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MODULATION OF GABA-GATED CHLORIDE CURRENTS BY INTRACELLULAR Ca^{2+} IN CULTURED PORCINE MELANOTROPHS

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SUMMARY

1. The modulatory role of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) on γ-aminobutyric acid type A ($GABA_A$) receptor-gated Cl^- currents was investigated in dialysed and intact cells of cultured porcine pituitary intermediate lobe (IL) cells using the patch-clamp technique. In order to isolate Ca^{2+} and Cl^- currents all other membrane currents were blocked pharmacologically. Isoguvacine, a specific $GABA_A$ receptor agonist, was used to activate selectively $GABA_A$ receptor-mediated whole-cell and single-channel Cl^- currents.

2. In the whole-cell recording (WCR) configuration inward Ca^{2+} currents triggered before and/or during the application of isoguvacine (100 μM), did not inhibit the $GABA_A$ receptor-mediated response. This lack of effect of calcium currents was obtained in all situations tested, i.e. when the intracellular Ca^{2+} concentration was only weakly buffered (0·5 mM-EGTA in the pipette solution), not buffered at all (no EGTA added to the pipette solution) or when the resting $[Ca^{2+}]_i$ was buffered at 10^{-7} M (pCa 7) with internal EGTA.

3. At pCa 7, simultaneous application of isoguvacine (100 μM) and caffeine (10 mM) resulted in a 47 ± 15% reduction of the whole-cell $GABA_A$ response. In the same conditions, a ten times lower concentration of caffeine (1 mM), induced a transient increase of the $GABA_A$ response which turned into a steady-state inhibition during the subsequent applications.

4. At pCa 7, when isoguvacine (100 μM) was applied together with 3Me-His^2-TRH (50 nM), a potent analogue of the calcium-recruiting thyrotrophin-releasing hormone, the $GABA_A$ receptor-gated Cl^- current was increased by 40 ± 8%. In the absence of the Ca^{2+} chelator EGTA in the pipette solution, either potentiating or inhibitory effects of 3Me-His^2-TRH on the $GABA_A$ response were observed.

5. If a high concentration (18 mM) of the calcium chelator EGTA was included in the pipette solution, caffeine and 3Me-His^2-TRH had markedly lower effects on the $GABA_A$ response than those observed at pCa 7, suggesting that the effect of both substances was mediated by an increase in $[Ca^{2+}]_i$.

6. In the absence of extracellular Ca^{2+}, the effects of caffeine and 3Me-His^2-TRH were not significantly different from those obtained in the presence of Ca^{2+} (5 mM), suggesting that Ca^{2+} influx was not the major route for increasing $[Ca^{2+}]_i$.

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7. In the cell-attached (CA) configuration, the presence of isoguvacine (3–5 μM) in the pipette solution triggered the opening of channels displaying multiple current levels. The three main current levels observed at a membrane potential hyperpolarized by 80 mV with respect to the resting potential were $0{\cdot}55 \pm 0{\cdot}04$, $1{\cdot}01 \pm 0{\cdot}02$ and $2{\cdot}09 \pm 0{\cdot}13$ pA.

8. In the cell-attached configuration, application of caffeine (10 mM) or 3Me-His^2-TRH (50 mM) to the cell resulted in a reversible inhibition of single $GABA_A$-gated Cl^- channel activity. Thus 3Me-His^2-TRH had opposite effects on the $GABA_A$ receptor-gated Cl^- currents under whole-cell recording at pCa 7 and in the CA mode.

9. It is concluded that a rise in $[Ca^{2+}]_i$ triggered by Ca^{2+} release from intracellular stores but not by Ca^{2+} influx through voltage-dependent Ca^{2+} channels affects the activity of the $GABA_A$ receptor–Cl^- channel complex in cultured porcine IL cells. The functional implications of this effect on the control of excitation–secretion coupling by GABA are discussed.

INTRODUCTION

The endocrine cells of the porcine pituitary intermediate lobe (IL) constitute a well-suited model for studying the modulation of excitation–secretion coupling by neurotransmitters.

In situ, this homogeneous population of cells is under tonic inhibitory control by dopamine and γ-aminobutyric acid (GABA). Both neurotransmitters are localized in hypothalamo-hypophyseal nerve fibres forming synapses with IL cells (Bjorklund, Falck, Hromek, Owan & West, 1973; Baumgarten, Bjorklund, Holstein & Nobin, 1982; Oertel, Mugnaini, Tappaz, Weise, Dahl, Schmechel & Kopin, 1982; Vincent, Hökfelt, & Wu, 1982). At the postsynaptic level GABA and dopamine regulate the expression of the proopiomelanocortin (POMC) gene (Cote, Felder, Kebabian, Sekura, Reisine & Affolter, 1986; Loeffler, Demeneix, Kley & Höllt, 1988) and inhibit the release of α-melanocyte stimulating hormone (α-MSH), the major secreted post-transcriptional product of the POMC mRNA (Tomiko, Taraskevich & Douglas, 1983; Demeneix, Taleb, Loeffler & Feltz, 1986). Recently excitatory effects of thyrotrophin-releasing hormone (TRH) and acetylcholine (ACh) have been described in these cells. TRH stimulates phospholipase C (Trouslard, Demeneix & Feltz, 1989*a*; Trouslard, Loeffler, Demeneix & Feltz, 1989*b*) and acetylcholine (ACh) binds to a nicotinic receptor and activates a monovalent cation conductance leading to membrane depolarization and an increase in cell firing (Zhang & Feltz, 1990). Thus both TRH and ACh are likely to stimulate α-MSH secretion by increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). However, a major difference between the actions of TRH and ACh is the source of Ca^{2+} which contributes to the increase in $[Ca^{2+}]_i$. Indeed TRH induces release of Ca^{2+} from intracellular stores such as the endoplasmic reticulum via the action of inositol trisphosphate (IP_3), but the depolarization and increase in spike discharge induced by ACh is likely to promote Ca^{2+} influx via voltage-dependent Ca^{2+} channels.

Recent results obtained on outside-out membrane patches from cultured porcine IL cells strongly suggest that an elevated intracellular Ca^{2+} concentration inhibits the activity of the $GABA_A$ receptor–Cl^- channel complex (Taleb, Trouslard, Demeneix, Feltz, Bossu, Dupont & Feltz, 1987*b*). Similar results have been obtained

on primary sensory neurones of the frog (Inoue, Oomura, Yakushi & Akaike, 1986; Behrends, Maruyama, Tokutomi & Akaike, 1988).

In our study we were particularly interested in the physiological aspects of this observation in porcine IL cells. We wondered if a rise in intracellular Ca^{2+} concentration, which stimulates secretion under physiological conditions, could inhibit the activity of the $GABA_A$ receptor–Cl^- channel complex. To test this possibility, we have used the whole-cell recording (WCR) and cell-attached (CA) configurations of the patch-clamp technique, in order to study this aspect in dialysed and intact cells. Our results suggest that Ca^{2+} release from intracellular stores but not Ca^{2+} influx through voltage-dependent Ca^{2+} channels inhibits the $GABA_A$ receptor activity. The functional implication of this finding is that during cell firing the $GABA_A$ receptor will probably retain its full inhibitory efficiency to modulate electrical activity of melanotrophs. However, during the action of neurotransmitters or hormones which act via the release of Ca^{2+} from intracellular reservoirs, the $GABA_A$ receptor activity will be strongly depressed. This could provide a possible positive feedback mechanism for α-MSH secretion.

METHODS

Cell culture

The technique for culturing porcine IL cells has been extensively described elsewhere (Demeneix *et al.* 1986).

In brief, neuro-intermediate lobes (NIL) of female pigs were collected at the slaughterhouse in Locks solution. The NILs were dissociated enzymatically using a two step protocol involving collagenase (type IV, Sigma; 0·5 mg ml^{-1}) and trypsin (Seromed; 0·5 mg ml^{-1}) treatment. After mechanical dissociation the cells were separated from cellular debris using a Percoll kit (Pharmacia, Sweden) or a bovine serum albumin gradient. The cells were resuspended in culture medium and plated in 35 mm culture dishes (Costar). The composition of the culture medium was the following: 70% (v/v) Dulbecco's modified Eagle's medium (DMEM; Gibco); 25% (v/v) HAM F12 (Gibco); 5% (v/v) fetal calf serum (Gibco); penicillin (100 u ml^{-1}, Gibco); streptomycin (0·1 mg ml^{-1}, Gibco) and kanamycin (0·2 mg ml^{-1}, Seromed). Cultures were maintained at 37 °C in a 95% air and 5% CO_2 humidified atmosphere. Electrophysiological experiments were performed on cells cultured for 3–10 days.

Electrophysiological experiments

Patch-clamp recordings were performed under voltage-clamp in the whole-cell recording and cell-attached configurations (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) using an EPC7 amplifier (List Electronics, Darmstadt, Germany). Electrodes were pulled from haematocrit glass tubes and had a final tip resistance of 3–6 MΩ for WCR and 8–10 MΩ for cell-attached recording. Electrodes were coated with a thick layer of a synthetic polymer (RTV 141, Rhône Poulenc) in order to reduce the capacitance of the glass. Current and voltage traces were stored without filtering on videotape (pulse code modulator, Sony 701 ES) with a DC to 10 kHz bandwidth. Whole-cell currents were analysed after low pass filtering at 1 kHz and plotted on paper using a chart recorder (Gould 2200S) or a Hewlett Packard plotter (HP 7470A) in combination with the program pclamp5 (Axon Instruments, USA). Alternatively data were stored under digital form on the hard disc on an IBM-compatible microcomputer (Olivetti M290).

For analysis, single-channel data were low-pass filtered at 1 kHz and digitized at 5 kHz. The analysis of the channel amplitudes and fitting of amplitude histograms were performed using an interactive program and a DEC compatible computer (Plessey 6220). Individual traces were plotted on the HP plotter.

Solutions

The extracellular medium for experiments in whole-cell recording (WCR) and cell-attached (CA) configurations was similar and had the following composition (in mM): choline chloride, 120; TEA-

Cl, 20; KCl, 5; Ca^{2+}, 2 (CA) or 5 (WCR); $MgCl_2$, 1; HEPES, 5; glucose, 10; Phenol Red (0·5%), 2 μl ml^{-1}; pH 7·3. Choline chloride was substituted for NaCl in order to block Na^+ currents and TEA-Cl was added to reduce K^+ currents.

For experiments in the WCR configuration the pipette solution contained (in mM): CsCl, 140; $MgCl_2$, 2; HEPES, 10; Phenol Red (0·5%), 2 μl ml^{-1}. The resting free Ca^{2+} concentration was usually adjusted to 10^{-7} M (pCa 7) and occasionally to 10^{-8} M (pCa 8) by adding 5 or 0·9 mM-$CaCl_2$ to 10 mM-EGTA, respectively. In some experiments 18 mM-EGTA (without added Ca^{2+}) was included in the pipette solution in order to prevent or minimize a rise in intracellular Ca^{2+} concentration following release of Ca^{2+} from intracellular stores.

In the cell-attached configuration the composition of the pipette solution was the same as that of the extracellular medium, except that isoguvacine (3–5 μM) alone or together with pentobarbitone (50–100 μM) was added. All experiments were performed at room temperature (20–22 °C).

Convention

In all figures inward currents are represented as downward (negative) deflections of the current trace. The same convention was applied to single-channel currents which were recorded in the CA configuration. In the WCR mode the values of pipette potential correspond to the real membrane holding potential (HP). In the figures illustrating recordings obtained in the CA mode, the potential values indicated correspond to the pipette potential. These values are expressed with respect to the resting membrane potential which corresponds to a pipette potential of 0 mV. Positive pipette potentials stand for hyperpolarizations and negative pipette potentials for depolarizations of the membrane patch

Application of substances

Drugs were applied by a microperfusion system (Krishtal & Pidoplichko, 1980). All substances were prepared as stock solutions in distilled water, except caffeine which was directly dissolved at final concentration in the extracellular solution.

The substances were diluted to their final concentration in extracellular medium just before use.

All statistical results are expressed as mean ± standard deviation.

RESULTS

In pars intermedia cells it has been previously shown that the activation of $GABA_A$ receptors increases a membrane conductance specific for Cl^- ions (Taraskevich, & Douglas, 1985; Demeneix *et al.* 1986; Taleb, Loeffler, Trouslard, Demeneix, Kley, Höllt & Feltz, 1987*a*; Taleb *et al.* 1987*b*). This effect is reproduced by isoguvacine a selective agonist of $GABA_A$ receptors which does not interfere with $GABA_A$ receptors (Hill & Bowery, 1981). In our experiments all membrane currents other than Cl^- and Ca^{2+} currents were blocked pharmacologically (see Methods) and the Cl^- equilibrium potential (E_{Cl}) was set at −2 mV, in the WCR configuration. In these conditions, the application of isoguvacine (100 μM) by microperfusion triggers an inward current at a holding potential (HP) of −80 mV, with a concomitant increase in membrane current noise (see Fig. 1*A*). This total current is characteristic of the activation of $GABA_A$ receptors and will be referred to as the $GABA_A$ response.

In the first part of this work, we have tested the effect of increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) on the $GABA_A$ receptor-gated whole-cell Cl^- currents. The rise in $[Ca^{2+}]_i$ was achieved either by promoting Ca^{2+} influx through voltage-dependent Ca^{2+} channels or by inducing Ca^{2+} release from intracellular stores using caffeine or 3Me-His^2-TRH, a selective agonist of thyrotrophin-releasing hormone (TRH) (Vale, Rivier, & Burgus, 1971).

Effect of Ca^{2+} influx through voltage-dependent Ca^{2+} channels on the $GABA_A$ response

Cultured pars intermedia cells possess several types of voltage-dependent Ca^{2+} channels (Taleb, Trouslard, Demeneix & Feltz, 1986) which are activated upon membrane depolarization. They contribute to the generation of Ca^{2+}-dependent action potentials which are important for promoting α-MSH secretion (Tomiko *et al.* 1983) by increasing intracellular Ca^{2+} concentration (Nemeth, Taraskevich & Douglas, 1990).

TABLE 1. Effect of Ca^{2+} influx through voltage-dependent Ca^{2+} channels on the amplitude of $GABA_A$ gated whole-cell Cl^- currents

$[Ca^{2+}]_i$ (M)	n	Amplitude of isoguvacine response: Control (pA)	Amplitude of isoguvacine response: Preceded by I_{Ca} (pA)	Amplitude of I_{Ca} (pA)
10^{-8}	10	100±80	102±82	45±33
10^{-7}	7	149±72	152±74	76±2

The recordings were performed in the whole-cell configuration and $GABA_A$ receptors were activated by isoguvacine (10^{-4} M). The resting calcium concentration ($[Ca^{2+}]_i$) was set at 10^{-8} M (pCa 8) or 10^{-7} M (pCa 7). The third and fourth columns represent the mean amplitude of the $GABA_A$ response in control conditions and when preceded by a series of three to eight calcium currents, respectively. Ca^{2+} currents (I_{Ca}) were activated by step depolarization from −80 to 0 mV for 100 ms. The last column represents the amplitudes of the Ca^{2+} currents recorded. All values are given as means±standard deviation, and 'n' represents the number of cells tested.

In our experimental conditions, Ca^{2+} currents were activated by depolarizing the membrane from a steady holding potential (HP) of −80 mV to a test potential of 0 mV during 100 ms.

We compared the amplitude of membrane responses to isoguvacine recorded in the absence of Ca^{2+} currents and when preceded by a single or up to eight Ca^{2+} currents (Fig. 1*A*). This protocol was performed in conditions where the resting intracellular Ca^{2+} concentration was set at 10^{-8} M (pCa 8) or 10^{-7} M (pCa 7). The results obtained are summarized in Table 1.

It can be seen that the amplitudes of the $GABA_A$ responses when preceded by Ca^{2+} currents are similar to those obtained in control conditions, i.e. in the absence of Ca^{2+} influx. These results suggest that Ca^{2+} influx through voltage-dependent Ca^{2+} channels does not inhibit $GABA_A$ receptor-gated Cl^- currents.

However, recent data obtained in primary sensory frog neurones (Inoue *et al.* 1986) suggest that the effect of Ca^{2+} influx is transient and fast, occurring within 1 s. In order to avoid any time lag between Ca^{2+} influx and application of isoguvacine, Ca^{2+} currents were activated every 5 s before and during a sustained application of isoguvacine (Fig. 1*A*, right).

A Ca^{2+}-dependent inhibition of the $GABA_A$ response should normally result in a transient reduction of the steady inward Cl^- current which would be monitored as an inward current relaxation after each Ca^{2+} current as described by Behrends *et al.* (1988).

Figure 1*B* illustrates, at a faster time scale, averaged Ca^{2+} currents recorded in the

same cell as in Fig. 1*A*. The amplitude of these Ca^{2+} currents are almost the same before, during and after application of isoguvacine. During the activation of $GABA_A$-gated Cl^- current (Fig. 1*B*, middle trace) an outward current relaxation is observed when the potential is stepped back from 0 to −80 mV. This relaxation is due in part

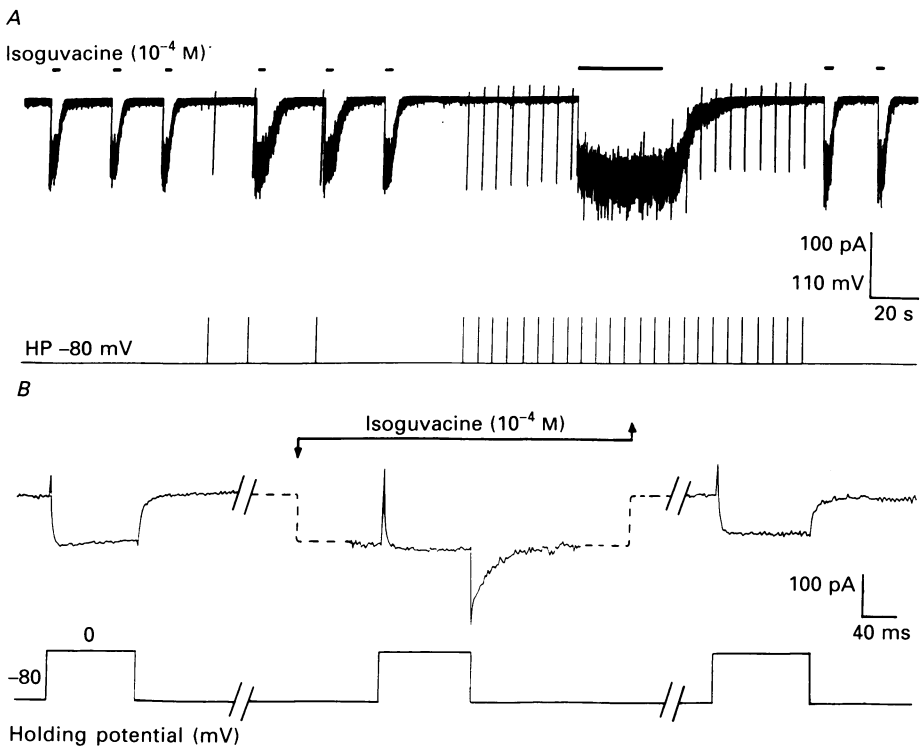

Fig. 1. Effect of calcium influx through voltage-dependent Ca^{2+} channels on the amplitude of the $GABA_A$ response. *A*, $GABA_A$ receptor-gated whole-cell inward Cl^- currents are evoked by transient and sustained applications of isoguvacine (10^{-4} M). Duration of application is shown by the horizontal bars. The steady-state holding potential was set at −80 mV. Voltage steps from −80 to 0 mV during 100 ms promote Ca^{2+} influx through voltage-dependent Ca^{2+} channels. The $GABA_A$ response is neither inhibited when preceded by a single or a series of Ca^{2+} currents, nor when Ca^{2+} currents are triggered during a sustained application of isoguvacine. *B*, averaged traces of the Ca^{2+} currents evoked before, during and after the sustained application of isoguvacine. During application of isoguvacine an outward current relaxation is observed when the membrane potential is stepped back from 0 to −80 mV. $GABA_A$-gated Cl^- currents are not inhibited during Ca^{2+} influx, since in this latter case a relaxation of the opposite polarity would have been expected. The difference in holding current (dashed line) corresponds to the amplitude of Cl^- current activated by isoguvacine. Note that the amplitudes of Ca^{2+} and Cl^- currents are in the same range. The resting free calcium concentration was set at 10^{-7} M (pCa 7).

to the deactivation of Ca^{2+} channels and probably to a slight voltage dependence of the $GABA_A$ receptor-gated Cl^- current (Gray & Johnston, 1985). An inhibitory effect of Ca^{2+} influx on the $GABA_A$ response would have resulted in a relaxation of the opposite polarity. We never observed such an inhibition (ten cells tested).

A possible explanation for the lack of effect of inward Ca^{2+} currents on the $GABA_A$ response could be that in our recording conditions the resting intracellular Ca^{2+} concentration was adjusted to 10^{-7} M (pCa 7) using a relatively large concentration of EGTA (10 mM) to which we added 5 mM-$CaCl_2$. This could result in a strong buffering of the increase in $[Ca^{2+}]_i$ triggered by Ca^{2+} influx through voltage-dependent Ca^{2+} channels. Therefore, we repeated the same type of experiments in conditions where (i) intracellular Ca^{2+} was only weakly buffered by 0·5 mM-EGTA in the pipette or (ii) in the absence of EGTA. Voltage steps from −80 to 0 mV lasting 300 ms were used to promote Ca^{2+} influx through voltage-dependent Ca^{2+} channels. Such Ca^{2+} currents were triggered at 5 s intervals before and during the application of isoguvacine (100 μM). In the experiments where 0·5 mM-EGTA was added to the pipette solution we recorded mean $GABA_A$ responses of 170 ± 84 pA ($n = 14$) in control conditions. The mean amplitude of these currents was 166 ± 76 pA ($n = 14$) when Ca^{2+} currents of 85 ± 59 pA were activated before and during the application of isoguvacine. In the absence of EGTA in the intracellular solution the mean amplitude of the $GABA_A$ receptor-gated Cl^- current was 81 ± 27 pA ($n = 5$) in control conditions and 85 ± 27 pA when calcium currents of 51 ± 18 pA ($n = 5$) were triggered before and during the application of isoguvacine. Thus either in the presence of a low concentration of EGTA or in the absence of EGTA in the pipette solution, inward Ca^{2+} currents failed to inhibit the $GABA_A$ response.

Taken together these results suggest that Ca^{2+} influx through voltage-dependent Ca^{2+} channels does not inhibit the activity of $GABA_A$ receptors in our preparation.

Effect of Ca^{2+}-mobilizing substances on the $GABA_A$ receptor-gated Cl^- current

Since Ca^{2+} influx had no effect on the $GABA_A$ response, we decided to examine the effect of increasing intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by triggering the release of Ca^{2+} from intracellular stores. Therefore we applied caffeine (Sato, Ozaki & Karaki, 1988; Thayer, Hirning & Miller, 1988*a*) and 3Me-His^2-TRH a potent structural analogue of TRH (Vale *et al.* 1971). Activation of TRH receptor is known to mobilize intracellular Ca^{2+} (Schlegel & Wollheim, 1984; Gershengorn & Thaw, 1985).

Effect of caffeine

Caffeine is a methylxanthine known to promote large increases of $[Ca^{2+}]_i$ by triggering the release of Ca^{2+} from endoplasmic reticulum in various cell types (Miller, 1988; Sato *et al.* 1988; Thayer *et al.* 1988*a*). We have used this substance in order to increase the $[Ca^{2+}]_i$ independently of Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

Figure 2*A* shows a typical result obtained. All recordings were made with a pipette solution where the resting Ca^{2+} concentration was buffered at 10^{-7} M (pCa 7). In the experimental protocol used, we applied first isoguvacine alone (100 μM) and then together with 10 mM-caffeine.

Caffeine inhibited the $GABA_A$-gated whole-cell Cl^- current by 45 ± 17% ($n = 9$, range 18–48%). A reduction of the $GABA_A$ response was observed in all cells tested ($n = 9$). The inhibition was reversible in five out of eight cells where it was examined.

In these five cells the amplitude of the response recovered to $91 \pm 16\%$ (range: 63–100%) of the control value (i.e. before application of caffeine).

Effect of 3Me-His²-TRH on $GABA_A$-gated Cl^- currents

In order to examine if Ca^{2+} release which occurs in these cells under physiological conditions has the same effect as caffeine, we applied a selective and potent agonist of thyrotrophin-releasing hormone (TRH): 3Me-His²-TRH.

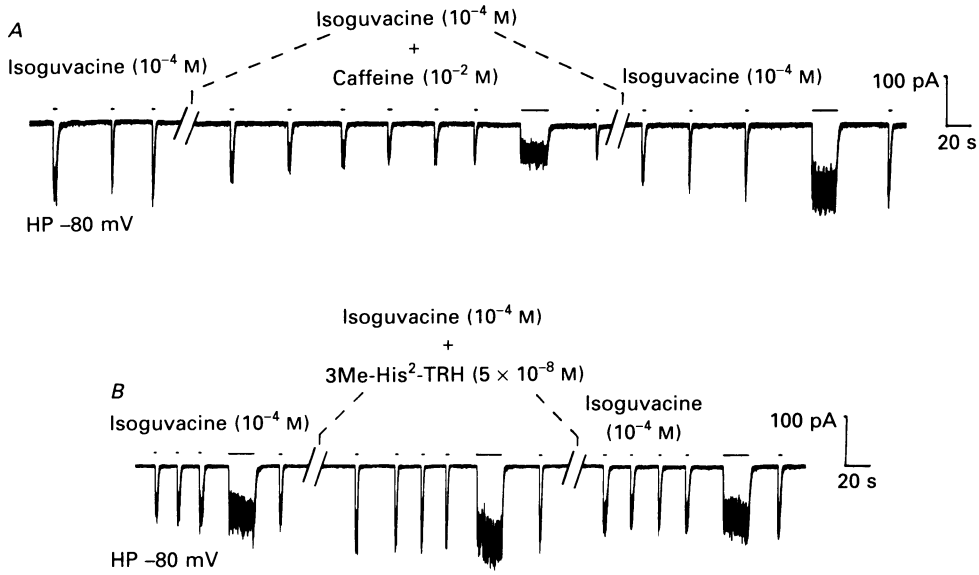

Fig. 2. Effects of caffeine (10^{-2} M) and 3Me-His²-TRH (5×10^{-8} M) on the $GABA_A$ response. *A*, application of isoguvacine (10^{-4} M) evokes an inward current (left), which is inhibited when isoguvacine is applied together with caffeine (middle trace). Note that the inhibitory effect of caffeine is progressive since maximal inhibition is only observed after the third application where it reaches a plateau. The effect is fully reversible (right). *B*, same protocol as in Fig. 2*A*, but isoguvacine is applied together with 3Me-His²-TRH, a potent thyrotrophin-releasing hormone (TRH) receptor agonist. 3Me-His²-TRH increases the amplitude of the $GABA_A$ response reversibly. In contrast to the inhibitory effect of caffeine, the potentiating effect of 3Me-His²-TRH is already maximal during the first application. The resting Ca^{2+} concentration was set at 10^{-7} M (pCa 7) in both experiments.

Membrane receptors for TRH are present on cultured porcine pars intermedia cells (Trouslard *et al.* 1989*a*, *b*) and their activation stimulates the break-down of membrane bound phosphoinositides (PIP_2) which generates inositol trisphosphate (IP_3). The latter will in turn trigger the release of calcium from intracellular stores, such as the endoplasmic reticulum (Berridge & Irvine, 1989). In these cells TRH and its agonist 3Me-His²-TRH have been shown to increase cell firing and promote α-MSH secretion (Trouslard *et al.* 1989*a*, *b*). In our preparation, the effect of 3Me-His²-TRH on phosphoinositide break-down is maximal at 50 nM. Therefore we used this concentration of agonist in our study.

In five out of seven cells tested, 3Me-His^2-TRH (50 nM) increased the amplitude of the $GABA_A$ response by 40±8% (range 34–50%). In four cells we observed a full recovery to the control value. In the remaining cell no recovery was observed, the amplitude of the Cl^- current being the same as in the presence of 3Me-His^2-TRH. These results were obtained when the resting $[Ca^{2+}]_i$ was set at pCa 7. In another set of experiments, we decided to test the effect of 3Me-His^2-TRH (50 nM) on the $GABA_A$ response in eleven cells where no EGTA was added to the intracellular solution. When 3Me-His^2-TRH (50 nM) was applied together with isoguvacine (100 μM), the $GABA_A$ receptor-gated Cl^- current was reversibly inhibited by 49±14% in three cells, increased by 66±32% in five other cells and was not affected at all in the three remaining cells. These results suggest that in the absence of the calcium chelator EGTA in the intracellular medium both inhibitory and potentiating effects of the $GABA_A$ response by 3Me-His^2-TRH are observed in IL cells.

Since caffeine and 3Me-His^2-TRH affected the amplitude of the $GABA_A$-gated Cl^- current, although in opposite directions at pCa 7, we wondered if both effects were mediated through a rise in $[Ca^{2+}]_i$, or if the action of 3Me-His^2-TRH might involve another pathway. Therefore, we repeated the same type of experiments, first in conditions where the rise in $[Ca^{2+}]_i$ was minimized or blocked by including in the pipette solution a high concentration (18 mM) of the Ca^{2+} chelator EGTA, and second in conditions where Ca^{2+} was replaced by Mg^{2+} in the extracellular medium.

Role of intracellular Ca^{2+} in the effect of caffeine and 3Me-His^2-TRH on the $GABA_A$ response

Effect of caffeine

The effect of caffeine on the amplitude of the $GABA_A$ response was tested in conditions where the resting calcium concentration was buffered at very low levels by including a high concentration (18 mM) of the Ca^{2+} chelator EGTA in the pipette solution. This should also prevent or minimize large increases in $[Ca^{2+}]_i$ triggered by caffeine application. In seventeen out of twenty cells tested, caffeine inhibited (n = 11) or did not affect at all (n = 6) the $GABA_A$ response. The mean inhibition of the $GABA_A$ receptor-gated Cl^- current was 18±15% (range 0–37%). This value is significantly different (t test, $P < 0{\cdot}1\%$) from the value obtained in control conditions, in which $[Ca^{2+}]_i$ was buffered at pCa 7 and in which the $GABA_A$ response was systematically inhibited in all cells tested (see previous section). These results are summarized in Fig. 3*A*. When the six cells in which the effect of caffeine was completely blocked by the high concentration of EGTA are not considered in our statistical analysis, the mean inhibitory effect of caffeine on the $GABA_A$ response is 28±9% (n = 11, range 16–37%). This value is still significantly different from control (t test, $P < 1\%$). Interestingly, in three cells out of twenty, caffeine increased the $GABA_A$ response by 21±12% (range 15–35%). The importance of this finding will be considered in the Discussion section.

Effect of 3Me-His^2-TRH

In the presence of 18 mM-internal EGTA, an effect of 3Me-His^2-TRH (50 nM) was observed in 46% of the cells tested (n = 9). In five cells 3Me-His^2-TRH had no effect on the $GABA_A$ response, in the four other cells it increased the response by only

$13 \pm 6\%$ (range 6–21 %). Again this result is significantly different (t test, $P < 0{\cdot}1\%$) from the effect of 3Me-His^2-TRH observed in control conditions (resting $[Ca^{2+}]_i$ buffered at pCa 7, see Fig. 3*B*)

Taken together these results show that the effects of both caffeine and 3Me-His^2-TRH on the $GABA_A$ response are mediated by a rise in intracellular Ca^{2+}

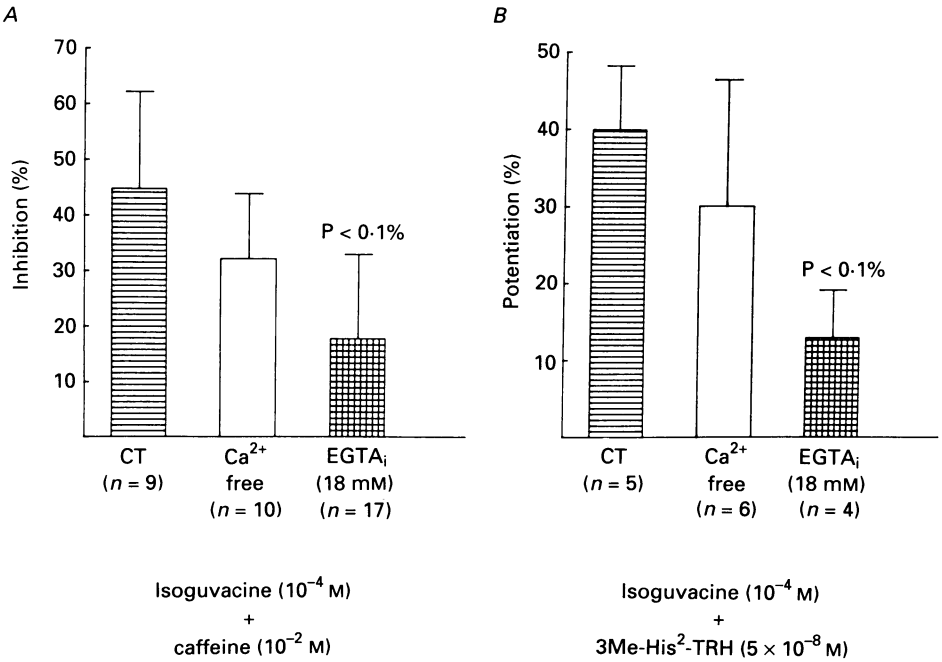

Fig. 3. Role of extracellular and intracellular Ca^{2+} in the modulation of $GABA_A$ response by caffeine (10^{-2} M) and 3Me-His^2-TRH (5×10^{-8} M). Inhibitory effect of caffeine (*A*) and potentiation effect of 3Me-His^2-TRH (*B*) on the amplitude of the $GABA_A$ response in control conditions (left column), in the absence of extracellular Ca^{2+} (middle column) and in the presence of a high concentration of EGTA (18 mM) in the intracellular solution (right column). The results are expressed as the percentage of inhibition (*A*) or potentiation (*B*), with respect to the control response, i.e. recorded in the absence of caffeine or 3Me-His^2-TRH. Each column represents mean values $\pm$ standard deviations. Removing extracellular Ca^{2+} does not significantly affect the mean effect of caffeine and 3Me-His^2-TRH. In the presence of 18 mM-internal EGTA, the inhibitory effect of caffeine and potentiation effect of 3Me-His^2-TRH are significantly reduced with respect to control (t test, $P < 0{\cdot}1\%$ and $P < 0{\cdot}1\%$ respectively). In the experiments concerning the effects of 3Me-His^2-TRH, only responsive cells were included in the figure, since it is not possible to distinguish between a lack of effect of 3Me-His^2-TRH or the absence of TRH receptors on a given cell.

concentration. A high concentration of EGTA (18 mM) in the intracellular medium reduced (i) the fraction of cells where these modulatory effects were observed and (ii) decreased significantly the amplitude of these effects in the cells where it could still be observed. In addition, with caffeine an increase of the $GABA_A$ response was observed in three out of twenty cells tested.

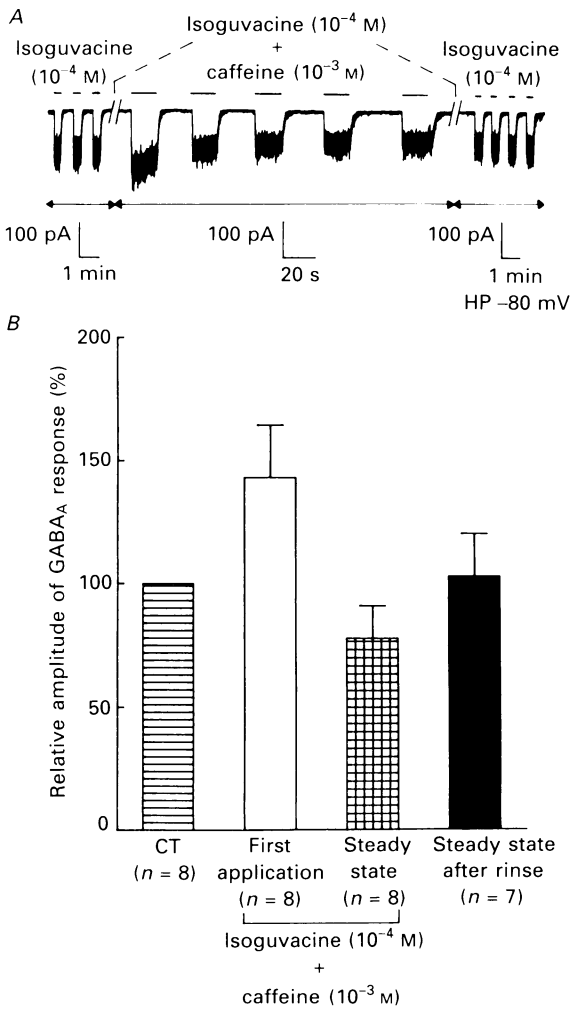

Fig. 4. Effect of a low concentration (1 mM) of caffeine on the $GABA_A$ response. Experiments were carried out in the WCR configuration with a resting $[Ca^{2+}]_i$ fixed at pCa 7. The holding potential was −80 mV. *A*, concomitant application of isoguvacine (10^{-4} M) and caffeine (1 mM) causes a transient increase of the $GABA_A$ response during the first application, which turns into an inhibition after the third application. The effect of caffeine is fully reversible. The potentiating effect of caffeine decreases already during the first application of caffeine. Note also the difference in time scales of the current traces. *B*, histogram summarizing the effect of 1 mM-caffeine on the $GABA_A$ response in eight different cells tested. The amplitude of the control $GABA_A$ response, recorded in the absence of caffeine, was normalized to 100% (first column). The second column shows the potentiation of the $GABA_A$ response measured at the peak of the whole-cell Cl^- current during the first caffeine application, and the third column the steady-state inhibition of the response after the third application. The last column illustrates that the effect of caffeine on the $GABA_A$ response is reversible.

Effect of a low concentration of caffeine (1 mM) on the $GABA_A$ response

In the previous section we reported that in the presence of 18 mM-EGTA in the pipette solution, the application of a high concentration of caffeine (10 mM) increased the $GABA_A$ response in three out of twenty cells. This suggested that when the rise in $[Ca^{2+}]_i$ is minimized in the presence of a high concentration of calcium buffer, the $GABA_A$ receptor activity was increased by caffeine. In order to check if a similar effect can be observed in our standard WCR recording configuration (resting $[Ca^{2+}]_i$ fixed at pCa 7), we decided to test the effect of a 10 times lower concentration of caffeine (1 mM) on the $GABA_A$ response. A typical result is illustrated in Fig. 4. Figure 4*A* shows that upon the first concomitant application of caffeine (1 mM) and isoguvacine (10^{-4} M) the peak of the $GABA_A$ receptor-gated Cl^- current increases but that the amplitude of this current decreases subsequently with time of application. This decrease continued to occur during the three subsequent co-applications of both substances and reached a steady-state level where the $GABA_A$ response was inhibited with respect to control responses. This effect of a low concentration of caffeine (1 mM) was fully reversible and was consistently observed in all eight cells tested. The histogram in Fig. 4*B* summarizes the effect of 1 mM-caffeine in the eight cells examined. The control $GABA_A$ response (first column) was normalized to 100%. During the first co-application of caffeine and isoguvacine we observed a 43 ± 22% increase of the $GABA_A$-gated Cl^- current peak (second column) which turned into a steady-state inhibition of 22 ± 13% with respect to the control response after the third application (third column). In seven cells in which it was tested, we observed a full recovery of the $GABA_A$ response after rinsing caffeine (fourth column).

Role of extracellular Ca^{2+} in the effects of caffeine and 3Me-His^2-TRH

In this set of experiments we investigated the possibility of a contribution of Ca^{2+} influx to the effects of caffeine and 3Me-His^2-TRH.

The experimental conditions were similar to those used to study the effects of caffeine and 3Me-His^2-TRH described previously, except that these substances were applied in an extracellular solution where Ca^{2+} (5 mM) was replaced by an equivalent amount of Mg^{2+}. We chose to substitute Ca^{2+} by Mg^{2+} because other divalent cations have been reported to block the $GABA_A$ response (Kaneko & Tachibana, 1986).

Effect of caffeine

In the absence of extracellular Ca^{2+}, caffeine inhibited the $GABA_A$ response by 32 ± 12% (range 17–52%) in all cells tested ($n = 10$). This effect is slightly but not significantly different (at a $P = 5$% confidence interval limit) from the inhibition observed in the presence of 5 mM-extracellular Ca^{2+} (Fig. 3*A*).

Effect of 3Me-His^2-TRH

In six out of seven cells where 3Me-His^2-TRH was tested in the absence of extracellular Ca^{2+}, we observed a mean potentiation of the $GABA_A$ response of 30 ± 17% (range 6–56%) compared to 40 ± 8% ($n = 5$) in control conditions. However, these values are not statistically different (*t* test at a confidence interval of 5%).

Therefore, our data suggest that caffeine and 3Me-His^2-TRH modulate the amplitude of $GABA_A$ receptor-gated Cl^- currents via an increase in $[Ca^{2+}]_i$ which seems primarily due to the release of Ca^{2+} from intracellular stores and not to involve Ca^{2+} influx.

Effect of $[Ca^{2+}]_i$ on the $GABA_A$ response in intact cells

The first part of our work, described in the previous section, was performed using the whole-cell recording (WCR) configuration of the patch-clamp technique. This situation leads to a dialysis of the intracellular medium by the pipette solution and will therefore affect the concentrations of metabolic factors and second messenger molecules present in the intracellular compartment. In this respect, we wanted to check if a rise in $[Ca^{2+}]_i$ triggered by caffeine and 3Me-His^2-TRH in an intact, non-dialysed, cell would have the same effect on $GABA_A$ receptor activity as in the WCR configuration.

Thus, in the second part of this study we have first characterized the single-channel current levels underlying the $GABA_A$ receptor activity in physiological Cl^- gradients and second, tested the effect of caffeine and 3Me-His^2-TRH on the activity of these single $GABA_A$-gated Cl^- channels. This study was performed in the cell attached configuration of the patch-clamp technique, keeping the intracellular medium intact especially with respect to Ca^{2+} buffering and second messenger systems.

Characterization of single $GABA_A$ receptor-gated Cl^- channels on intact IL cells

In order to observe $GABA_A$ receptor-gated Cl^- channel currents in isolation, Na^+ channel currents were blocked by substituting choline chloride for NaCl in the extracellular solution. A large fraction of the voltage-dependent K^+ channels were blocked by TEA (see Methods section). Most of our recordings were made at a pipette potential of +80 mV, i.e. at 80 mV more hyperpolarized than the resting membrane potential. Hyperpolarization of the membrane patch prevented the activation of voltage-dependent channels which were obviously not all blocked in our experimental conditions. The unitary currents through these channels were outward at all depolarized potentials, suggesting that these channels were probably K^+ channels (see Fig. 5*B*).

Application of large hyperpolarizations to the membrane patch were also necessary in order to improve resolution of the most frequently occurring current sublevel of the $GABA_A$-gated Cl^- channel, which was of small amplitude (see below).

Figure 5*A* illustrates a typical trace obtained at a pipette potential of +80 mV in a IL cell with 3 μM-isoguvacine present in the electrode in order to activate selectively $GABA_A$-gated Cl^- channels. Figure 5*B* shows a recording obtained in conditions in which the pipette did not contain isoguvacine. When the membrane patch was hyperpolarized (pipette potentials of +80 or +100 mV) no channel openings were observed. However, at depolarized potentials (pipette potentials of −80 or −100 mV) unitary outward currents were detected, suggesting that the membrane patch contained voltage-dependent channels.

As illustrated in Fig. 5*A*, the presence of isoguvacine in the pipette solution triggered the opening of $GABA_A$ receptor-gated Cl^- channels which displayed

multiple current levels. The openings of these channels were typically of short duration, a condition which rendered a detailed analysis of their individual amplitudes difficult. As pentobarbitone was shown to prolong openings of $GABA_A$ receptor-gated Cl^- channels without affecting their conductance (MacDonald,

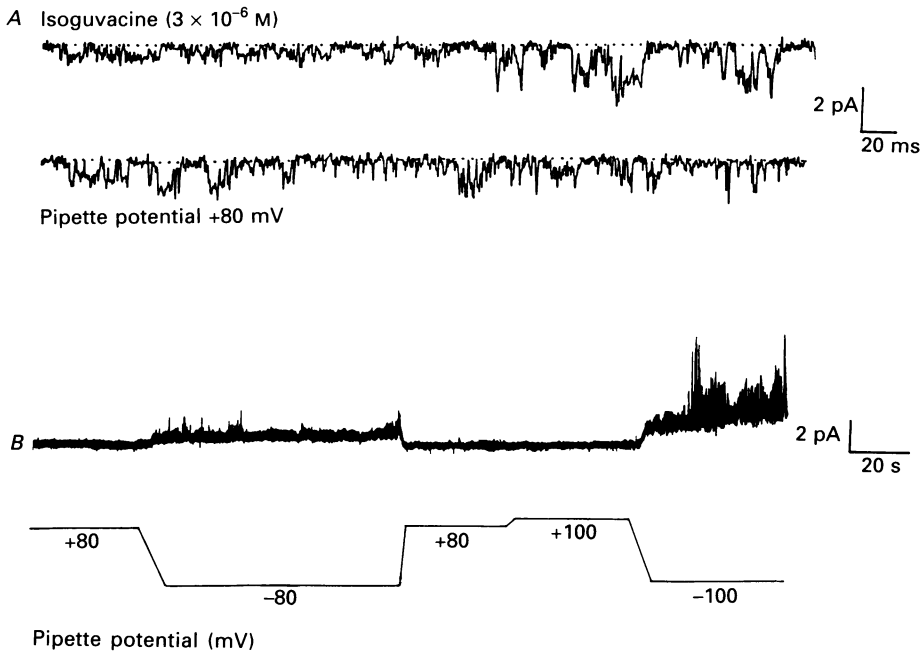

Fig. 5. Single-channel activity recorded in the cell-attached configuration in the presence (*A*) and in the absence (*B*) of isoguvacine (3 μM) in the pipette solution. *A*, typical pattern of activity recorded in the presence of isoguvacine at a pipette potential of +80 mV, i.e. 80 mV more hyperpolarized than the resting potential, in order to avoid activation of voltage-dependent channels. The $GABA_A$ receptor-gated channel activity is characterized by brief openings displaying multiple current levels. *B*, single-channel activity recorded in the absence of isoguvacine in the pipette. Note that at hyperpolarized potentials (+80 and +100 mV) no channel activity is recorded. However, when the membrane patch is depolarized (−80 or −100 mV), unitary outward currents are recorded corresponding to the activation of K^+ channels. This shows that the unitary currents observed in Fig. 4*A* correspond to single $GABA_A$ receptor-gated channels. The latter can be observed in isolation when the membrane patch is hyperpolarized with respect to the resting potential.

Rogers & Twyman, 1989), this substance was added to the pipette solution in order to facilitate analysis of the isoguvacine-gated channel amplitudes. Figure 6*A* shows, on a slow and fast time scale elementary currents with 5 μM-isoguvacine and 75 μM-pentobarbitone present in the pipette.

Figure 6*B* illustrates the amplitude histogram obtained at a pipette potential of +60 mV (60 mV hyperpolarized with respect to resting potential). This histogram could be fitted with the sum of three Gaussian functions suggesting the presence of three current levels of 0·51, 0·84 and 1·68 pA respectively in the case of this membrane patch.

Figure 6*C* shows the current-to-voltage relationship of these unitary current levels in the same cell. It can be seen that the amplitude of these current levels decreases when the pipette potential is progressively lowered from +80 to +40 mV. At −40 mV (40 mV depolarized with respect to rest) the single-channel current is outward, i.e. has reversed polarity.

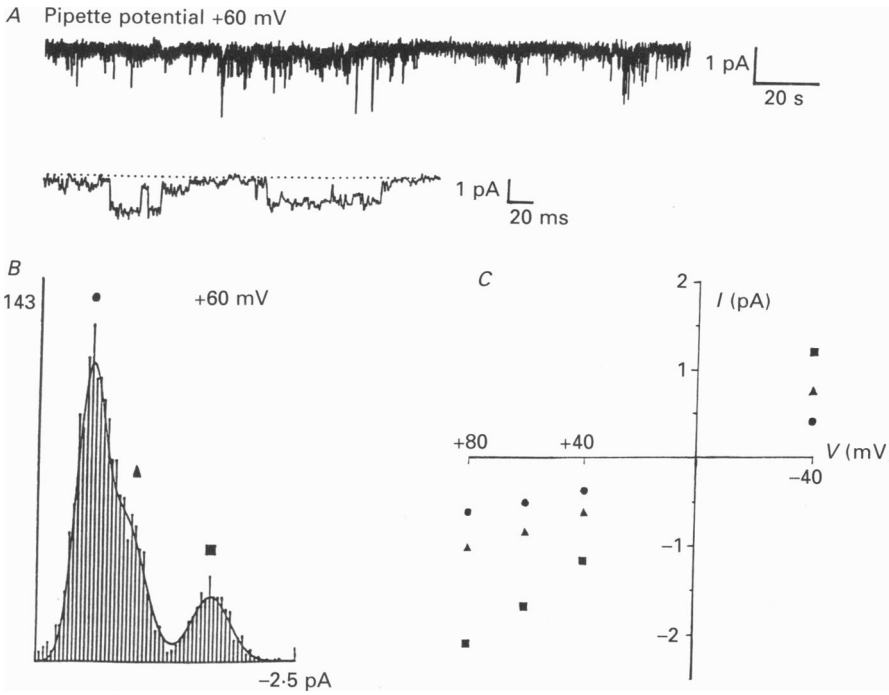

Fig. 6. Characterization of the main conductance states of $GABA_A$-gated Cl^- channel in physiological Cl^- ion gradient. The recording was performed in the cell-attached configuration in the presence of isoguvacine (5 μM) and pentobarbitone (75 μM) in order to prolong channel open time. *A*, typical current trace shown at a slow and fast time scale. The recording was obtained at a pipette potential of +60 mV (60 mV hyperpolarized with respect to resting potential). Note that in the presence of pentobarbitone the duration of channel openings has increased (compare with Fig. 5*A*). *B*, amplitude histogram of the channel activity illustrated in Fig. 6*A* at a pipette potential of +60 mV. The histogram could be fitted with a sum of three Gaussian functions corresponding to three main current sublevels of 0·51 (●), 0·84 (▲) and 1·68 pA (■). *C*, current to voltage (*I/V*) relationship of the three current sublevels shown in *B*. The amplitude of the three current sublevels is plotted as a function of pipette potential. Positive potential values correspond to hyperpolarizations and negative potentials stand for depolarizations.

In five cell-attached patches examined in more detail, the main values of the most frequently occurring current sublevels were 0·63 ± 0·04, 1·04 ± 0·02 and 2 ± 0·05 pA at a pipette potential of +80 mV (hyperpolarized). In three patches which were tested only in the presence of isoguvacine (3 μM), i.e. without pentobarbitone, the current sublevels had values of 0·55 ± 0·04, 1·01 ± 0·02 and 2·09 ± 0·13 pA at a pipette

potential of +80 mV. It should be noticed that these three main current levels could be subdivided in additional sublevels and that occasionally, currents larger than 2·1 pA were observed. However, these states were too infrequent to be analysed.

These results indicate that $GABA_A$ receptor-gated Cl^- channels display three main current levels which are the same in the presence or absence of pentobarbitone.

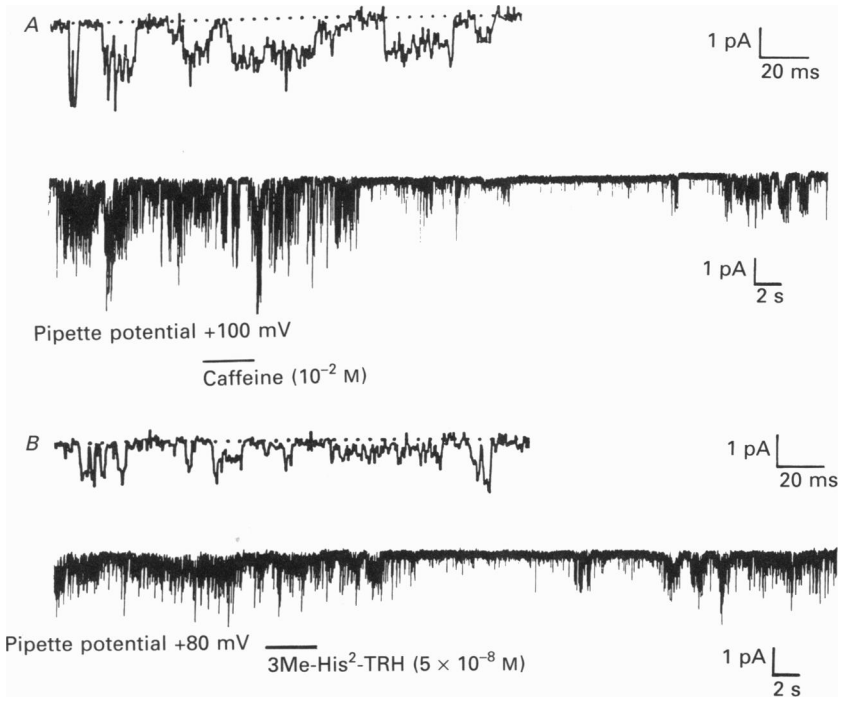

Fig. 7. Effect of caffeine (10^{-2} M) and 3Me-His²-TRH (5×10^{-8} M) on the activity of $GABA_A$ receptor-gated Cl^- channels in the cell attached configuration. The channels were activated by isoguvacine (3 μM). Application of caffeine (10^{-2} M; *A*) or 3Me-His²-TRH (5×10^{-8} M; *B*) to the cell during 5 s reduces reversibly the activity of single $GABA_A$ receptor-gated channels. The recordings were obtained at a pipette potential of +100 mV (*A*) and +80 mV (*B*). Inset shows an example on single-channel activity at a faster time scale.

However, in order to prevent any interaction of pentobarbitone with the effect of intracellular Ca^{2+} concentration on $GABA_A$ receptor-gated Cl^- channels, we have tested the effect of caffeine and 3Me-His²-TRH in conditions where only isoguvacine (3 or 5 μM) was present in the pipette. In addition, the effects of these substances were examined at hyperpolarized patch potentials in order to avoid activation of voltage-dependent channels which would be eventually present in the membrane patch and not be blocked in our experimental conditions.

Effect of caffeine and 3Me-His²-TRH on single $GABA_A$ receptor-gated Cl^- channels

In these experiments the substance tested was applied to the cell by micro-perfusion. Since the recording was made in the cell-attached configuration, the

observed effects could not be due to a direct interaction of the substances with the $GABA_A$ receptors present under the electrode.

Effect of caffeine

In six out of seven cells tested, the application of caffeine (10 mM) during 5 s strongly decreased or completely stopped the $GABA_A$ receptor–Cl^- channel complex activity as shown in Fig. 7*A*. The effect of caffeine was maximal 20–30 s after application and was reversible in two out of six cells tested.

Effect of 3Me-His²-TRH

Application of 3Me-His²-TRH (50 nM) induced a decrease in the activity of single $GABA_A$ receptor-gated Cl^- channel activity in five cells (Fig. 7*B*) and had no effect in the remaining five cells tested.

DISCUSSION

The present results suggest that, in cultured porcine intermediate lobe (IL) cells of the pituitary, the concentration of free intracellular calcium ($[Ca^{2+}]_i$) controls the activity of $GABA_A$ receptor-gated chloride currents.

The effect of increasing $[Ca^{2+}]_i$ on the $GABA_A$ response was investigated in situations where the intracellular medium was dialysed by the pipette solution (WCR configuration) and in conditions where the cytoplasmic compartment was kept intact (CA configuration). The rise in $[Ca^{2+}]_i$ was induced in conditions which stimulate hormonal secretion in intact cells, i.e. activation of voltage-dependent Ca^{2+} channels (Douglas & Taraskevich, 1980) and/or stimulation of TRH receptors which induces Ca^{2+}-release from intracellular stores via the phosphatidyl inositol pathway (Trouslard *et al.* 1989*b*). We also tested the effect of caffeine which triggers intracellular calcium release by acting directly at the level of the endoplasmic reticulum (Thayer *et al.* 1988*a*; Trouslard *et al.* 1989*b*).

Our data suggest that the $GABA_A$ response is not affected when Ca^{2+} enters the cell during the activation of voltage-dependent Ca^{2+} channels. However, when Ca^{2+} is released from intracellular stores by caffeine, we observe a strong inhibition of the $GABA_A$ receptor-gated Cl^- current both in dialysed and in intact cells. An interesting finding is that upon TRH receptor activation, apparently opposing effects are recorded in the WCR configuration at pCa 7 and the CA configuration. In addition, our results suggest that the effects of both caffeine and 3Me-His²-TRH are mediated by a rise in $[Ca^{2+}]_i$ which seems independent of Ca^{2+} influx. All these results will now be discussed.

Role of Ca^{2+} influx through voltage-dependent Ca^{2+} channels on the control of $GABA_A$ responses

A major finding of our work is that Ca^{2+} influx through voltage-dependent Ca^{2+} channels does not affect the amplitude of $GABA_A$ receptor-gated chloride currents. Since IL cells display Ca^{2+}-dependent action potentials (Douglas & Taraskevich, 1980; Trouslard *et al.* 1989*a*), it is likely that the $GABA_A$ response will not be

inhibited during cell firing. However, it could be argued that in our experimental conditions (pCa 7), an inhibition of the $GABA_A$ response is not observed because of the small amplitude of the Ca^{2+} currents (I_{Ca}) triggered by our voltage protocol. In this respect, it is important to notice that we did not observe any effect in cells where the Ca^{2+} currents had the same amplitude (see Fig. 1*A*) or were up to three times larger than the $GABA_A$ receptor-gated Cl^- currents. In addition, even in the case of a weak effect of voltage-dependent Ca^{2+} influx, a transient inhibition of the $GABA_A$ response should have been observed when Ca^{2+} currents were activated during sustained applications of isoguvacine (see Fig. 1*A* right and 1*B* middle). In this situation an inward relaxation of the chloride current should have been recorded after the termination of each calcium current as it was described in frog sensory neurones (Behrends *et al.* 1988). Such an effect was never observed in our experiments.

On the other hand, a voltage step from −80 to 0 mV during 100 ms should be sufficient to evoke a significant increase in $[Ca^{2+}]_i$ since substantial elevations in $[Ca^{2+}]_i$ are observed in GH_3B_6 cells after a single action potential (Schlegel, Winiger, Mollard, Vacher, Wuarin, Zahnd, Wollheim & Dufy, 1987). In the latter case it was also shown that the maximal increase in $[Ca^{2+}]_i$ occurs with a delay of 100–200 ms. If the same observation applies to IL cells an inhibition of the $GABA_A$ receptor-gated chloride current (monitored as an inward relaxation) should have been recorded with a comparable delay after termination of the Ca^{2+} current. In addition, all other attempts to increase Ca^{2+} influx through voltage-dependent Ca^{2+} channels, e.g. by prolonging the duration of the voltage step or increasing the frequency at which the voltage steps were delivered, failed to inhibit the $GABA_A$ response.

An important point, when examining the effect of Ca^{2+} influx through voltage-dependent Ca^{2+} channels, concerns the use of the calcium chelator EGTA in order to adjust the resting $[Ca^{2+}]_i$ to a given level. Most of our experiments have been carried out at a resting $[Ca^{2+}]_i$ of 10^{-7} M (pCa 7). This was achieved by including 10 mM-EGTA and 5 mM-$CaCl_2$ in the pipette solution. Therefore it could be argued that increases in $[Ca^{2+}]_i$ normally occurring during the activation of voltage-dependent Ca^{2+} currents could be prevented or minimized by the relatively large amount of EGTA present in the intracellular medium. However, when only a low concentration of EGTA (0·5 mM) was used or when no calcium buffer at all was present in the pipette solution, Ca^{2+} currents still failed to inhibit the $GABA_A$ response. In addition the latter sets of experiments were carried out using voltage-steps lasting 300 ms, i.e. 3 times longer than those used in the experiments where the resting $[Ca^{2+}]_i$ was set at pCa 7. This should allow substantially larger calcium influx to occur since the Ca^{2+} currents were in the same range of amplitude as those recorded at pCa 7.

Thus we conclude that Ca^{2+} influx through voltage-dependent Ca^{2+} channels does not inhibit the $GABA_A$ receptor-gated chloride current in porcine IL cells.

Effect of intracellular Ca^{2+} release on the $GABA_A$ response

Since Ca^{2+} influx through voltage-dependent Ca^{2+} channels failed to inhibit the $GABA_A$ response, we decided to test the effect of calcium release from intracellular stores on the $GABA_A$ receptor-gated Cl^- current. This aspect was investigated in the WCR and CA configurations of the patch-clamp technique. Intracellular calcium release was triggered by application of caffeine or 3Me-His^2-TRH a potent and

selective agonist of thyrotrophin-releasing hormone (TRH) on TRH receptors (Vale *et al.* 1971).

Caffeine is one of the most potent pharmacological agents known to trigger large increases in $[Ca^{2+}]_i$ in various preparations (Miller, 1988; Sato *et al.* 1988; Thayer *et al.* 1989*a*) by acting directly at the level of the endoplasmic reticulum. On the other hand, stimulation of TRH receptors provides a physiological means of increasing $[Ca^{2+}]_i$ in IL cells. Activation of TRH receptors stimulates the activity of phospholipase C (PLC) and generates inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Trouslard *et al.* 1989*b*). IP_3 in turn will cause the release of Ca^{2+} from intracellular stores by acting at specific intracellular IP_3 receptor sites (see review by Berridge & Irvine, 1989). In addition, experiments carried out on cultured sensory neurones suggest that the calcium pools involved in the increase in $[Ca^{2+}]_i$ induced by caffeine and IP_3 are not the same (Thayer, Perney & Miller, 1988*b*). In IL cells, the activation of TRH receptors causes an increase in electrical activity and promotes α-MSH secretion in IL cells of both lower vertebrates (Tonon, Leroux, Leboulenger, Delarue, Jegou & Vaudry, 1986) and mammals (Trouslard *et al.* 1989*a*, *b*). In order to stimulate these receptors we used 3Me-His^2-TRH which is known to activate maximally PLC at a concentration of 50 nM in our model (Trouslard *et al.* 1989*b*).

In the CA configuration both caffeine (10 mM) and 3Me-His^2-TRH (50 nM) caused a reversible inhibition of the activity of single $GABA_A$ receptor-gated Cl^- channels. More intriguing was the finding that caffeine and 3Me-His^2-TRH had apparently opposite effects on the whole-cell $GABA_A$ response when the resting $[Ca^{2+}]_i$ was buffered at pCa 7 (see below).

Variability of the effects of Ca^{2+} *elevation on the* $GABA_A$ *response*

Indeed, both caffeine and 3Me-His^2-TRH had differing effects on the sensitivity of the $GABA_A$ receptor depending on the recording configuration used, CA *versus* WCR, and in the latter case on the calcium buffering capacity. In the CA mode, the intracellular compartment is kept intact particularly with respect to its calcium buffering capacity. In these conditions, both caffeine (10 mM) and 3Me-His^2-TRH (50 nM) inhibited the activity of $GABA_A$ receptor-gated Cl^- channels. In the WCR configuration most experiments were performed in conditions where the resting $[Ca^{2+}]_i$ was adjusted at pCa 7. This was achieved by including 10 mM-EGTA and 5 mM-$CaCl_2$ in the pipette solution. In this situation, caffeine (10 mM) inhibited the $GABA_A$ response by 45% whereas 3Me-His^2-TRH (50 nM) increased the response by 40%. Although these effects are opposite in sign, they were clearly mediated by a rise in $[Ca^{2+}]_i$. This conclusion can be drawn from our results obtained in WCR conditions in which we used a high concentration of EGTA (18 mM) in the pipette solution and added no calcium to this solution. This situation leads to a strong buffering of the resting $[Ca^{2+}]_i$ to a low and unknown level and minimizes the rise in $[Ca^{2+}]_i$ during the application of caffeine and 3Me-His^2-TRH. Under these experimental conditions the effects of both substances were either blocked or strongly reduced. This suggested that both the effects of caffeine and 3Me-His^2-TRH were mediated by a rise in $[Ca^{2+}]_i$.

An interesting finding was that in three out of twenty cells in which we

investigated the effect of 10 mM-caffeine in the presence of 18 mM-EGTA in the pipette solution, we observed a potentiation of the $GABA_A$ response instead of an inhibition. This effect resembled the effect of 3Me-His^2-TRH when the resting $[Ca^{2+}]_i$ was fixed at pCa 7. Another important finding was that at pCa 7, a low concentration of caffeine (1 mM) also potentiated, although only transiently, the $GABA_A$ response. This potentiation was observed only during the first application of caffeine (1 mM) and the effect turned into a steady-state inhibition after the third application of caffeine. In addition, when we tested the effect of 3Me-His^2-TRH in the absence of EGTA in the pipette solution we observed either a potentiation or an inhibition of the $GABA_A$ response. Taken together these results suggest that the magnitude and the sign of the caffeine and 3Me-His^2-TRH effects depend probably on both the initial value of the resting $[Ca^{2+}]_i$ and on the calcium buffering capacity of the cell under different experimental conditions.

A possible explanation for these complex results may be provided by previous observations from our laboratory (Taleb, Trouslard, Demeneix, Feltz, Bossu, Dupont & Feltz, 1987) which provided evidence that the activity of $GABA_A$ receptor-gated Cl^- channels, recorded in outside-out patches of IL cells, presents a maximum near 10^{-7} M. At lower concentrations the activity of these channels increases as a function of $[Ca^{2+}]_i$, but at $[Ca^{2+}]_i$ concentrations in the micromolar range this channel activity is almost completely abolished. These results suggest a 'bell-shaped' relationship between $[Ca^{2+}]_i$ and the $GABA_A$ receptor-gated Cl^- channel activity. Let us now reconsider our experimental results by speculating that such a 'bell-shaped' relationship exists in IL cells and that the maximal activity of the $GABA_A$ receptor-gated Cl^- channels is observed at a concentration of $[Ca^{2+}]_i$ somewhat higher than 10^{-7} M, e.g. 3×10^{-7} M. In our standard WCR conditions, the resting $[Ca^{2+}]_i$ was set at pCa 7. Therefore one can expect that modest rises in $[Ca^{2+}]_i$ reaching a final $[Ca^{2+}]_i$ concentration close to 3×10^{-7} M would increase rather than decrease the $GABA_A$ response. Such a potentiation of the $GABA_A$ response was observed with 3Me-His^2-TRH (50 nM). In the latter situation a modest rise in $[Ca^{2+}]_i$ could be explained by (i) a dilution of the amount of IP_3 triggered during TRH receptor activation by the bulk solution of the pipette and/or (ii) a dilution or a reduction of the amount of Ca^{2+} released by IP_3. With caffeine similar but transient potentiating effects of the $GABA_A$ response are obtained with a low concentration of caffeine (1 mM) or occasionally with a higher concentration (10 mM) but only in conditions in which the rise in $[Ca^{2+}]_i$ is minimized (18 mM-EGTA in the pipette solution). On the other hand, one can speculate that when large rises in $[Ca^{2+}]_i$ are allowed to occur, final $[Ca^{2+}]_i$ concentrations in the micromolar range are likely to be reached. According to our hypothesis, this would lead to an inhibition of the $GABA_A$ response. Such effects were observed in the WCR configuration with 10 mM-caffeine and also with both caffeine and 3Me-His^2-TRH in the CA configuration (a situation in which the intracellular calcium buffering capacity of the cells is not influenced by EGTA).

It is important to mention that in hippocampal pyramidal cells apparently opposite effects of glutamate on $GABA_A$ responses have been described. A potentiating effect of glutamate is observed in dialysed cells (WCR configuration) under conditions where the variations in $[Ca^{2+}]_i$ are minimized (Stelzer & Wong,

1989). However, in non-dialysed hippocampal neurones glutamate decreases GABAergic postsynaptic potentials (Stelzer, Slater & Tenbruggencate, 1987), although it is not clear if a change in $[Ca^{2+}]_i$ is responsible for this inhibitory effect. These apparently paradoxical effects of glutamate resemble those mediated by 3Me-His^2-TRH on the $GABA_A$ response in IL cells.

In cultured melanotrophs, it should be possible to test our hypothesis concerning the 'bell-shaped' relationship between $GABA_A$ receptor activity and $[Ca^{2+}]_i$ in future work by monitoring the magnitude and kinetics of changes in $[Ca^{2+}]_i$ using fluorescent Ca^{2+}-sensitive dyes and by examining the effect of such changes in $[Ca^{2+}]_i$ on the whole-cell or single-channel $GABA_A$ receptor-gated Cl^- currents recorded in the same cell.

Other possible effects of caffeine on the $GABA_A$ response

Caffeine is a methylxanthine and therefore has phosphodiesterase blocking activities (see references in Sato *et al.* 1988). Since it is known that cyclic AMP is an important second messenger in IL cells (Frey, Cote, Grewe & Kebabian, 1982), it could be possible that part of the effect of caffeine was due to an interaction with the cyclic AMP pathway. A role of cyclic AMP in the modulation of the $GABA_A$ response has not yet been described in IL cells, but is obvious in neurones of the central nervous system (Gyenes, Farrant & Farb, 1988; Chen, Stelzer, Kay & Wong, 1990). In hippocampal neurones, phosphorylation is necessary for maintaining the $GABA_A$ response, since the latter disappears rapidly and almost completely within 10 min of whole-cell recording as the intracellular medium is dialysed with the pipette solution (Stelzer, Kay & Wong, 1988; Chen *et al.* 1990). In the WCR configuration cyclic AMP is likely to be strongly diluted. Despite this, the amplitude of the $GABA_A$ response was stable over our recording period (10 min). This suggests that cyclic AMP is probably not the major factor controlling the $GABA_A$ receptor-gated Cl^- current in IL cells. Nevertheless, if one supposes that cyclic AMP has the same role in our system as in central neurones, one would expect caffeine to potentiate the $GABA_A$ response via the elevation of cyclic AMP levels mediated by an inhibition of phosphodiesterases. If such an effect would be involved, we should have observed systematically a potentiating effect of the $GABA_A$ response by 10 mM-caffeine in the presence of 18 mM-EGTA in the pipette, a condition in which large rises in $[Ca^{2+}]_i$ do not occur. On the other hand, in the experiments carried out at pCa 7 a transient potentiation of the $GABA_A$-gated Cl^- current was observed with 1 mM-caffeine but not with 10 mM-caffeine. If this potentiation of the $GABA_A$ response by 1 mM-caffeine would be due to a phosphodiesterase blocking effect, it should have been larger with 10 mM than with 1 mM-caffeine. This was not the case.

Physiological significance

The goal of our study was to investigate the role of a rise in intracellular Ca^{2+} concentration, similar to that occurring during secretion, on the efficiency of $GABA_A$ receptors to modulate the excitation–secretion coupling in IL cells of the pituitary.

Our results suggest that large increases in $[Ca^{2+}]_i$ will inhibit the activity of the $GABA_A$ receptor–Cl^- channel complex, thus reducing the efficiency of tonic GABAergic inhibition in IL cells, at least with respect to the component mediated

by $GABA_A$ receptors. Interestingly, our data show that Ca^{2+} influx through voltage-dependent Ca^{2+} channels, such as that occurring during spiking activity, will not affect the amplitude of the $GABA_A$ response. This receptor will therefore probably retain its full potential to regulate spike discharges and the related spike-triggered α-MSH release.

On the other hand, when a large rise in intracellular Ca^{2+} is observed, for example upon stimulation of TRH receptors, $GABA_A$ receptors are likely to be strongly inhibited and to partially lose their modulatory role on electrical activity. The activation of TRH receptors has a biphasic effect on membrane potential (Louiset, Cazin, Lamacz, Tonon & Vaudry, 1989; Trouslard *et al.* 1989*a*). The early response consists of a hyperpolarization due to the activation of Ca^{2+}-dependent K^+ channels by a rise in $[Ca^{2+}]_i$. The second phase consists of a sustained depolarization associated with an increase in spike discharge. If $GABA_A$ receptor activity is still depressed during the latter phase, one can expect a reduced efficiency of GABAergic control on electrical activity. However, one must keep in mind that GABA acts on IL cells via a dual population of receptors: $GABA_A$ and $GABA_B$ (Demeneix *et al.* 1986). Therefore it remains to be determined if inhibition of $GABA_A$ receptors during TRH receptor stimulation will be sufficient to trigger additional secretion of α-MSH.

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