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Dual population of $GABA_A$ and $GABA_B$ receptors in rat pars intermedia demonstrated by release of αMSH caused by barium ions

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1 We have studied the effects of selective $GABA_A$ and $GABA_B$ agonists on α-melanophore stimulating hormone (αMSH) release from intact rat neurointermediate lobes (NIL) *in vitro*. Agonist effects were tested against either basal αMSH output or $BaCl_2$ (5 mM)-evoked release.

2 GABA (50 μM) produced a biphasic effect on basal release, with an enhancement followed by inhibition of release. The enhancement but not the inhibition was blocked by bicuculline methiodide (100 μM).

3 Baclofen (10 μM), a specific $GABA_B$ agonist, reduced the basal and Ba^{2+}-evoked hormonal release in a stereospecific manner. (−)-Baclofen (5 μM) was active whereas the (+)-isomer was inactive at the same concentration.

4 Isoguvacine (50 μM) a specific $GABA_A$ agonist, potentiated the Ba^{2+}-evoked release of αMSH. GABA (50 μM) mimicked this effect, and its action was antagonized by bicuculline methiodide (200 μM).

5 The results suggest that both $GABA_A$ and $GABA_B$ receptors are present on the endocrine cells of the intermediate lobe.

Introduction

Evidence is accumulating that γ-aminobutyric acid (GABA) may play an important role in excitation-secretion coupling in the neurointermediate lobe of the pituitary (NIL). Immunohistochemical studies of glutamate decarboxylase localization show the existence of a central GABAergic innervation of the intermediate lobe (IL) (Oertel, *et al.*, 1982; Vincent *et al.*, 1982). Taraskevich & Douglas (1982) have reported the presence of bicuculline-sensitive GABA receptors on the glandular cells of the IL. Activation of these receptors causes cellular depolarization. In their subsequent biochemical studies they observed a dual effect of GABA on α-melanophore stimulating hormone (αMSH) secretion from these cells: a stimulation of basal release, as expected from membrane depolarization produced by GABA, and an inhibition of the release evoked by large concentrations of K^+ (50 mM) (Tomiko *et al.*, 1983).

The apparently paradoxical observation that GABA can either stimulate or inhibit release has been observed on other preparations, e.g. enhanced output of dopamine by means of presynaptic depolarization of dopaminergic terminals (Giorguieff *et al.*, 1978; Reimann *et al.*, 1982) and release of [^{3}H]-noradrenaline from perfused cerebellar slices, (Bowery *et al.*, 1980). These authors (Bowery *et al.*, 1980) observed that GABA produced either facilitation or inhibition of release, according to dose and experimental conditions, whereas baclofen was never observed to stimulate release. From these and subsequent studies it has become clear that two classes of GABA receptors act on the process of excitation secretion coupling: $GABA_A$ and $GABA_B$.

$GABA_B$ receptors are insensitive to bicuculline antagonism. Activation of $GABA_B$ sites results in an inhibition of neurotransmitter release (Bowery, 1982). Such inhibitory effects are particularly evident if tested against evoked release. This is consistent with observations showing that GABA shortens

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the duration of the Ca^{2+} component of action potentials (Dunlap 1981, Désarmenien *et al.*, 1984). A specific agonist for the $GABA_B$ receptor is β-*p*-chlorophenyl GABA (baclofen) (Hill & Bowery, 1981; Bowery *et al.*, 1983). The $GABA_A$ site is bicuculline-sensitive. Receptor activation results in a selective increase in membrane Cl^- conducance (see Nistri & Constanti, 1979). Action potentials will thus either be suppressed by the decrease in membrane resistance, or their amplitude diminished by the depolarization caused by outward Cl^- movements (eg. if intracellular Cl^- is high). In this latter case inhibition of release is expected, but the depolarization itself may cause an influx of Ca^{2+} and thus facilitate release. It is therefore important to test $GABA_A$ and $GABA_B$ agonists not only against basal release but also in experimental conditions where cell firing is modified.

As to $GABA_B$ sites in the IL, there is only one brief report of a radio-ligand study concerning GABA receptors assayed on membrane fractions of rat NIL (Anderson & Mitchell, 1983). We examined the problem, firstly with an electrophysiological approach (preliminary accounts in Désarmenien *et al.*, 1983; Loeffler *et al.*, 1983), and in the detailed study of hormone release *in vitro* described here. Rather than stimulating the cells with large concentrations of K^+ (Tomiko *et al.*, 1981; Keith *et al.*, 1983) which would inhibit the spontaneous firing of the melanotrophs, we tested $GABA_A$ and $GABA_B$ agonists against Ba^{2+}-evoked αMSH release. Douglas *et al.* (1983) have recently presented a detailed study showing that Ba^{2+} has a very efficient secretagogue action on intermediate lobe cells and that part of this effect corresponds to enhanced cell firing.

Methods

Perfusion procedures

Charles River rats (150–200 g) of either sex were stunned and decapitated. The NIL was quickly removed, washed in standard perfusion medium at room temperature, then transferred to a perfusion chamber. The perfusion apparatus was based on the model used by Van der Schueren *et al.* (1982). Five lines were run in parallel, each chamber containing one intact NIL. The NIL rested on a disc of Whatman glass microfibre paper. Lobes were perfused for 2 h before experimentation was begun. Flow rate was adjusted to either 0.1 ml min^{-1} or 0.5 ml min^{-1}. The fast perfusion rate was used to avoid desensitization of GABA responses, as deduced from electrophysiological observations in the NIL (Loeffler *et al.*, 1982). During perfusion the NILS remained covered with 250 μl medium. All experiments were carried out at 36°C.

Solutions

The perfusion medium contained (mM): NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES (4-12-hydroxyethyl)-1-piperazine-ethane sulphonic acid) buffer 5, and glucose 1.8 g l^{-1}, bovine serum albumin 1.0 g l^{-1} and Bacitracin (Sigma) 100 mg l^{-1} adjusted to pH 7.4 with NaOH. The solution was bubbled with O_2 before adding the albumin. During experimentation an atmosphere of 100% O_2 was maintained over the solution. Barium was added as the chloride salt at a standard concentration of 5 mM, in the presence of 2 mM Ca^{2+}.

Drugs used were: γ-aminobutyric acid (GABA, Sigma); 1,2,3,6 - tetrahydropyridine - 4 - carboxylic acid (isoguvacine, kindly supplied by Dr Krogsgaard-Larsen, Copenhagen); β-(*p*-chlorophenyl)-GABA (baclofen, Ciba-Geigy) and the corresponding (+)- and (−)-stereoisomers of baclofen (kindly provided by Ciba-Geigy). A methiodide derivative of bicuculline (Sigma and gift of Synthelabo, Paris) was used at physiological pH.

αMSH radioimmunoassay

The effluent from each chamber was usually collected in 5 min fractions. Effluent was assayed in duplicate for αMSH content using the assay developed by Schmitt *et al.* (1979). The synthetic αMSH was kindly supplied by Ciba-Geigy and the antibody by Dr Eberle (Basel). The assay has a lower limit of sensitivity of 7.8 pg per tube. In our hands the intra and inter-assay coefficients of variation were less than 5 and 7% respectively.

Treatment and presentation of results

The majority of the data presented relate to measurement of release evoked by a Ba^{2+} (5 mM) stimulus. Owing to the differences in spontaneous rates of αMSH secretion, results are presented in two ways. In some figures, absolute concentrations (ng MSH ml^{-1}), over one representative perfusion, drawn from a minimum of five separate perfusions are presented. On the other hand, for the purpose of statistical evaluation, we calculated the percentage variation with respect to basal release, using as the reference level the mean spontaneous hormonal release in the 3 fractions preceding the Ba^{2+} stimulus.

Mean values of perfusion data treated in this manner are given in the histograms.

Peak secretory ratios were calculated from individual perfusion data, also treated as percentages of basal release, using in each case the highest concentration seen following the stimulus. Peak secretion usually was seen in the second or third fraction following the Ba^{2+} stimulus.

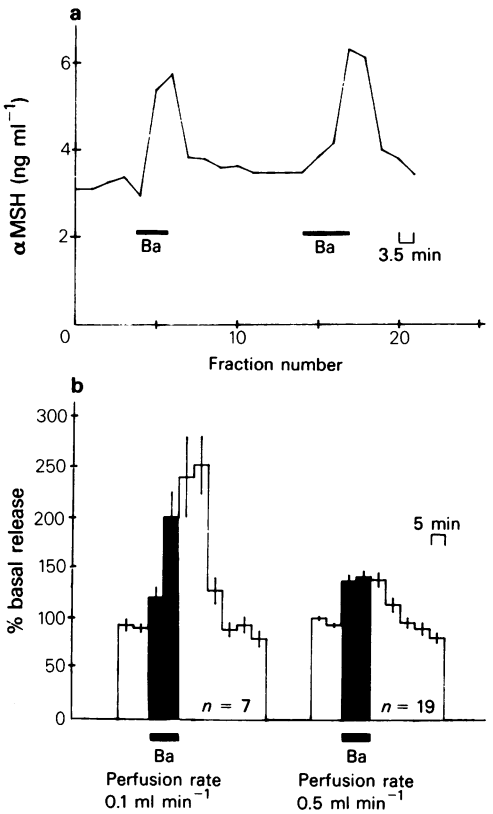

Figure 1 (a) Typical individual experiment showing the stimulation of αMSH secretion by the successive pulses of 5 mM barium (bars indicate time of application); fractions indicate collection periods of the superfusate (3.5 min); the flow rate was low (0.1 ml min^{-1}). Note the 20 min stimulus interval allowing full recovery and reproducible responses. The profile of the basal release was not always as constant, it may decline slowly with time (as much as 20% from initial level). (b) Statistical representation of Ba^{2+}-evoked stimulation of MSH release (% of basal release) at slow (left) or fast (right) perfusion rates. Dark areas indicate fractions collected during Ba^{2+} application. Means are given, and s.e.means are represented by the vertical bars.

Results

The secretagogue effect of Ba^{2+} on intact NIL

Our intention was to use the secretagogue effect of Ba^{2+} in such as way that the stimulus (Ba^{2+}, 5 mM) and the GABA-like compounds could be applied and withdrawn simultaneously over short periods of time (10 min). A rapid rate of drug delivery was required as GABA$_A$ effects are especially prone to desensitization, (see review by Nistri & Constanti, 1979). Using electrophysiological methods we have already shown that this is the case in the NIL (Loeffler *et al.*, 1982). Two flow rates were therefore used not only to avoid desensitization, but also to take into account that Ba^{2+} modifies the modes of cell firing (Ca^{2+} spikes, pace-maker activity, depolarization) and subsequent hormone release by diverse mechanisms, as discussed by Douglas *et al.* (1983). The results presented in Figure 1a are in accordance with these authors' observations and show reproducible increases in αMSH output from intermediate lobe cells. The intensity of the response varied with the perfusion rate (Figure 1b). This was probably due to differences in both the diffusion rate of Ba^{2+} into the tissue and to the renewal rate of medium in the chamber. At the slow flow rate, Ba^{2+} evoked a peak secretory rate that was 314.6±23% (mean ±

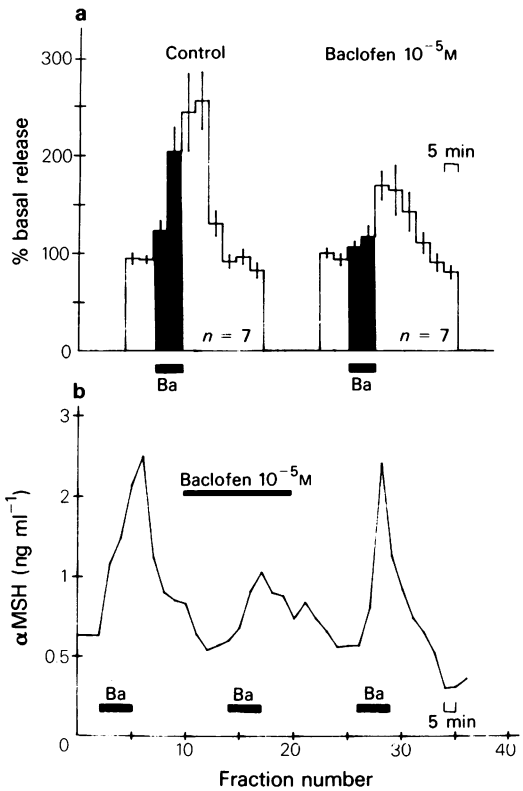

Figure 2 (a) The depressant effect of (±)-baclofen 10^{-5} M as monitored against Ba^{2+}-evoked αMSH release. (Low perfusion rate: 0.1 ml min^{-1}). In these experiments baclofen was applied simultaneously with Ba^{2+}. Mean values are given and s.e.means represented by vertical bars. (b) Hormone output as a function of time in a typical experiment at fast perfusion rate (0.5 ml min^{-1}) in which (±)-baclofen was added before and during the Ba^{2+} stimulus (as show by horizontal bars). Note reduced peak response (middle) and also a decline in basal release during baclofen perfusion.

s.e.mean, $n = 7$) of the basal secretory rate; with the fast flow rate the peak secretory rate was $154.3 \pm 6\%$ ($n = 19$) of basal output.

Inhibitory effects of baclofen

This part of the study was designed to examine the possibility that the $GABA_B$ receptor is present on the glandular cells. In these experiments the receptor was not visibly subject to desensitization and so both the fast and slow perfusion rates could be used. As seen in Figure 2a, with the slow perfusion rate the stimulant effect of Ba^{2+} was much reduced in the presence of baclofen 10^{-5} M (peak secretory rate was $314.6 \pm 23\%$ ($n = 7$) of basal rate for controls versus $181.4 \pm 22\%$ ($n = 7$) with baclofen, ($P < 0.01$, unpaired Student's t test)).

When perfusion was carried out at the higher flow rate, baclofen was added 20 min before the Ba^{2+} pulse. Under these conditions baclofen strongly inhibited the Ba^{2+} evoked release of αMSH (Figure 2b). In 4 separate experiments the peak secretory rate evoked by Ba^{2+} in the presence of baclofen (10^{-5} M) rose to $149.0 \pm 9\%$ of the basal rate before drug application, whereas the first control peak response evoked by Ba^{2+} alone was $257.5 \pm 11\%$ basal output. The response was bicuculline-resistant (see Figure 6 and subsequent commentary).

Baclofen inhibits basal hormone release: stereospecificity of the effect

In order to characterize the specificity of the inhibition seen with baclofen, we examined the effect of (+)- and (−)-baclofen (5×10^{-6} M) on basal αMSH output. In 8 trials within 5 independent experiments (+)-baclofen was devoid of any effect on basal hormonal output, whereas (−)-baclofen application decreased output by $22.9 \pm 1.5\%$ of the basal secretory rate. Typical profiles of such perfusions are depicted in Figure 3a. Figure 3b shows that GABA (5×10^{-5} M), in the presence of bicuculline (10^{-4} M)

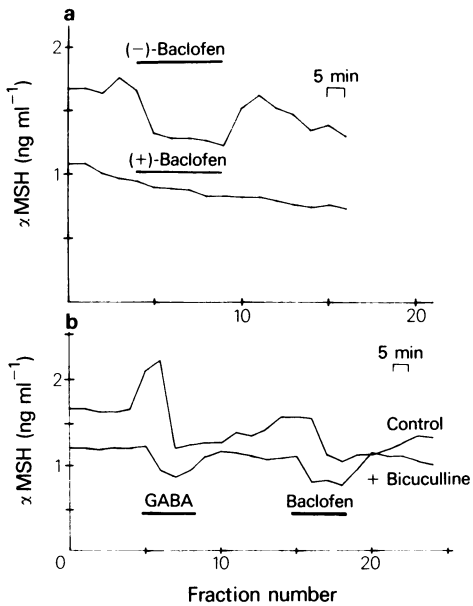

Figure 3 (a) Typical perfusions showing depression of basal release of αMSH in the presence of (−)-baclofen (5×10^{-6} M) but not when the (+)-stereoisomer (5×10^{-6} M) was added (lower trace). Note further that the application of (−)-baclofen for 25 min, causes a depressant effect which does not diminish. Fast flow rate (0.5 ml min^{-1}). (b) Typical profiles of perfusions showing the depressant effects of GABA (5×10^{-5} M) and baclofen (5×10^{-5} M) on basal hormone release. Note the stimulatory effect of GABA on hormone release (upper graph) is completely abolished by bicuculline (10^{-4} M), but the depressant effect is bicuculline-resistant. Fast flow rate (0.5 ml min^{-1}).

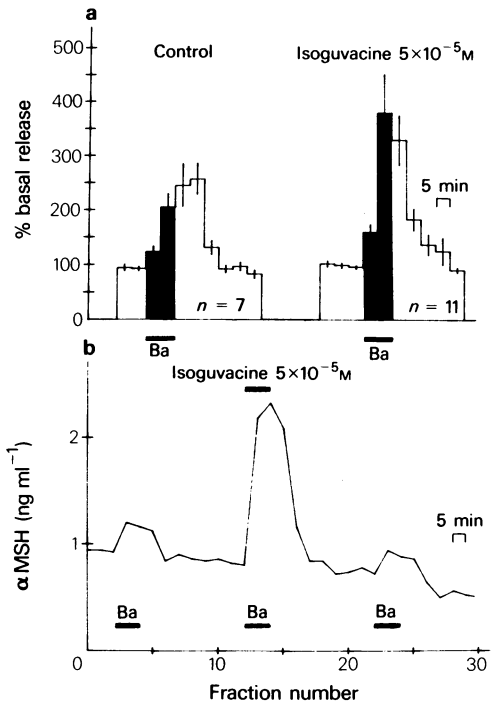

Figure 4 (a) Facilitation of αMSH release when isoguvacine is applied simultaneously with Ba^{2+} (right) as compared to Ba^{2+} controls (left). Fast perfusion rate (0,5 ml min^{-1}). Each result is the mean with s.e.mean indicated by vertical bars. (b) Typical profile of MSH release stimulated by Ba^{2+} at the fast perfusion rate, (0,5 ml min^{-1}) showing facilitation by isoguvacine when applied simultaneously with Ba^{2+} (central peak).

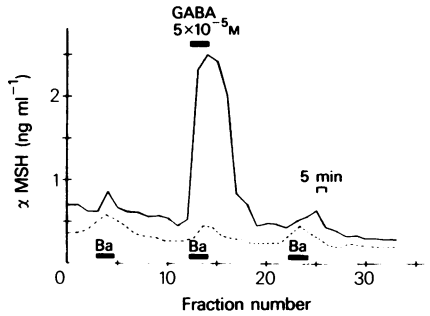

Figure 5 Facilitation of Ba^{2+}-evoked αMSH release by GABA. Profile of release in response to 3 successive applications of Ba^{2+} showing facilitatory effect of 5×10^{-5} M GABA. Experimental procedure as described in Figure 4b. Note the GABA effect is completely inhibited by bicuculline (2×10^{-4} M). Solid line, control; broken line, bicuculline 2×10^{-4} M.

reproduced the inhibitory effect of (−)-baclofen (5×10^{-5} M) on basal secretion. In the absence of bicuculline the inhibitory effect of GABA was preceded by a stimulation of release (Figure 3b, upper graph).

Isoguvacine potentiates a Ba^{2+} evoked release

As the GABA$_A$ receptor is particularly susceptible to desensitization (Loeffler *et al.*, 1983a) the majority of experiments designed to demonstrate GABA$_A$ effects were carried out at the fast flow rate. Under such conditions, isoguvacine potentiated the secretagogue effect of Ba^{2+} (Figure 4a, b). However, at the slow perfusion rate isoguvacine (5×10^{-5} M) produced no significant alteration in the response to Ba^{2+} (controls peak release rate: 314.6 ± 23% basal rate; peak release rate in the presence of isoguvacine (5×10^{-5} M): 355.7 ± 47%). At the fast perfusion rate, isoguvacine (5×10^{-5} M) applied simultaneously with the Ba^{2+} greatly potentiated release (Figure 4b). In 4 experiments carried out under these conditions the peak αMSH release for the first control Ba^{2+} stimulus was 129.5 ± 3% of basal output, but in the presence of isoguvacine, Ba^{2+}-evoked release rose to 230.5 ± 17% of basal rate.

Bicuculline antagonism

The last series of experiments was designed to test the bicuculline sensitivity of the GABA and baclofen effects: bicuculline being a competitive antagonist for the GABA$_A$ receptor site. Bicuculline was perfused throughout the test experiments, either at 10^{-5} M or at 2×10^{-4} M). In five experiments where Ba^{2+} was applied simultaneously with GABA (5×10^{-5} M) the evoked hormonal output rose to 531.2 ± 95% of the basal secretory rate. In the presence of bicuculline (10^{-5} M) the release evoked with Ba^{2+} and GABA was partially reduced to 295 ± 35% of basal release ($n = 3$). With bicuculline used at a higher concentration (2×10^{-4} M) (Figure 5), the potentiation by GABA of evoked release was completely eliminated, peak secretory rate being 139.8 ± 7% of basal rate

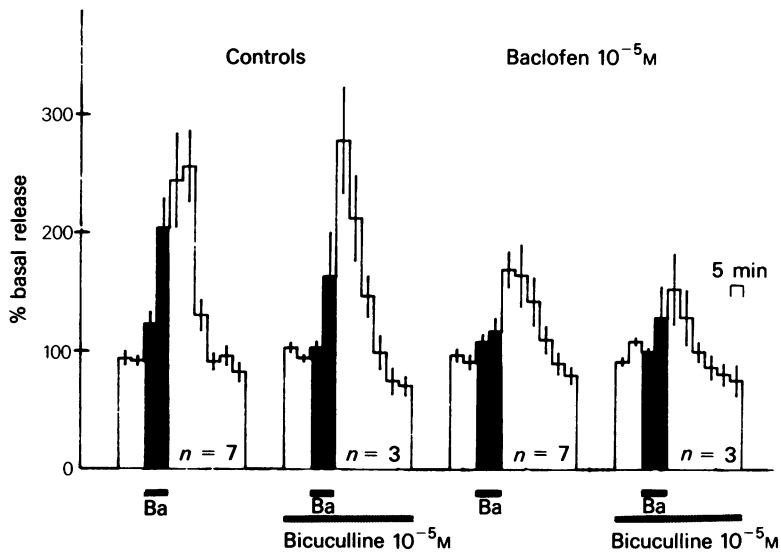

Figure 6 Histograms showing the secretagogue effect of Ba^{2+} (extreme left) to be unaltered by continuous perfusion of bicuculline (centre left). Right pair of histograms show the depressant effect of (±)-baclofen and its resistance to bicuculline antagonism. Means are given and s.e. means are indicated by the vertical lines.

($n = 4$), which was not different from the peak secretory rate evoked in controls (Ba^{2+} alone, but in the presence of bicuculline 2×10^{-4} M: 139.8% basal output; Ba^{2+} alone but in the presence of bicuculline 10^{-5} M; $146.5 \pm 7\%$ basal output). In contrast, bicuculline (10^{-5} M) did not modify the peak response evoked by Ba^{2+} applied with baclofen (10^{-5} M) (Figure 6).

Discussion

This paper demonstrates the coexistence of two types of GABA receptor on the endocrine cells of the IL. Douglas and his coworkers have already reported that bicuculline-sensitive receptors are involved in the rapid depolarization and transient release of αMSH seen following GABA application to these cells (Taraskevich & Douglas, 1982; Tomiko *et al.*, 1983). We show that activation of these same bicuculline-sensitive receptors potentiates a Ba^{2+}-evoked release of αMSH and that the other class of receptors, bicuculline-insensitive, are implicated in inhibition of both basal and evoked peptide release. The magnitude of αMSH output in the presence of a given drug will depend on whether Ca^{2+} is entering the cells in increased amounts or whether the inward Ca^{2+} currents are being counteracted (Tomiko *et al.*, 1981). Ca^{2+} entry is in turn related to the rate of cell firing and to slow membrane depolarizations, both of which are modified by $GABA_A$ agonists. Experimentally it is difficult to evaluate the relative contribution of each of these factors to changes in hormone output as the melanotrophs display tetrodotoxin (TTX)-resistant action potentials (Douglas & Taraskevich, 1980). Thus the study of $GABA_A$ effects on the phenomenon of excitation-secretion coupling in these cells becomes prone to errors of interpretation. The Ba^{2+} stimulus as used here, not only facilitates sustained cell firing (Douglas & Taraskevich, 1982) but also accentuates the development of long lasting Ca^{2+}-dependent action potentials. Possibly part of Ba^{2+} action of IL cells is similar to that seen in sympathetic ganglia or CNS neurones, where Ba^{2+} depresses M currents (outward voltage-dependent K^+ currents, Constanti *et al.*, 1981; Adams *et al.*, 1982; Halliwell & Adams, 1982). In the depolarized state, induced by $GABA_A$ receptor activation, the effect of Ba^{2+} will be fully expressed (either by maintaining potentiation or by allowing continuous firing). Thus the surge of hormone release we have measured in the presence of Ba^{2+} given simultaneously with a non-desensitizing application of GABA is probably the result of two mechanisms keeping the membrane in a depolarized state. However, one has to await a detailed electrophysiological study of Ca^{2+} and K^+ currents, ideally in a culture system, to understand more fully these facilitatory effects of $GABA_A$ receptor activation on hormone release.

As to the $GABA_B$ receptor, baclofen consistently caused depression of either spontaneous or evoked release. It is worth noting that in other preparations baclofen does not modify membrane potential, (Fox *et al.*, 1978; Dunlap, 1981; Schofield, 1983; Désarmenien *et al.*, 1984). More important, it does not suppress spikes but rather alters the ratio of Ca^{2+} and K^+ conductances. If the drug were to interfere mainly with basal release, which probably depends on spontaneous firing of these melanotrope cells (see Douglas & Taraskevich, 1982), $GABA_B$ receptor activation would result in a decreased Ca^{2+} component of each action potential (Dunlap, 1981; Désarmenien *et al.*, 1984). Consequently less Ca^{2+} would penetrate the cells, so reducing the efficiency of the excitation-secretion coupling. Our finding that the basal release declines by about 25% in the presence of (−)-baclofen argues in favour of such an interpretation: minimum changes of firing rate and alterations of Ca^{2+}-dependent action potentials being observed in other preparations (see Dunlap, 1981; Scholfield, 1983). In principle, when tested against the Ba^{2+} stimulus, one would predict an equivalent decrease in hormone output (also reflecting the average firing rate). Having observed somewhat larger depressions of evoked release than basal release (about 45%, see Figure 2b), we suspect that baclofen might have an additional effect on the basic mechanisms by which Ba^{2+} promotes cell firing and subsequent hormone release (See Douglas *et al.*, 1983). It is noteworthy to recall that even larger inhibitions of αMSH release (up to 60%) were observed in experiments performed at the slow perfusion rate. In this light we are currently investigating baclofen depression of Ca^{2+} influx in the NIL, using ion-sensititive microelectrodes (Loeffler *et al.*, 1983).

A possible limitation to this sort of study on intact NILs might be indirect effects on αMSH secretion through GABA action on neurosecretory fibres in the neurohypophysis or on nerve terminals in the IL. As to GABA modulating Ba^{2+}-evoked release of neurohypophyseal hormones, we have found (unpublished data) that Ba^{2+} (5 mM) itself has no secretagogue effect on oxytocin release from fibres of perfused neural lobes in our experimental conditions. Turning to the possibility that synapses in the IL might be affected, it is established that the innervation of the rat pars intermedia includes a large population of dopaminergic fibres having an inhibitory role in modulating αMSH secretion. Using extracellular and intracellular recordings, Douglas & Taraskevich (1978, 1980) have shown that dopamine decreases the frequency of spontaneous action potentials and might block voltage-dependent Ca^{2+} channels in melanotrophs. Were GABA to depolarize the

dopaminergic terminals, dopamine release would be enhanced (Giorguieff *et al.*, 1978, Reimann *et al.*, 1982) and a subsequent inhibition of αMSH release result. Thus, our observation that the specific $GABA_A$ agonist, isoguvacine, facilitates αMSH release, would argue in favour of a direct depolarizing effect of GABA on the IL cells. Moreover, using *in vitro* techniques, Sharman *et al.* (1982) have shown that GABA (baclofen was not tested) does not modfy K^+-evoked release of dopamine in the pars intermedia. The use of cultured melanotrophs, devoid of nerve terminals, should allow us to establish the localization of the $GABA_A$ and $GABA_B$ receptors on the intermediate lobe cells.

In conclusion GABA, and specific $GABA_A$ and $GABA_B$ agonists, modulate the release of αMSH from intact NILs. We propose that GABA is acting via a dual population of receptors, each modifying either cell firing patterns or individual action potentials and Ca^{2+} uptake by distinct mechanisms. The use of melanotrophs in a culture system should confirm the presence of these receptors on the endocrine cells and will allow more insight into the electrophysiological mechanisms involved in $GABA_A$ and $GABA_B$ modulation of excitation-secretion coupling in the intermedia lobe.

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