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Multiple modulatory effects of the neuroactive steroid pregnanolone on $GABA_A$ receptor in frog pituitary melanotrophs

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1. The effects of the neuroactive steroid pregnanolone (5β-pregnan-3α-ol-20-one) on the electrical response to GABA were investigated in cultured frog pituitary melanotrophs using the patch-clamp technique.

2. Low concentrations of pregnanolone (0·01–1 μM) in the extracellular solution enhanced the current evoked by submaximal concentrations of $GABA_A$ receptor agonists and prolonged the GABA-induced inhibition of the spontaneous action potentials in a dose-dependent manner.

3. Pregnanolone augmented the opening probability of the single GABA-activated channels but did not modify the conductance levels.

4. Pregnanolone (1 μM) shifted the GABA dose–response curve towards the low GABA concentrations, reducing the EC_{50} from 4·2 to 1·8 μM.

5. Internal cell dialysis with pregnanolone (1 or 10 μM) did not alter the GABA-evoked current.

6. Pregnanolone accelerated the desensitization of both the current and conductance increases caused by GABA.

7. High concentrations of pregnanolone (30 μM) markedly and reversibly diminished the current evoked by 10 μM GABA.

8. At high concentrations (10–30 μM), pregnanolone induced an outward current which reversed at the chloride equilibrium potential.

9. It is concluded that, in frog pituitary melanotrophs, pregnanolone exerts a dual inverse modulation and a direct activation of the $GABA_A$ receptor–channel depending on the concentrations of both GABA and steroid. Pregnanolone acts on an extracellular site on the $GABA_A$ receptor inducing conformational changes of the receptor–channel complex, resulting in a desensitized less-conducting state.

The $GABA_A$ receptor is a hetero-oligomeric protein complex comprising several (probably five) subunits which form an intrinsic chloride channel (Schofield *et al.* 1987; Nayeem, Green, Martin & Barnard, 1994). Molecular cloning of the $GABA_A$ receptor subunits has revealed the existence of multiple isoforms which might assemble in various combinations to form functional receptors (Verdoorn, Draguhn, Ymer, Seeburg & Sakmann, 1990; Rabow, Russek & Farb, 1995; Davies, Hanna, Hales & Kirkness, 1997). $GABA_A$ receptors are subject to modulation by a variety of endogenous or extrinsic compounds including benzodiazepines (Sigel & Baur, 1988; Louiset, Valentijn, Vaudry & Cazin, 1992), barbiturates (Thompson, Whiting & Wafford, 1996) and ethanol (Wafford *et al.* 1991). Certain naturally occurring or synthetic steroids termed neuroactive steroids (Paul & Purdy, 1992) are also potent stereoselective modulators of $GABA_A$ receptors (Harrison & Simmonds, 1984; Majewska, Harrison, Schwartz, Barker & Paul, 1986). In particular, progesterone and deoxycorticosterone metabolites such as allopregnanolone (5α-pregnan-3α-ol-20-one), pregnanolone (5β-pregnan-3α-ol-20-one), 5α,3α-THDOC (5α-pregnan-3α,21-diol-20-one) and 5β,3α-THDOC (5β-pregnan-3α,21-diol-20-one) allosterically potentiate the responses mediated by $GABA_A$ receptors in neurones and

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chromaffin cells (Harrison, Majewska, Harrington & Barker, 1987; Peters, Kirkness, Callachan, Lambert & Turner, 1988; Twyman & Macdonald, 1992). Investigations on the effects of neuroactive steroids on recombinant $GABA_A$ receptors have led to contrasting results emphasizing the heterogeneity of these receptors whose pharmacological properties depend on the various combinations of polypeptide subunits (Puia *et al.* 1990; Hauser, Chesnoymarchais, Robel & Baulieu, 1995; Zhu, Wang, Krueger & Vicini, 1996; Davies *et al.* 1997).

At the pituitary level, it has been reported that 3α-hydroxypregnanes enhance the inhibition of prolactin release caused by a $GABA_A$ receptor agonist (Vincens, Shu, Moguilewsky & Philibert, 1989). However, electrophysiological studies aimed at elucidating the mechanism of action of neuroactive steroids on $GABA_A$ receptors in the pituitary have been hampered by the heterogeneity of the cell types found in the anterior lobe. Unlike the pars distalis, the frog pars intermedia is composed of a homogeneous population of endocrine cells (Benyamina *et al.* 1986) and thus represents a valuable model in which the modulatory effects of neuroactive steroids on native $GABA_A$ receptors can be investigated. In these cells, GABA, acting on a typical $GABA_A$ receptor, exerts a modulatory effect on the secretory (Adjeroud *et al.* 1986; Desrues, Vaudry, Lamacz & Tonon, 1995) and electrical (Louiset, Mei, Valentijn, Vaudry & Cazin, 1994) activity. We have recently demonstrated that, in frog melanotrophs, the $GABA_A$ receptor is modulated by various steroids (Le Foll, Louiset, Castel, Vaudry & Cazin, 1997). Among these steroids, pregnanolone has been shown from binding studies to be a multiple-component modulator of the $GABA_A$ receptor (Hawkinson, Kimbrough, McCauley, Bolger, Lan & Gee, 1994; Zhong & Simmonds, 1996). Since the key enzyme (5β-reductase) of the pregnanolone biosynthesis pathway has been found in hypothalamic neurones as well as in pituitary cells of non-mammalian vertebrates (Schlinger, Fivizzani & Callard, 1989), it is likely that, *in vivo*, pregnanolone exerts multiple modulations of the response to GABA in pituitary. Herein, we have therefore explored the effects of separate and combined applications of GABA and pregnanolone, in a broad range of concentrations, on the bioelectrical activity of cultured frog melanotrophs. We also investigated the location of the site of action of the steroid.

METHODS

Animals

Adult male frogs (*Rana ridibunda*; body weight, 30–40 g) were obtained from a commercial supplier (Couétard, Saint-Hilaire de Riez, France). Frogs were housed in a temperature-controlled room (8 °C) under an established photoperiod of 12 h of light–day (lights on from 06.00 h to 18.00 h). The animals had free access to running water and were maintained under these conditions for at least 1 week before use. Animal manipulations were performed according to the recommendations of the French Ethical Commitee and under the supervision of authorized investigators.

Reagents and test subtances

Leibovitz L-15 medium, MS-222, antibiotics, GABA and pregnanolone were purchased from Sigma. Fetal calf serum was supplied from Biosys (Compiègne, France); isoguvacine was from Research Biochemicals International (Natick, MA, USA).

Cell culture

To prepare primary cultures of pituitary melanotrophs, the method of Louiset *et al.* (1994) was adopted. In short, after anaesthetization with 1% MS-222, the animals were killed by cervical dislocation and decapitated. Eight neurointermediate lobes were dissected and pituitary cells were dissociated by enzymatic digestion and mechanical dispersion. After centrifugation, the cells were suspended in Leibovitz L-15 medium adjusted to *R. ridibunda* osmolality. The medium was supplemented with fetal calf serum (10% v/v) and an antibiotic solution (0·1 mg ml^{-1} kanamycin, 100 U ml^{-1} penicillin and 0·1 mg ml^{-1} streptomycin). The cells were plated at a density of 250 000 cells ml^{-1} on 35 mm plastic culture dishes and cultured at 22 °C in a humidified atmosphere.

Patch-clamp recordings

Electrophysiological studies were performed at room temperature (20–22 °C) on 5- to 15-day-old cells using the standard patch-clamp technique. Culture dishes were placed on the stage of an inverted microscope (Labovert FS, Leitz, Germany). For the whole-cell experiments, the cells were continuously superfused with the standard extracellular solution containing (mM): NaCl, 112; KCl, 2; $CaCl_2$, 2; Hepes, 15; K_2-ATP, 2; pH 7·4. The patch pipettes were fabricated from 1·5 mm (outer diameter) thin-wall microhaematocrit glass tubes on a two-step vertical pipette puller (L/M-3P-A, List-Medical, Darmstadt, Germany) and fire polished. The potassium glutamate solution used to fill the patch pipettes had the following composition (mM): potassium glutamate, 100; $CaCl_2$, 1; $MgCl_2$, 2; Hepes, 10; EGTA, 10; pH 7·4. The resistance of the electrodes filled with this solution was 3–5 MΩ. The liquid junction potential error was measured and a correction of −13 mV was applied prior to gaining the whole-cell configuration. For the outside-out recordings, the extrapatch solution contained (mM): NaCl, 100; KCl, 4; $CaCl_2$, 1; $MgCl_2$, 6; Hepes, 15; pH 7·4. The patch pipettes were filled with a KCl solution containing (mM): KCl, 100; $MgCl_2$, 1; Hepes, 10; EGTA, 10; pH 7·4. Whole-cell signals or single-channel currents were recorded using an Axopatch 200A amplifier (Axon Instruments) and filtered at 2 kHz (−3 dB, four-pole, low-pass Bessel filter). In some experiments, electrical stimulations were applied to the cells by means of a SMP-300 stimulator (Biologic, Claix, France). In the voltage-clamp mode, GABA currents were measured at a holding potential of 0 mV unless otherwise indicated. Cell capacitance (19·7 ± 0·6 pF, $n = 79$) and series resistance (11·8 ± 0·6 MΩ, $n = 79$) were measured and corrected using the on-board cancellation and compensation circuitry of the amplifier. Signals were stored on a DTR 1200 digital tape recorder (Biologic) at a sampling rate of 48 kHz and later replayed on a 2200S chart recorder (Gould, Valley View, OH, USA) for further off-line analysis. When directly digitized, the currents were sampled at either 0·27 kHz (whole-cell currents) or 5 kHz (single-channel currents) using pCLAMP 6.0.2 programs through a Digidata 1200 interface (Axon Instruments) connected to a personal computer. The single-channel traces were low-pass filtered at 1 kHz.

Drug application

GABA or isoguvacine were dissolved in the extracellular solution and focally applied by pressure ejection from a micropipette. To avoid uncontrolled drug leakage, the pipette was brought in the vicinity of the recorded cell just before microejection. Stock

solutions of pregnanolone were prepared in ethanol (final concentration < 0·1 %) or in hydroxypropyl-β-cyclodextrin (final concentration < 0·4 % w/v) and diluted in the extracellular solution as needed. Pregnanolone was expelled near the recorded cell, at a flow rate of 3 ml min^{-1}, via a gravity-fed rapid solution exchange system. Only one cell per culture dish was studied, to avoid long-term influence of test substances.

Data analysis

The amplitude of the GABA-evoked current (I_{GABA}) was determined by the analysis software. When pregnanolone and GABA were coapplied, in order to subtract any intrinsic effect of the steroid, the current amplitude was evaluated by measuring the difference between the baseline before GABA application and the peak current. Quantitative data are expressed as means ± S.E.M., calculated from at least four independent experiments. Statistical analysis was carried out using Student's *t* test. Peak currents evoked by various concentrations of drugs were fitted by an equation of the form:

$$I_{GABA} = I_{max}[GABA]^n/([GABA]^n + EC_{50}{}^n),$$

using the Marquardt–Levenberg algorithm of the SigmaPlot 5.0 software (Jandel Scientific, Sausalito, CA, USA), where [GABA] is the GABA concentration, I_{max} is the maximum amplitude of the peak current, EC_{50} is the concentration of GABA that induces 50 % of the maximum response and n is the slope factor, corresponding to the pseudo-Hill coefficient. The decaying phases of the whole-cell currents or the all-points amplitude histograms of the single-channel data were fitted to a monoexponential function or a multiorder Gaussian distribution, respectively, using the Simplex routines of the pCLAMP software.

RESULTS

Effects of pregnanolone on GABA-evoked responses

The mean resting potential of cultured melanotrophs was −45·6 ± 1·0 mV ($n = 79$). A large proportion of the cells (59/79) exhibited spontaneous action potentials (Fig. 1). At the beginning of each set of experiments, the cells were exposed to three successive pulses of GABA at 2 min intervals. Most of the cells exhibited a reproducible inhibition in response to iterative GABA ejections. Only those cells were used in the present study. Pulses of GABA (3 μM, 10 s) caused a reversible hyperpolarization accompanied by an arrest of spontaneous firing. Concurrently, a

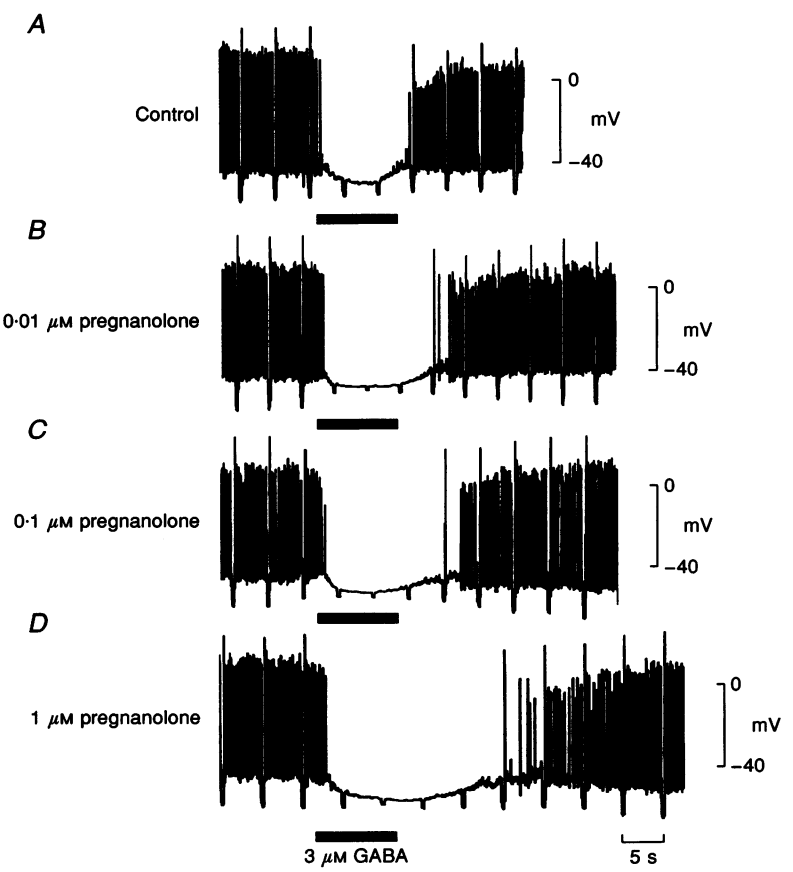

Figure 1. Effect of pregnanolone on the electrical response evoked by GABA, recorded in a single melanotroph in the whole-cell current-clamp mode

The resting potential was −40 mV. Hyperpolarizing pulses (−20 pA, 400 ms, 0·2 Hz) were applied to monitor the cell input resistance. GABA (3 μM) was ejected in the vicinity of the cell for 10 s, as indicated by the filled bars below the traces. The recordings were obtained in the absence (*A*, Control) or presence of increasing concentrations of pregnanolone (0·01–1 μM) in the bath solution (*B–D*). Pregnanolone provoked a dose-related prolongation of the inhibitory effect of GABA on the spontaneous spiking activity.

decrease in the cell input resistance from 1·10 ± 0·26 to 0·27 ± 0·04 GΩ ($n = 5$) was observed (Fig. 1*A*). Bath perfusion of pregnanolone (0·01–1 μM) prolonged the hyperpolarization and the inhibition of action potentials provoked by GABA, in a dose-dependent manner (Fig. 1*B–D*). The effect of pregnanolone (1 μM) on the modifications of membrane potential evoked by GABA was associated with a decrease in the membrane resistance, from 0·27 ± 0·04 GΩ (in the presence of GABA alone) to 0·11 ± 0·05 GΩ ($n = 5$). In cells studied in the voltage-clamp mode, the effect of pregnanolone on the GABA-evoked current was investigated at a holding potential of 0 mV in order to maximize the chloride driving force. Microejection of GABA (3 μM, 10 s) elicited a robust outward current (Fig. 2*A*), named I_{GABA}. Bath perfusion of pregnanolone (1 μM) had no effect on the basal membrane current, but provoked a marked increase in I_{GABA} from 101·8 ± 12·3 to 242·6 ± 32·4 pA ($n = 13$; Fig. 2*A*). Pregnanolone induced both a potentiation of the current amplitude and an acceleration of the current desensitization during the exposure to GABA. In frog melanotrophs, the selective $GABA_A$ receptor agonist isoguvacine (3 μM, 10 s) induced a modest outward current (Fig. 2*B*). Bath perfusion of pregnanolone (1 μM) caused a strong potentiation of the isoguvacine-evoked current (5·7 ± 1·2-fold, $n = 6$; Fig. 2*B*). To investigate the voltage dependence of the cell response to GABA in the absence or presence of pregnanolone, the amplitude of I_{GABA} was measured at different holding potentials. The current–voltage relationship of the response to GABA exhibited a pronounced outward rectification and reversed at −74·7 ± 1·5 mV ($n = 16$), a value corresponding to the chloride equilibrium potential under our recording conditions ($E_{Cl} = -75$ mV). In the presence of pregnanolone (1 μM), the reversal potential of I_{GABA} was not modified (Fig. 3).

The effect of pregnanolone on the electrical response to increasing concentrations of GABA was studied in a total of sixty-five melanotrophs. In the absence of pregnanolone, low concentrations of GABA (0·1–1 μM) generated a weak steady-state current, whereas higher concentrations of GABA (3–100 μM) elicited a large decaying current.

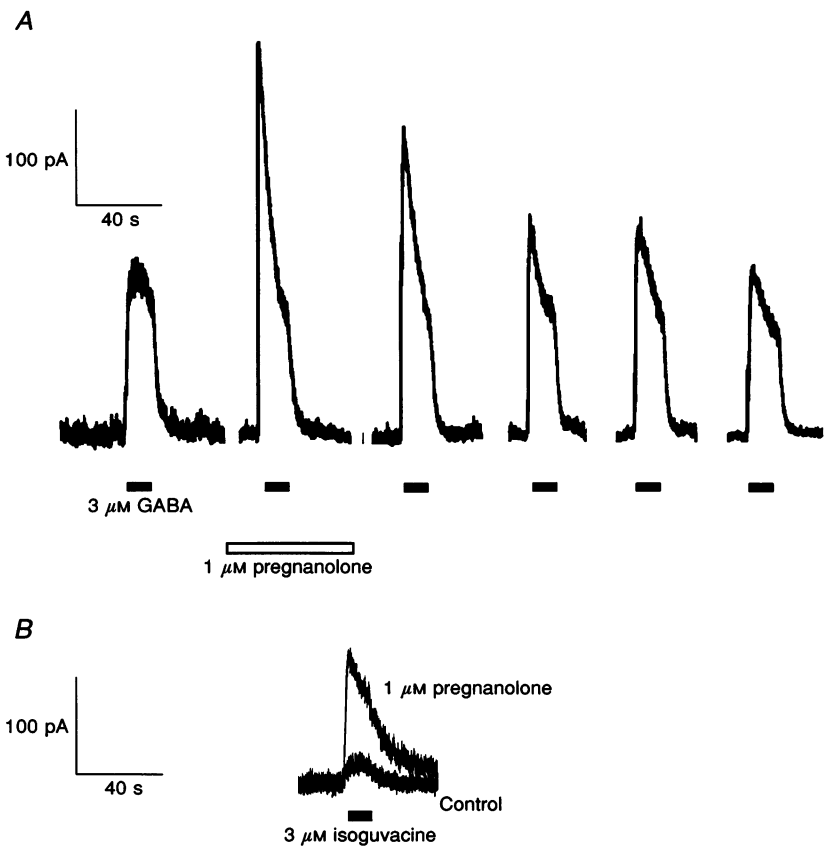

Figure 2. Effect of pregnanolone on the GABA- and isoguvacine-evoked currents recorded in two melanotrophs clamped at 0 mV

A, outward current elicited in a cell repeatedly exposed to GABA (3 μM, 10 s, filled bars) at 2 min intervals. Perfusion of 1 μM pregnanolone (open bar) reversibly enhanced the peak current amplitude and increased the current desensitization rate. Recovery of the control current amplitude occurred after extensive (8 min) washout. *B*, superimposed outward currents evoked in another cell by 3 μM isoguvacine (10 s, filled bar) in the absence (Control) or presence of 1 μM pregnanolone. Note that the isoguvacine-evoked current was strongly potentiated by pregnanolone.

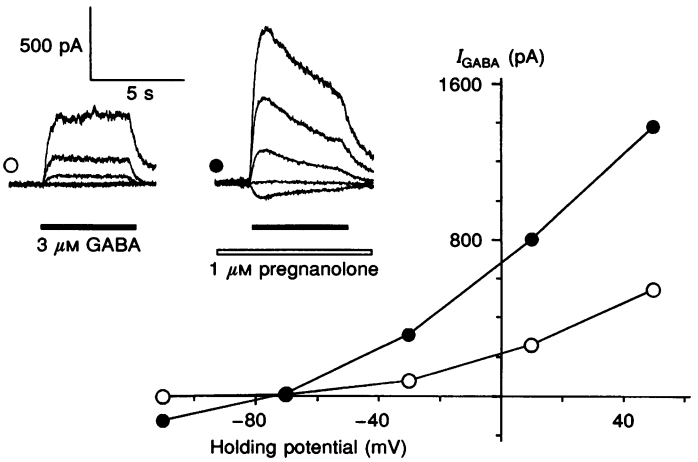

Figure 3. Effect of pregnanolone on the voltage dependence of the GABA-evoked current in a frog melanotroph

GABA (3 μM, 5 s) was delivered in the vicinity of the cell clamped at holding potentials ranging between −110 and +50 mV. Peak current amplitude was plotted against holding potential in the absence (○) or presence (●) of pregnanolone (1 μM). Note that bath perfusion of pregnanolone increased the slope of the current–voltage curve but did not shift the reversal potential (−71 mV). The inset shows the current traces recorded from the same cell.

Addition of pregnanolone (1 μM) enhanced the current evoked by low concentrations of GABA. In contrast, pregnanolone did not affect the response of melanotrophs to a maximum effective concentration of GABA (Fig. 4*A*). Figure 4*B* shows that pregnanolone caused a shift of the GABA dose–response curve to the left, with a reduction of the EC_{50} from 4·2 to 1·8 μM. In contrast, the Hill coefficient and the maximum peak current amplitude were not affected by pregnanolone.

The effect of increasing concentrations of pregnanolone on the current responses evoked by different concentrations of GABA is shown in Fig. 5. Threshold concentrations for detecting potentiation by pregnanolone were between 0·01 and 0·1 μM. At low concentrations (0·1 and 1 μM), pregnanolone induced a dose-related potentiation of I_{GABA} (Fig. 5*A*). At a higher concentration (30 μM), it provoked a marked potentiation of the response to 1 μM GABA (12·9 ± 3·6-fold, $n = 10$), but only weakly enhanced the

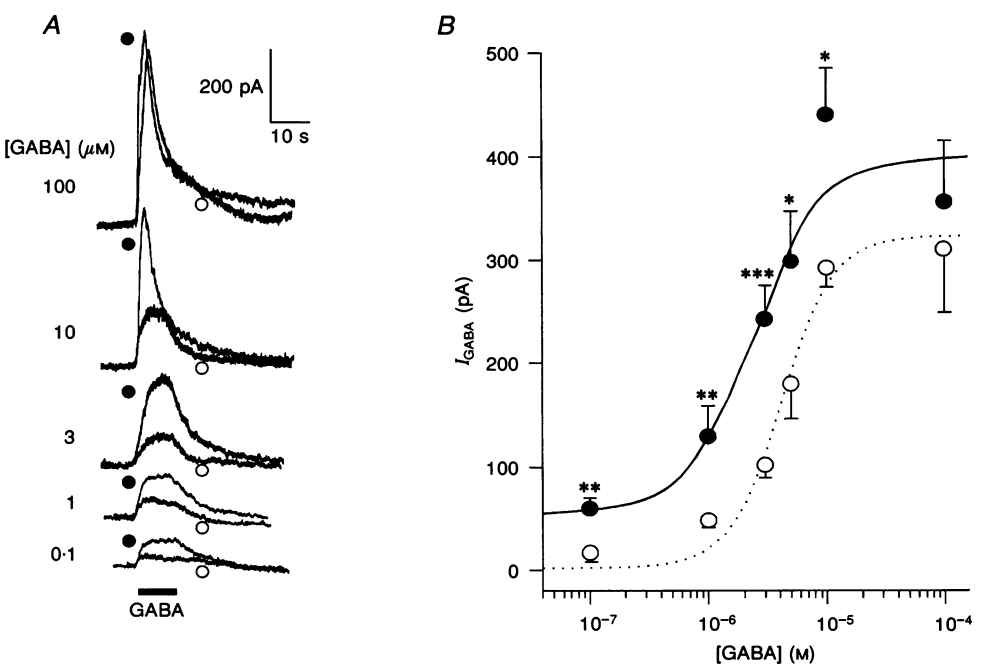

Figure 4. Effect of pregnanolone on the response of melanotrophs to increasing concentrations of GABA

A, superimposed currents evoked by increasing concentrations of GABA (0·1–100 μM, filled bar) recorded in a single cell in the absence (○) or presence (●) of pregnanolone (1 μM). Holding potential was 0 mV. *B*, semi-logarithmic curves showing the effect of increasing concentrations of GABA on the outward current in the absence (○) or presence (●) of 1 μM pregnanolone. Each point represents the mean (± S.E.M.) of 4 to 13 independent recordings. * $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.

response to 3 μM GABA (3·35 ± 0·5-fold, $n = 5$) and strongly inhibited the response to 10 μM GABA (0·51 ± 0·06-fold, $n = 10$; Fig. 5*A*). Both the potentiating and the inhibitory effects of pregnanolone were reversible during washout (data not shown). The dual effects of pregnanolone on I_{GABA} are summarized in a tridimensional dose–response representation (Fig. 5*B*).

Effects of pregnanolone on the GABA-evoked current and conductance desensitization

The question of whether the acceleration of the current desensitization caused by pregnanolone was based on a fading of the membrane chloride conductance was addressed by applying two successive protocols on each tested cell. The first approach consisted of recordings of I_{GABA} evoked by 15 s ejections of GABA (3 μM) at 0 mV. The second approach involved the measurement of the membrane conductance (g_{GABA}) during the 15 s GABA administrations. In the latter case, the cells were voltage clamped at the reversal potential of I_{GABA} (−75 mV) in order to prevent any chloride flux that could contribute to the current desensitization (Huguenard & Alger, 1986; Akaike, Inomata & Tokutomi, 1987). The membrane conductance was monitored by delivering repeated hyperpolarizing voltage pulses. The current or conductance traces were best approximated by a single exponential function fitted to the decay phase of I_{GABA} or g_{GABA} giving time constants τ_I or τ_g, respectively. As shown in the representative example of Fig. 6*A*, I_{GABA} desensitized much more rapidly than g_{GABA}. The curves fitted to the traces yielded time constants with values of τ_I and τ_g of 6·1 ± 0·3 s ($n = 19$) and 11·6 ± 0·6 s ($n = 20$), respectively. Pregnanolone (1 μM) drastically accelerated the decay of both I_{GABA} and g_{GABA} (Fig. 6*B*). In the presence of the steroid, desensitization kinetics were significantly faster ($P < 0·001$) than those of the control, yielding values of τ_I and τ_g of 2·1 ± 0·3 s ($n = 9$) and 2·1 ± 0·2 s ($n = 19$), respectively. Interestingly, whereas τ_I was significantly lower than τ_g in the control ($P < 0·001$), the time constants did not differ when pregnanolone was present in the bath solution ($P > 0·1$).

Effect of internal cell dialysis with pregnanolone on I_{GABA}

To further determine whether the site of action of pregnanolone is intra- or extracellularly located, experiments were carried out on melanotrophs using a pregnanolone-containing pipette solution (Fig. 7). Immediately after gaining the whole-cell configuration, I_{GABA} was repeatedly elicited by brief pulses of GABA (3 μM, 3 s) at 1 min intervals. After 20 min of cell dialysis with pregnanolone (1 or 10 μM), the amplitude of I_{GABA} did not significantly differ from that observed just after the whole-cell access ($n = 8$). In contrast, an additional extracellular application of the steroid to the pregnanolone-containing cells resulted in an enhancement of I_{GABA} similar to that observed in cells recorded with the standard pipette solution.

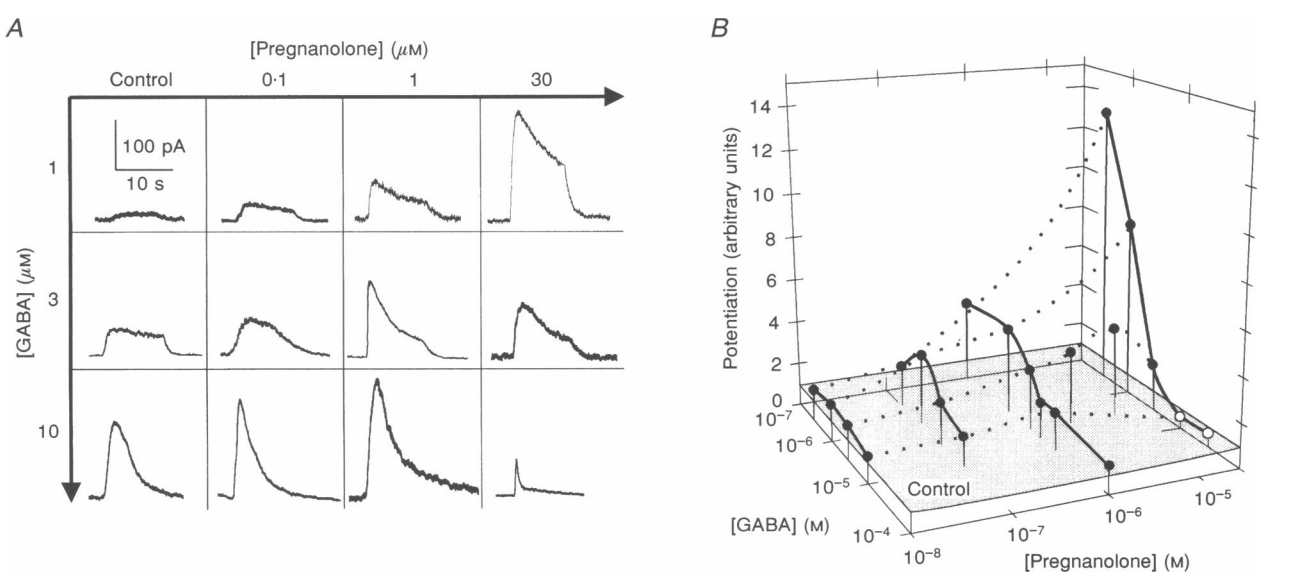

Figure 5. Effect of increasing concentrations of pregnanolone on the currents evoked by different concentrations of GABA

A, typical recordings illustrating the effects of various concentrations of GABA in the absence (Control) or presence of increasing concentrations of pregnanolone (0·1–30 μM). Holding potential was 0 mV. *B*, dose–response curves depicting the potentiating effect of increasing concentrations of pregnanolone on the currents evoked by various concentrations of GABA. Each point represents the mean of 4 to 19 independent recordings. All points above the shaded level (●) represent potentiation of the GABA-evoked response and points below the shaded level (○) correspond to inhibition.

Effect of pregnanolone on single-channel currents evoked by GABA

The direct modulation of the $GABA_A$ receptor–chloride channel complex by pregnanolone in frog melanotrophs was investigated in outside-out patches held at −80 mV, a value corresponding to the potassium equilibrium potential under the present recording conditions. Figure 8 shows unitary currents activated by GABA (1 μM) in the absence (Fig. 8*A*) or presence (Fig. 8*B*) of pregnanolone (1 μM). In all patches, no single-channel activity was detected before GABA application. A microejection of GABA generated inward single-channel currents which persisted over the period of GABA exposure. Typically, the opening events were organized in brief bursts separated by pauses. Pregnanolone conspicuously enhanced GABA-induced channel activity by increasing the duration of the bursts and causing

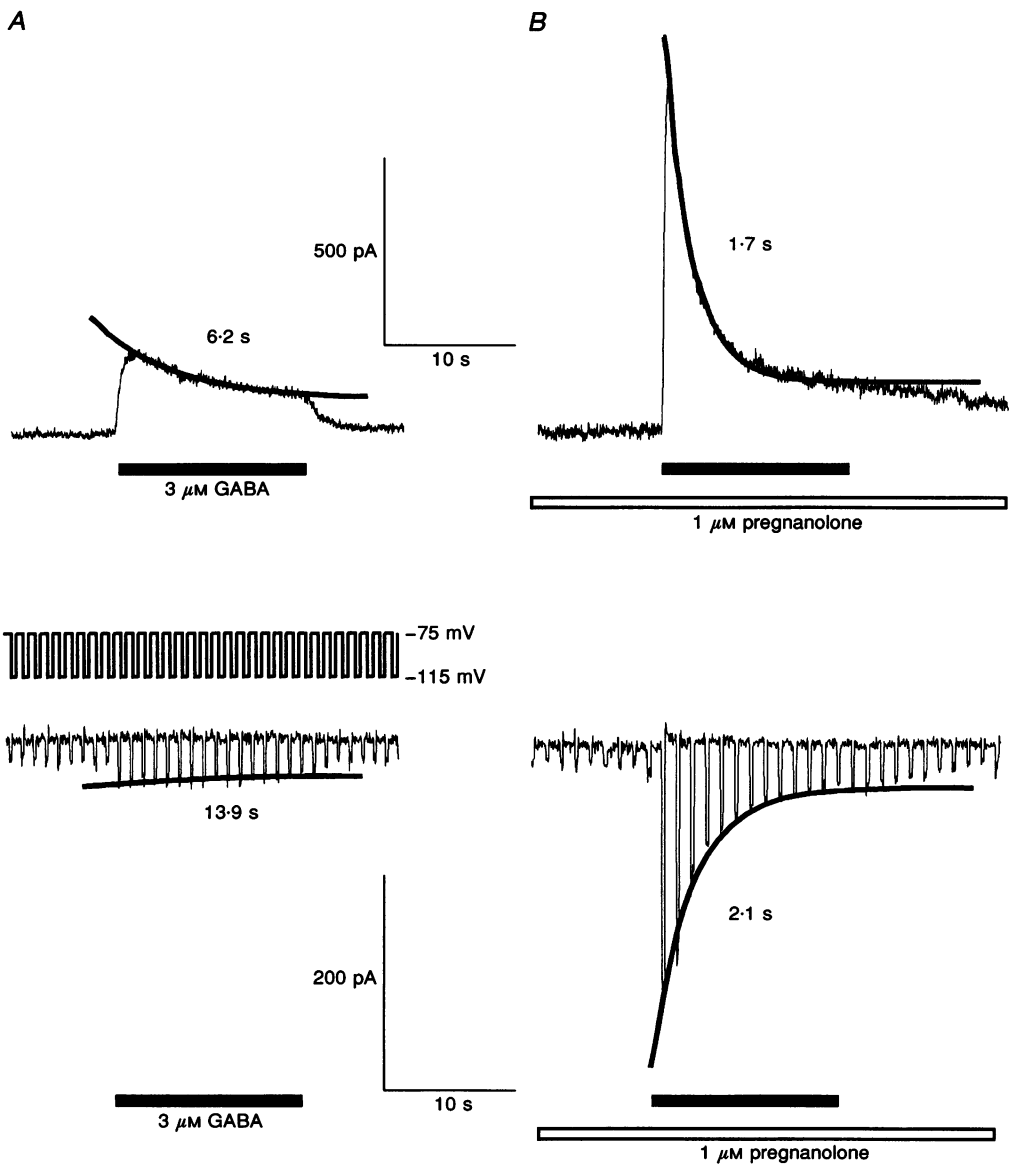

Figure 6. Effects of pregnanolone on the desensitization of both current and conductance during exposure of a single melanotroph to GABA

GABA (3 μM) was applied for 15 s as indicated by the filled bars. The desensitization time courses were fitted with a single exponential function by non-linear regression. Top traces, GABA-evoked currents at a membrane potential of 0 mV. The exponential curves fitted to the current traces yielded time constants of 6·2 or 1·7 s, respectively, during the exposure to GABA in the absence (*A*) or presence (*B*) of 1 μM pregnanolone (open bar). Bottom traces, GABA-evoked conductances at a membrane potential of −75 mV corresponding to the reversal potential of I_{GABA}. The cell membrane conductance was monitored by repeated constant voltage pulses (−40 mV, 200 ms, 1·33 Hz). The exponential curves, fitted to the peaks of the current jumps provoked by the voltage pulses, yielded time constants of 13·9 or 2·1 s, respectively, during the exposure to GABA in the absence (*A*) or presence (*B*) of 1 μM pregnanolone (open bar).

simultaneous openings of several channels (Fig. 8). The fitting of the all-points amplitude histograms to the sum of Gaussian distributions (Fig. 8*C* and *D*) yielded a peak amplitude of 1·9 pA for the control current (Fig. 8*C*). In the presence of pregnanolone, two distinct main current levels of 1·9 and 4 pA were measured (Fig. 8*D*). Dividing by the driving force for chloride ions gave a 25 pS conductance for the control and 25 and 52 pS conductances in the presence of pregnanolone. Pregnanolone provoked a reduction in the area under the peak corresponding to the closed state (0 pA) concommitantly with an increase in the area under the peaks corresponding to one or more channel openings (1·9 and 4 pA).

Intrinsic effects of pregnanolone on membrane potential and current

In order to investigate a possible intrinsic action of pregnanolone on the bioelectrical activity of melanotrophs, voltage and current recordings were performed in cells exposed to the steroid at concentrations ranging between 0·01 and 30 μM. At low concentrations (0·01–1 μM), pregnanolone had no effect on both spontaneous action potentials and overall membrane current. In contrast, at high concentrations (10–30 μM), pregnanolone inhibited spontaneous firing (Fig. 9*A*) and generated an outward current which gradually rose and reached a plateau. The amplitude of the stabilized current elicited by 10 μM pregnanolone was 51 ± 9 pA ($n = 10$). The potentiation of I_{GABA} by pregnanolone increased during the slowly developing pregnanolone-induced current (Fig. 9*B*). The current–voltage relationship of the pregnanolone-induced current was linear. The deduced reversal potential was −72 mV, a value corresponding to the chloride equilibrium potential under the present recording conditions (Fig. 9*C*).

DISCUSSION

Previous studies have shown that GABA regulates both the bioelectrical and secretory activity of frog melanotrophs through activation of a neurone-like $GABA_A$ receptor (Adjeroud *et al.* 1986; Louiset *et al.* 1994). Herein, we have characterized, using the model of the frog melanotroph, the action exerted by pregnanolone on the bioelectrical response to GABA.

Modulatory effects of pregnanolone on GABA-evoked responses

The present work provides evidence that low pregnanolone concentrations (0·01–1 μM) altered the effect of GABA on the membrane potential of melanotrophs by extending the GABA-induced inhibition of the spontaneous activity. To our knowledge, such a control by pregnanolone of the voltage response to GABA had so far never been observed in spontaneously spiking cells. The prolongation of the arrest of action potential discharge was probably underlaid by the remarkable drop in the membrane resistance that occurred

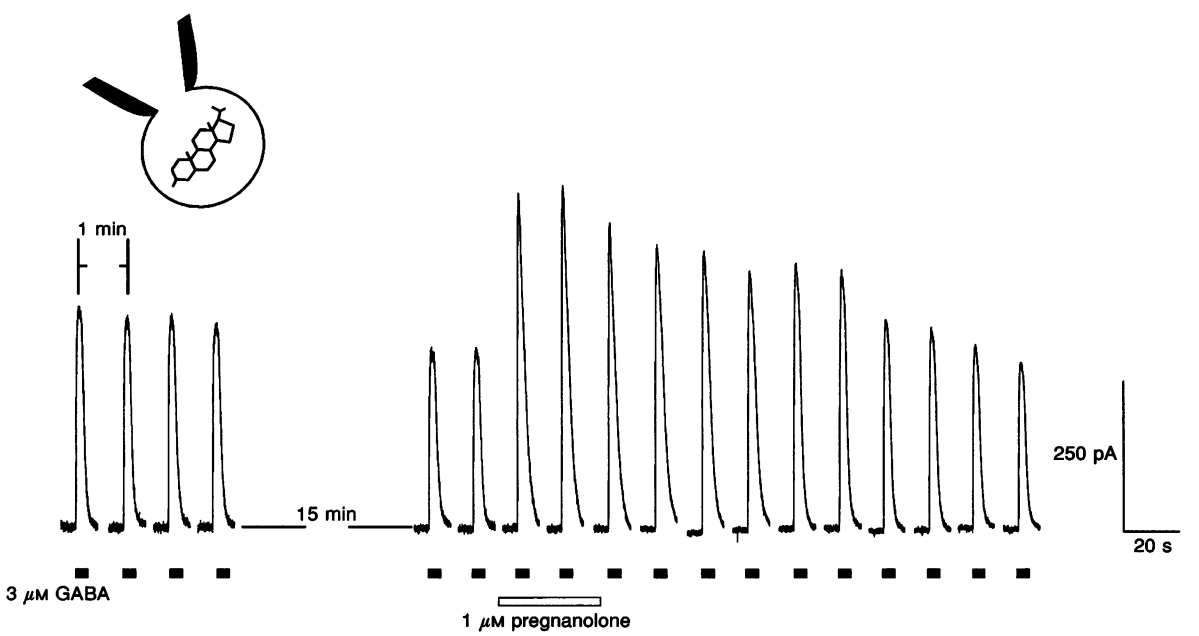

Figure 7. Effect of internal cell dialysis with pregnanolone on the GABA-evoked current in a single melanotroph

GABA (3 μM, 3 s, filled bars) was repeatedly applied at 1 min intervals immediately after gaining the whole-cell configuration over a period of 30 min. The patch pipette contained 1 μM pregnanolone. Holding potential was 0 mV. Note that the steroid cell dialysis did not affect the GABA-evoked current. After 20 min recording of the pregnanolone-loaded cell, an additional extracellular perfusion of 1 μM pregnanolone (open bar) produced a potentiation of the GABA-evoked current. The level of the control evoked current recovered after 9 min washout.

when pregnanolone was coapplied with GABA. In concert with this effect, pregnanolone potentiated the GABA-evoked current. The results obtained with the type A receptor agonist isoguvacine suggest that the potentiation of I_{GABA} exerted by pregnanolone can be ascribed to a specific action on the $GABA_A$ receptor. Isoguvacine elicited relatively weak currents in comparison with those generated by equimolar GABA concentrations. Currents of the same amplitude induced by isoguvacine or GABA were enhanced by pregnanolone with similar potentiating factors. Threshold concentrations of pregnanolone for detecting a potentiating effect ranged between 0·01 and 0·1 μM. Similar findings have been reported in rat hypothalamo-hypophyseal co-cultures (Poisbeau, Feltz & Schlichter, 1997), bovine chromaffin cells (Peters *et al.* 1988) and rat hippocampal neurones (Kokate, Svensson & Rogawski, 1994).

In frog melanotrophs, chloride ions appear to be the exclusive charge carriers of I_{GABA} since the current reversed exactly at a potential corresponding to the chloride equilibrium potential. Pregnanolone did not modify the reversal potential of I_{GABA}. As a consequence, it can be assumed that the steroid changes neither the ionic selectivity of the $GABA_A$ receptor–channel nor the chloride driving force. Rather, pregnanolone might increase the affinity of the

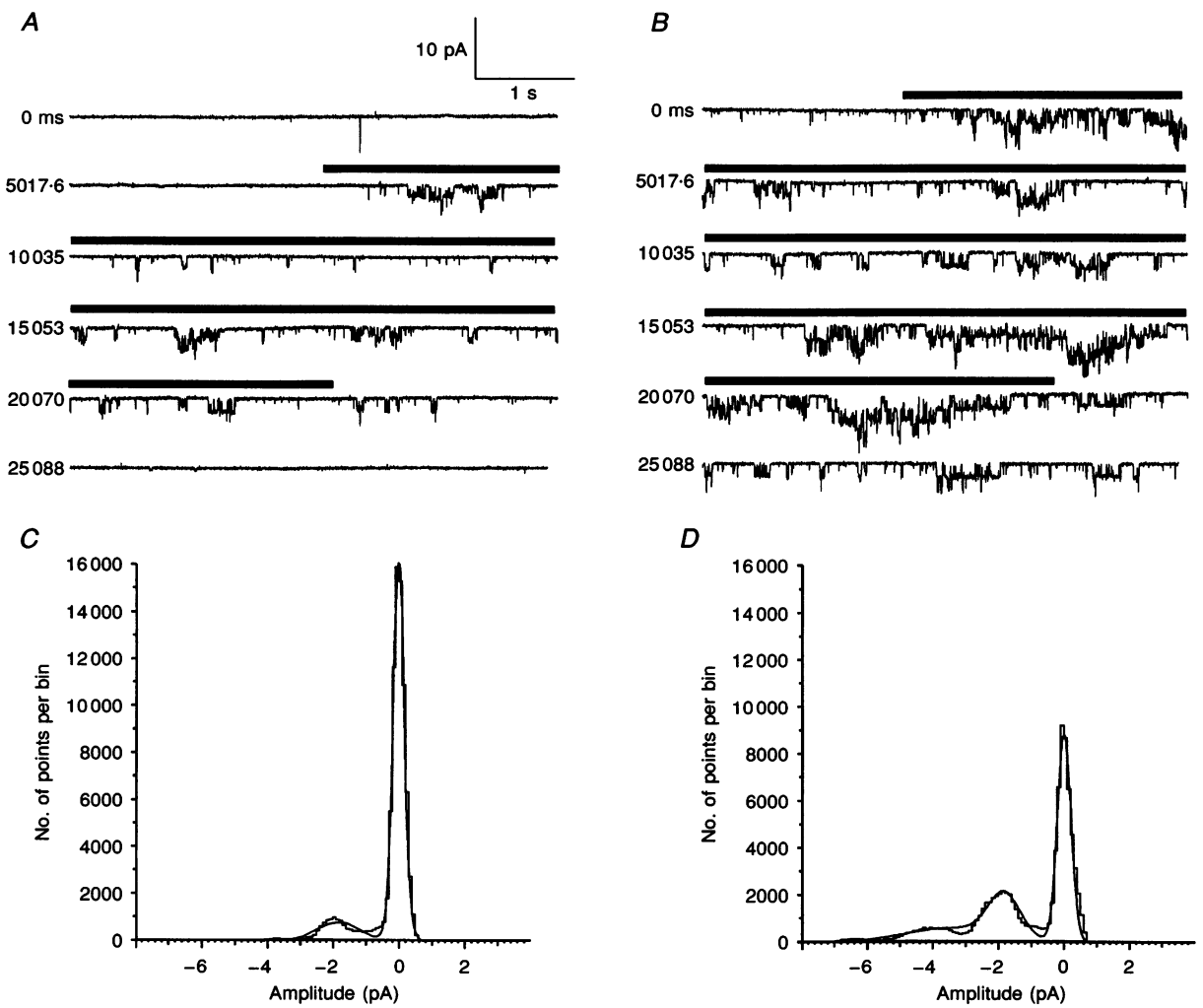

Figure 8. Effect of pregnanolone on single-channel currents evoked by GABA, recorded in the outside-out configuration

A and *B*, continuous single-channel recordings obtained from the same patch held at −80 mV. Elapsed time of recording (in ms) is indicated at the begining of each trace. GABA (1 μM) ejection is indicated by the filled bars above the current traces. Downward deflections correspond to channel openings evoked by GABA in the absence (*A*) or presence (*B*) of pregnanolone (1 μM) in the bath solution. *C* and *D*, all-points amplitude histograms of single-channel currents based on 17 s segments of recording, binned into 128 levels. Data originating from *A* and *B* are analysed in *C* and *D*, respectively. In both histograms, the peak centred on 0 pA corresponds to the closed state of the channels. The current amplitude distributions were fitted by the sum of Gaussian distributions. Note the decrease in the area under the peak corresponding to the closed state and the increase in the area under the peaks corresponding to one or more simultaneous channel openings in the presence of pregnanolone.

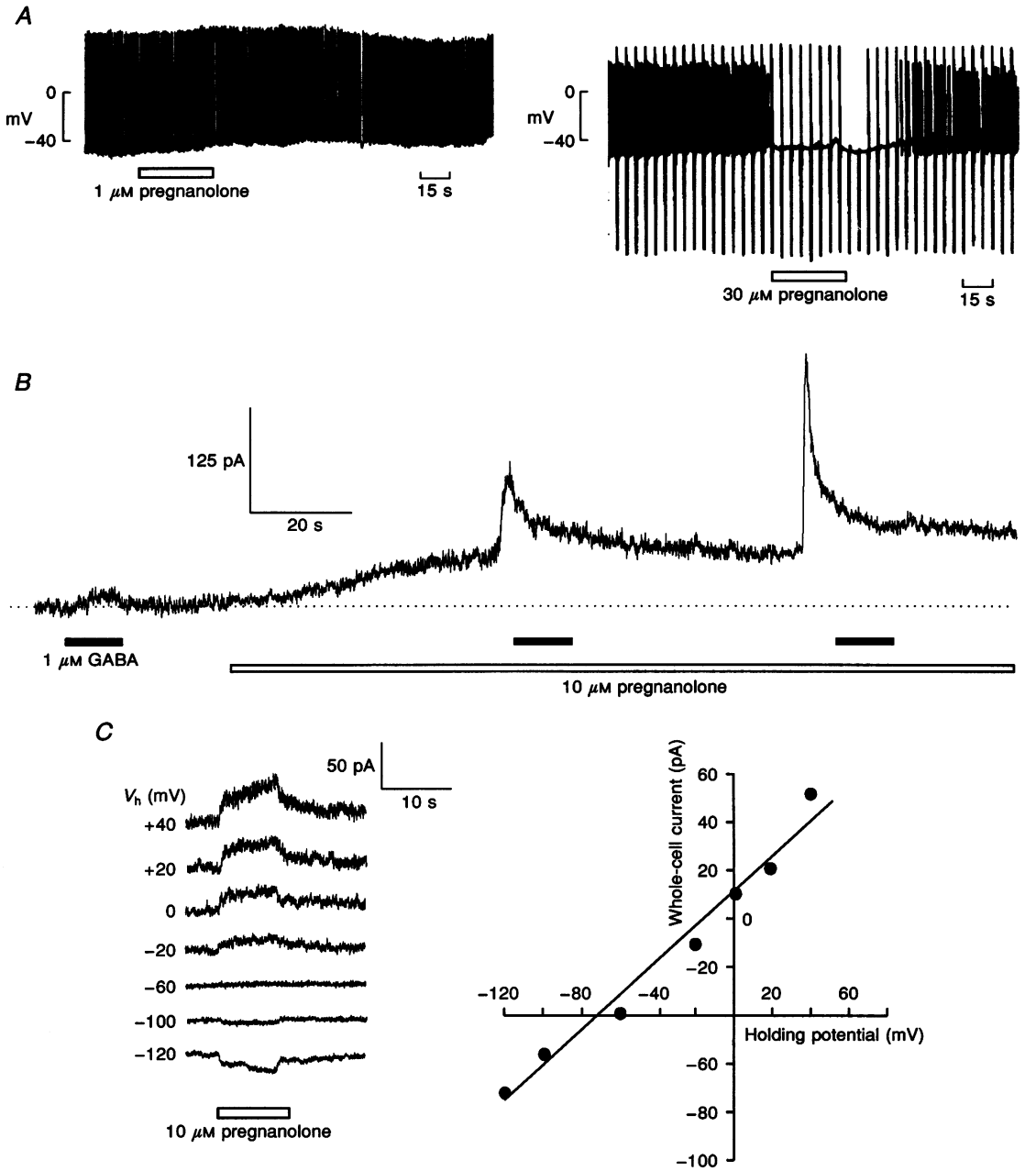

Figure 9. Intrinsic effect of high concentrations of pregnanolone on spontaneous action potentials and membrane current of melanotrophs

A, effect of a 30 s microejection of pregnanolone on spontaneous firing in two distinct melanotrophs. Resting potential was −40 mV in both cells. Left, at a low concentration (1 μM), pregnanolone had no effect on action potential discharge. Right, in another cell, at a higher concentration (30 μM), pregnanolone inhibited spontaneous activity without apparent changes in the membrane input resistance. Membrane input resistance was monitored by applying hyperpolarizing pulses (−20 pA, 400 ms, 0·2 Hz). Spikes off caused by the pulses persisted during the pregnanolone-induced inhibition. *B*, slowly developing outward current elicited in a cell held at 0 mV and exposed to 10 μM pregnanolone (open bar). The cell was submitted to successive 10 s pulses of 1 μM GABA (filled bars) before and during bath perfusion of pregnanolone. Note that the higher the pregnanolone-induced current, the more pronounced the potentiation of the GABA-evoked current. *C*, current–voltage relationship of the pregnanolone-induced current recorded in a single melanotroph. Left, currents provoked by 10 μM pregnanolone (10 s, open bar) at various holding potentials (V_h) as indicated. Right, the peak amplitude of the pregnanolone-evoked current was plotted against holding potential. Zero-current was reached at −72 mV, a value which corresponded to the chloride equilibrium potential under our recording conditions.

$GABA_A$ receptor. Consistent with this hypothesis, studies have revealed that pregnanolone enhanced [^{3}H]muscimol binding in pig (Peters *et al.* 1988) and rat (Goodnough & Hawkinson, 1995) brain membranes at concentrations similar to those that induced a potentiation in our experiments. Also in agreement with an allosteric modulation of the affinity of the GABA binding site, we found that pregnanolone reduced the EC_{50} of GABA but did not change I_{max}. The shift of the GABA dose–response curve towards the low GABA concentrations results in a decrease in the concentration of GABA required to generate a liminar response.

One of the main findings reported here is that, in the melanotrophs, pregnanolone caused a dual inverse modulation of I_{GABA}, i.e. potentiation or inhibition, depending on the concentration of both GABA and pregnanolone. It was shown that, at low micromolar concentrations, pregnanolone potentiated currents evoked by submaximal concentrations of GABA. In contrast, with increasing concentrations of pregnanolone, the potentiating effect diminished at high GABA concentrations. In addition a clear inhibitory action of the steroid on I_{GABA} was observed at high concentrations of both GABA and pregnanolone. The present work also provides evidence that high concentrations of pregnanolone gated a chloride current in the absence of GABA by direct activation of the $GABA_A$ receptor. It can therefore be expected that, at low GABA and high pregnanolone concentrations, the overall membrane current resulted from the combination of both the steroid-potentiated GABA-evoked current and the pregnanolone-activated current. At saturating concentrations of GABA and pregnanolone, the response probably involved the recruitment of all $GABA_A$ receptors. Under these conditions, because of the direct activation of the receptors, a decrease in the GABA-activated component occurred and led to an apparent reduction of the potentiating effect of the steroid. However, in our experiments, the current directly activated by pregnanolone was rather weak (50 pA) in comparison with that evoked by saturating concentrations of GABA (400 pA). Thus, it seems very unlikely that the pregnanolone-activated current can account for the whole decrease in the potentiation of I_{GABA} by pregnanolone. In very much the same way, the inhibitory action of the steroid on I_{GABA} must be independent of the direct effect of pregnanolone. Alternatively, the dual effect of pregnanolone on I_{GABA} can be ascribed to opposite actions of the steroid on the $GABA_A$ receptor through distinct binding sites. Recently, authors have reported the existence of low- and high-affinity binding sites for pregnanolone in brain membranes (Hawkinson *et al.* 1994; Zhong & Simmonds, 1996). It is still unclear whether the two populations of sites are present on the same receptor complex or on structurally distinct receptors. The ratio of low- and high-affinity sites has been shown to depend on the GABA concentration. It can therefore be suggested that low- and high-affinity sites are responsible for the functionally opposite effects of pregnanolone. Such a 'bell-shaped' effect of steroids has also been described in a study performed in human embryonic kidney (HEK 293) cells expressing the α_6-subunit of the $GABA_A$ receptor (Hauser *et al.* 1995), a particular isoform only present in the adult rat cerebellum. Taken together, our data indicate that the dual inverse action of pregnanolone on I_{GABA} is not restricted to the recombinant α_6-subunit-containing $GABA_A$ receptor but is also a feature of native receptors expressed by pituitary cells.

In the melanotrophs, as in other cell types, I_{GABA} decayed in spite of the continuous presence of the agonist (Akaike *et al.* 1987; Louiset *et al.* 1994). The current desensitization can be attributed to a local intracellular accumulation of chloride ions resulting in a transient decrease in the chloride driving force (Huguenard & Alger, 1986). We observed that pregnanolone markedly accelerated the current desensitization. In order to elucidate the mechanism by which pregnanolone modified the current kinetics, we compared I_{GABA} and g_{GABA}. The conductance was measured at the zero-current membrane potential to abolish the chloride flux. Our results indicate that I_{GABA} declined more rapidly than g_{GABA}, suggesting that the desensitization phenomenon can be ascribed, at least in part, to the decrease in the chloride driving force. In the presence of pregnanolone, desensitization time courses of I_{GABA} and g_{GABA} were conspicuously accelerated and did not differ from each other. Thus, the pronounced desensitization of the current caused by the steroid appears to be mainly due to a fall in g_{GABA}. Pregnanolone probably provokes conformational changes of the $GABA_A$ receptor–chloride channel complex, resulting in a less-conducting desensitized state. This desensitization may represent an additional process involved in the decrease in I_{GABA} observed when pregnanolone was present at high concentrations.

Site of action and intrinsic effect of pregnanolone

To determine whether the site of action of pregnanolone is located in the intra- or extracellular domain of the $GABA_A$ receptor, we have performed whole-cell recordings with the steroid within the patch pipette solution. Internal cell dialysis with pregnanolone, even at a high concentration (10 μM), failed to modify the GABA-evoked current. This result strongly suggests that pregnanolone binds to an extracellular site and discards the possibility of a passive diffusion of the steroid through the cell membrane. The single-channel recordings of the present study revealed that the external application of pregnanolone to outside-out patches increased the opening probability of the GABA-activated channel but did not alter the main conductance. These findings, which are in good agreement with data obtained in mouse spinal cord neurones (Twyman & Macdonald, 1992), are in favour of a direct extracellular allosteric modulation of the $GABA_A$ receptor–channel by steroids.

When applied at high concentrations in the absence of GABA, pregnanolone inhibited the spontaneous action

potentials by activating a transmembrane current which reversed at a potential corresponding to E_{Cl}. The present results suggest a direct 'agonist-like' action of pregnane steroids on the gating mechanism of the chloride channel associated with the $GABA_A$ receptor. Similar findings have been reported in bovine chromaffin cells (Peters *et al.* 1988) and rat hippocampal neurones (Rodgers-Neame, Covey, Hu, Isenberg & Zorumski, 1992). In addition, we also observed that intracellular application of pregnanolone did not elicit any current, indicating that the intrinsic effect of the steroid results from a direct action on an extracellular site. According to recent studies conducted in transfected HEK 293 cells, the modulatory and intrinsic effects of pregnanes on the $GABA_A$ receptor–channel are mediated through distinct sites of action (Zhu *et al.* 1996; Davies *et al.* 1997).

Physiological significance

In the pituitary pars intermedia, the melanotrophs secrete several biologically active peptides, including α-melanocyte-stimulating hormone (α-MSH). Although the hormonal function of α-MSH remains obscure in mammals, in amphibians α-MSH causes skin darkening through pigment dispersion in dermal melanophores, and thus plays a pivotal role in the physiological process of background colour adaptation. In frog melanotrophs, $GABA_A$ receptor agonists exert a biphasic effect on α-MSH release, i.e. an early transient stimulation and a delayed prolonged inhibition (Adjeroud *et al.* 1986; Desrues *et al.* 1995). The present study demonstrates that pregnanolone produces a dual inverse modulation and direct activation of the $GABA_A$ receptor–channel depending on the concentration of both GABA and steroid. To date, the action of neuroactive steroids on the secretory response to GABA of melanotrophs has not yet been documented. Nevertheless, one can speculate that, as observed with benzodiazepines in perifusion experiments (Adjeroud *et al.* 1987), at low concentrations, pregnanolone potentiates both phases of the secretory response to GABA. At higher concentrations, pregnanolone is supposed to antagonize the effect of GABA on α-MSH release. An important issue is whether the effects of pregnanolone are likely to occur *in vivo*. Several lines of evidence indicate that the concentration of GABA in the synaptic cleft is high enough to saturate postsynaptic receptors (Mody, De Koninck, Otis & Soltesz, 1994). In this case, low concentrations of pregnanolone would not be capable of influencing the maximal response to GABA. However, it is also assumed that transmitter overspill diffuses from the synaptic cleft into the extrasynaptic space (Clements, 1996). As a consequence, efficient but sub-maximal concentrations of GABA could reach neighbouring synapses as well as extrasynaptic $GABA_A$ receptors in which pregnanolone could reinforce the GABAergic inputs. Thus, it can be suggested that pregnanolone plays the role of a neuromodulator contributing to the tonic inhibitory control of melanotrophs. In addition, as recently shown in rat hypothalamo-hypophyseal co-cultures (Poisbeau *et al.* 1997), pregnanolone also acts at the presynaptic level, causing facilitation of GABA release. At higher concentrations, pregnanolone could directly inhibit the effect of GABA on α-MSH release by diminishing the chloride current.

In the pituitary pars intermedia, pregnanolone may originate from several sources. Mensah-Nyagan *et al.* (1994) have recently reported the presence of the enzyme (3β-hydroxysteroid dehydrogenase) that synthesizes progesterone in cell bodies of frog hypothalamic nuclei known to project on pituitary intermediate lobe (Tonon, Bosler, Stoeckel, Pelletier, Tappaz & Vaudry, 1992). Moreover, a 5β-reductase activity responsible for the conversion of progesterone into 5β-pregnan-3,20-dione, the precursor of pregnanolone, has been characterized in the bird adenohypophysis (Schlinger *et al.* 1989). Another source of steroids may be represented by folliculo-stellate cells which are present in a small proportion in the pars intermedia. These cells, as glial cells, could contribute to a local synthesis of steroids (for review, see Robel & Beaulieu, 1994). It can then be expected that, in the neuroendocrine synapses of the intermediate lobe, pregnane steroids are present at concentrations sufficient to potentiate the GABA-evoked responses. The pregnanolone-induced inhibition of the $GABA_A$ receptor activity observed in the melanotrophs could alternatively be caused by an increase in the steroid concentration originating from the adrenal gland (Holzbauer, Birmingham, De Nicola & Oliver, 1985) in particular situations such as stress.

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