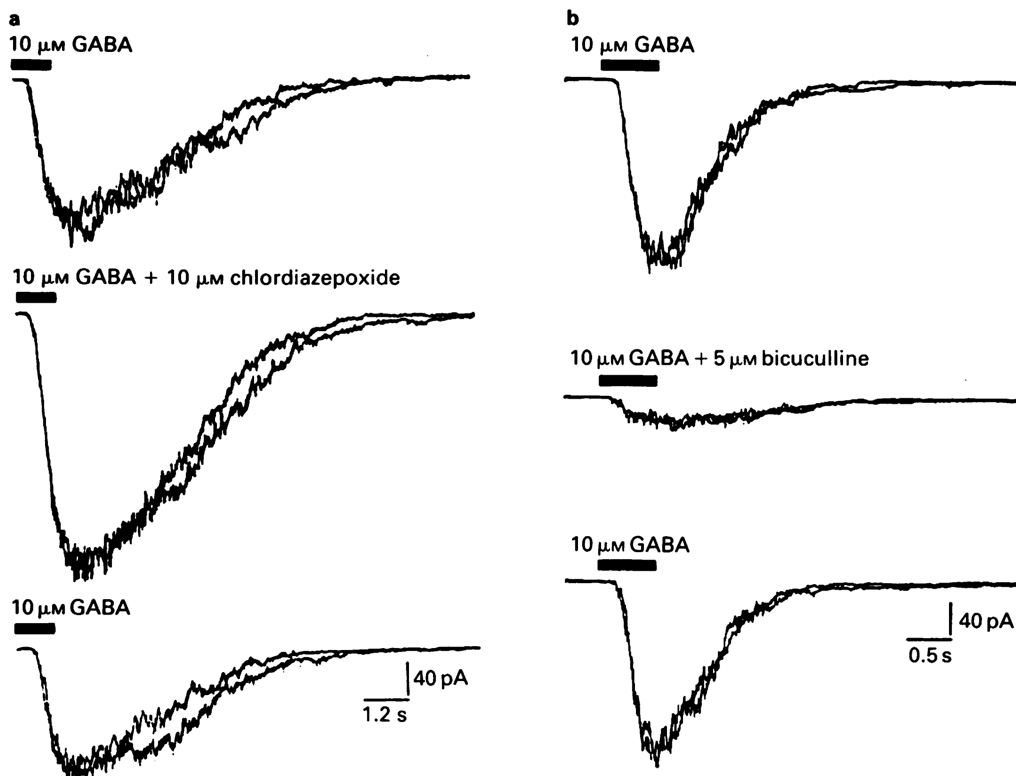

Figure 6 From above downwards each column indicates control, treatment and recovery traces of membrane currents. To illustrate the consistency of drug applications involving the U-tube tool, each record is comprised of two consecutive superimposed responses. (a) After switching to a solution containing GABA as well as chlordiazepoxide there was a nearly two fold increase of the peak current amplitude. $V_H = -60$ mV. (b) In another cell 5 μM bicuculline methiodide caused a reversible 85% reduction of the current elicited by 10 μM GABA. $V_H = -70$ mV. In both cells E_{Cl} was -3.5 mV (IS1/ES1).

tion a value of -40 mV was found for responses to exogenous GABA using intracellular electrodes containing 4 M potassium acetate. However in cells in the excised intact gland there was a value near -60 mV for the bicuculline-sensitive i.p.s.p. evoked by stimulation of fibres in the pituitary stalk and recorded using electrodes containing 2 M potassium acetate. In either case it would be predicted that G_{GABA} would increase quite dramatically when the cell was depolarized beyond E_{Cl} and would therefore provide a powerful braking effect on further depolarization of the membrane.

The experimentally-determined values of 1.3–1.7 for the Hill coefficient suggest that some link in the chain of reactions occurring between the application of GABA and the increase of membrane conductance is cooperative. The simplest interpretation of this data is that channel opening is initiated by the binding of two molecules of GABA to the receptor. Hill coefficients of 1.83, 1.9 and 2 have been found for GABA responses in chick spinal cord (Choi *et al.*, 1981), bovine chromaffin cells (Bormann & Clapham, 1985) and cat sensory neurones (Gallagher *et al.*, 1978), respectively.

During prolonged applications of GABA at concentrations above 10 μM there was a progressive decline of the membrane current. This fading appeared to reflect a decrease of G_{GABA} rather than a shift of E_{Cl}. That E_{Cl} was not affected by relatively large chloride currents is probably due to rapid equilibration of the solution in the intracellular compartment with that in the pipette. It is quite conceivable, however, that in the absence of this artificial 'buffering' system substantial shifts of E_{Cl} might occur *in vivo* during high frequency synaptic input. The decline of currents seen with the prolonged applications of GABA was consistently faster at

positive holding potentials; though this may be of only a limited significance to normal physiological function, where the concomitant activation of outward potassium currents would impose significant constraints on the capacity of the cell to remain depolarized for any appreciable period.

It seems likely, on the basis of biochemical studies by others, that $GABA_B$ receptors also contribute to the modulation by GABA of the secretory activity of melanotrophs. The results of binding assays have indicated that $GABA_B$ type receptors are present on cultured porcine melanotrophs and in this same preparation baclofen inhibits the spontaneous or barium-induced release of α-MSH (Demeneix et al., 1984; 1986). Indirect evidence for a similar relationship between $GABA_B$ receptors and hormone release in rat melanotrophs is provided by the observations of Taraskevich & Douglas (1985), that the selective $GABA_A$ agonist isoguvacine reproduces the stimulatory but not the inhibitory effect of GABA itself. This inhibition of α-MSH release by baclofen is reminiscent of its action to reduce neurotransmitter release from synaptic terminals in the CNS (Bowery et al., 1980).

The electrophysiological basis for the baclofen-mediated reduction of secretion from melanotrophs is not clear. Using the patch recording technique Taraskevich & Douglas (1985) observed a hyperpolarizing action of baclofen which was associated with a slight increase of the membrane resistance. A direct baclofen-induced hyperpolarization has also been demonstrated in the hippocampus (Gahwiler & Brown, 1985; Newberry & Nicoll, 1984) and substantia nigra (Pinnock, 1984), but this effect was associated with a decrease of the input resistance and was ascribed to the activation of a potassium conductance. In the present study we could not detect any conductance change with baclofen, nor could Demeneix et al. (1986) in similar investigations using porcine melanotrophs.

There are at least two possible explanations for the fact that we did not detect any direct effect of baclofen. The first possibility is that although the cells may possess $GABA_B$ receptors the normal consequences of their activiation have been abrogated by the wash-out of intracellular components required for the transduction of the signal. This conjecture is based on recent reports that in dorsal root ganglion (DRG) and hippocampal neurones the actions of baclofen are linked to one or more types of G-binding proteins (Andrade et al., 1986; Dolphin & Scott, 1986; Holz et al., 1986). This wash-out would have had to occur very quickly (<2 min) since baclofen was ineffective even when applied shortly after obtaining the whole cell recording. A second and more likely explanation, we feel, is that baclofen does indeed have no direct effect on membrane conductance but instead causes an inhibition of α-MSH release by reducing voltage-dependent calcium currents. This is based on preliminary observations that baclofen reduces the calcium- but not the sodium-dependent component of the action potential in cells where potassium currents are blocked with internal caesium (Kehl & McBurney, unpublished observations). If this hypothesis is correct then melanotrophs will, in this respect, resemble DRG neurones where baclofen has no hyperpolarizing action but does block calcium currents (Dunlap, 1981; Holz et al., 1986). Voltage clamp experiments are currently being undertaken to assess the effect of baclofen on calcium currents in melanotrophs and the role, if any, for guanyl nucelotides and G-binding proteins in the actions of baclofen.

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