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Direct channel-gating and modulatory effects of triiodothyronine on recombinant $GABA_A$ receptors

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Abstract

We have previously shown that triiodothyronine (T3) inhibits γ-aminobutyric acid type A ($GABA_A$) receptors in synaptoneurosomes and transfected cells. To further characterize this phenomenon, the effect of T3 on recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes was investigated using the two-electrode voltage-clamp method. T3 inhibited GABA-gated chloride currents in a non-competitive manner and yielded an IC_{50} of 7.3 ± 0.8 μM in oocytes coexpressing $\alpha_1\beta_2\gamma_{2L}$ receptor subunits. T3 had no inhibitory effect on $\alpha_6\beta_2\gamma_{2L}$ or $\beta_2\gamma_{2L}$ receptor constructs, indicating that the α_1 subunit imparts T3 sensitivity to the receptor. In addition to the inhibitory effect of T3 on GABA responses, T3 alone induced a current in oocytes expressing $\alpha_1\beta_2\gamma_{2L}$, $\alpha_6\beta_2\gamma_{2L}$ and $\beta_2\gamma_{2L}$ constructs. This current displayed a reversal potential identical to that of GABA-gated chloride currents, and was blocked by picrotoxin (10 μM), but not by bicuculline (50 μM), indicating that T3 gates the chloride channel by binding to a site other than the GABA-binding site. The direct channel-gating action of T3 was concentration-dependent, with an EC_{50} of 23 ± 5 μM. The actions of T3 are unique in that T3 acts as a noncompetitive antagonist in the presence of GABA but can directly gate the chloride channel in the absence of GABA.

Keywords: $GABA_A$ receptor; Triiodothyronine; *Xenopus* oocyte; Voltage-clamp, two-electrode

1. Introduction

GABA (γ-aminobutyric acid) is the primary inhibitory neurotransmitter in the central nervous system. It exerts its inhibitory effects primarily through the $GABA_A$ receptor, a ligand-gated chloride channel. Activation of the receptor by GABA opens the channel, resulting in membrane hyperpolarization and decreased neuronal excitability. The receptor is thought to be pentameric, with over 15 subunits cloned and sequenced (α_{1-6}, β_{1-4}, γ_{1-4}, δ, $\rho_{1,2}$ as well as alternatively spliced variants) (for review, see Rabow et al., 1995). The properties of the receptor, including channel gating (Sigel et al., 1990), allosteric modulation (Zhu et al., 1996; Hill-Venning et al., 1997) and desensitization (Verdoorn et al., 1990) are determined by the subunit composition of the receptor. The $GABA_A$ receptor contains binding sites for a large number of allosteric modulators, including therapeutic agents such as benzodiazepines, barbiturates and anaesthetic drugs. These modulators exert their effects by potentiating the GABA response, and, in some cases, at higher concentrations by directly activating the $GABA_A$ receptor. The $GABA_A$ receptor is also a site of action of endogenous compounds such as neurosteroids, which are thought to be involved in mediating stress responses through either inhibition or potentiation of the GABA response (Majewska, 1992; Lan and Gee, 1994).

Recently, we have shown that thyroid hormones modulate $GABA_A$ receptors (Martin et al., 1996). Thyroid hormones including L-triiodothyronine (T3) and L-thyroxine affect [^{35}S]*t*-butylbicyclophosphorothionate binding, and inhibit both recombinant $GABA_A$ receptors expressed in human embryonic kidney (HEK) 293 cells and GABA-stimulated $^{36}Cl^-$ uptake into rat brain synaptoneurosomes (Martin et al., 1996). The rapid in vitro effects of the thyroid hormones on $GABA_A$ receptor activity are evidence of a non-genomic action of the thyroid hormones, probably due to a direct interaction of the hormones with the $GABA_A$ receptor protein.

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An early observation was a subunit specificity of the inhibition of GABA-stimulated chloride currents in recombinant $GABA_A$ receptors expressed in HEK-293 cells (Martin et al., 1996). Recombinant receptors expressing α_6 subunits were not inhibited by thyroid hormones, while receptors containing α_1 subunits were inhibited by micromolar levels of thyroid hormones. From these experiments, however, it was not clear whether the α_6 subunit conferred insensitivity to thyroid hormones or whether the α_1 subunit conferred sensitivity. In current studies, we further examine the role of α_1/α_6 subunits in T3 inhibition of GABA responses and report, for the first time, the direct gating of the $GABA_A$ receptor chloride channel by T3.

2. Materials and methods

2.1. Materials

GABA, picrotoxin and bicuculline methiodide were obtained from Research Biochemicals International (Natick, MA); all other reagents used were analytical grade and were purchased from Sigma (St. Louis, MO). T3 was dissolved in 0.1 M NaOH to make a stock concentrate of 10 mM. This was diluted to working concentrations in buffer immediately before use and brought to a final pH of 7.5 with 0.1 M HCl.

Xenopus laevis were obtained from Xenopus I (Ann Arbor, MI) and maintained in plastic tanks at 17–18°C on a 12 h light/dark cycle and fed a diet of crickets and Nasco frog brittle.

2.2. Oocyte preparation

X. laevis were anesthetized by placing them in 0.2% tricaine for 15–20 min. A small incision was made in the abdomen and lobes of ovary were removed. Sections of ovary were separated by hand into small clumps and incubated with gentle agitation at room temperature with collagenase solution (2 mg/ml in NaCl 83 mM, KCl 2 mM, $MgCl_2$ 1 mM, HEPES 5 mM, pH = 7.5) until the ovarian epithelium, the theca and the follicular cell layer were dissolved (2–3 h). Defolliculated stage V and VI oocytes were placed in ND96 solution (NaCl 96 mM, KCl 2 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, HEPES 5 mM, pH = 7.5) and the oocyte nucleus was injected with cDNAs encoding human $GABA_A$ receptor subunits (30 nl of sterile H_2O containing 0.5 ng cDNA/subunit). Injected oocytes were maintained at room temperature in ND96 supplemented with 2 mM pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin and 0.5 mM theophylline.

2.3. Two-electrode voltage-clamp recording

At least 24 h following cDNA injection, oocytes were placed in a plexiglass recording chamber (100 μl volume) and continuously perfused with ND96. All drugs were applied by bath perfusion with either a gravity flow system (5 ml/min) or roller pumps (1 ml/min), as indicated. Cells were impaled with two glass microelectrodes (0.5–1.5 MΩ) filled with 3 M KCl and voltage clamped at −60 mV with a Model OC-725B Oocyte Clamp (Warner, Hamden, CT). GABA or T3-gated chloride currents were measured using a chart recorder. The peak amplitudes of responses were used for data. Statistical analysis is as noted in the results section. EC_{50} and IC_{50} values were generated using the curve-fitting function of Sigmaplot version 5.0.1 (Jandel, Corte Madera, CA).

Experiments began after three consecutive GABA or T3 responses did not vary by more than 10%. GABA was applied until the peak amplitude of the response was reached, at 10 min intervals. To assess the concentration-dependence of T3 effects on the GABA response, oocytes were preincubated with T3 (0.5–100 μM) for 20 s, followed immediately by a coapplication of T3 and GABA until the peak amplitude of the response was reached. The response to GABA under these conditions is expressed as a percent of the response to GABA alone. For these experiments, a roller pump system was used and solutions entering the perfusion chamber were changed by manually moving the intake tubing from one solution reservoir to another.

To assess the effects of T3 on the GABA concentration–response curve, oocytes expressing the $\alpha_1\beta_2\gamma_{2L}$ construct were perfused with GABA (1–1000 μM) in both the presence or absence of T3 (50 μM). For T3 application, oocytes were preincubated with T3 (50 μM) for 20 s, followed immediately by a coapplication of T3 and GABA until the peak amplitude of the response was reached. GABA responses are presented as a percent of response to 1000 μM GABA in the absence of T3, which was taken as 100%.

To examine the effects of T3 in the absence of GABA, oocytes were perfused with T3 (0.5–100 μM) until the peak current amplitude was reached. T3 was applied at 5 min intervals. To examine the effects of picrotoxin (10 μM) or bicuculline (50 μM) on the T3 or GABA response, these antagonists were applied for 30–60 s prior to their coapplication with T3 (50 μM) or GABA (100 μM). GABA and T3 responses in the presence of picrotoxin or bicuculline are expressed as a percent of the GABA or T3 response immediately preceding the coapplication of antagonist.

To determine the current/voltage relationships of GABA and T3 responses, applications of GABA (100 μM) or T3 (50 μM) were alternated at 5 min intervals. Oocytes were voltage-clamped at −80 to +40 mV at 20 mV intervals, and responses to GABA and T3 were determined at each voltage. Responses to each agonist were measured at −60 mV between voltage steps to ensure that responses to GABA and T3 remained stable throughout the experiment.

3. Results

The effect of T3 on GABA responses in *Xenopus* oocytes expressing $\alpha_1\beta_2\gamma_{2L}$, $\alpha_6\beta_2\gamma_{2L}$ or $\beta_2\gamma_{2L}$ constructs is shown in Fig. 1. In a representative tracing, the response to GABA of an oocyte expressing the $\alpha_1\beta_2\gamma_{2L}$ construct is shown to be dramatically reduced in the presence of 100 μM T3 (Fig. 1A). For this construct, the GABA response was reduced in the presence of T3 to 61 ± 4% of control, with a T3 IC_{50} of 7.3 ± 0.8 μM (Fig. 1B). Although 100 μM T3 appears to exert a near-maximal effect, higher concentrations of T3 could not be tested due to difficulty solubilizing T3 at higher concentrations. No inhibition of the GABA response was seen in oocytes expressing either $\alpha_6\beta_2\gamma_{2L}$ or $\beta_2\gamma_{2L}$ constructs (Fig. 1B).

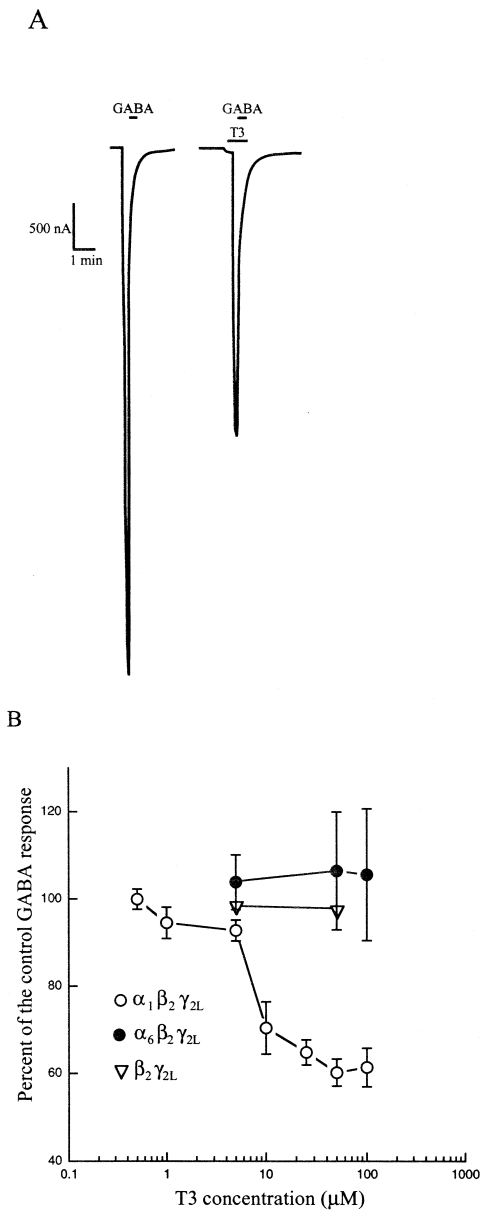

Fig. 1. T3 inhibition of GABA responses. (A) A representative tracing of T3 inhibition of the GABA response in an oocyte expressing the $\alpha_1\beta_2\gamma_{2L}$ construct. GABA (10 μM) was applied alone and then in the presence of T3 (100 μM) following a 20 s pretreatment with T3. Note the slight stimulatory T3 effect prior to the application of GABA. (B) Oocytes expressing $\alpha_1\beta_2\gamma_{2L}$, $\alpha_6\beta_2\gamma_{2L}$ and $\beta_2\gamma_{2L}$ constructs were perfused with 0.5–100 μM T3 for 20 s, followed by perfusion with T3 (0.5–100 μM) and GABA (10 μM). T3 inhibits the GABA response in $\alpha_1\beta_2\gamma_{2L}$ constructs only, with an IC_{50} of 7.3 ± 0.8 μM. Results are presented as a percent of the GABA response in the absence of T3 (mean ± S.E.M., $n = 3$).

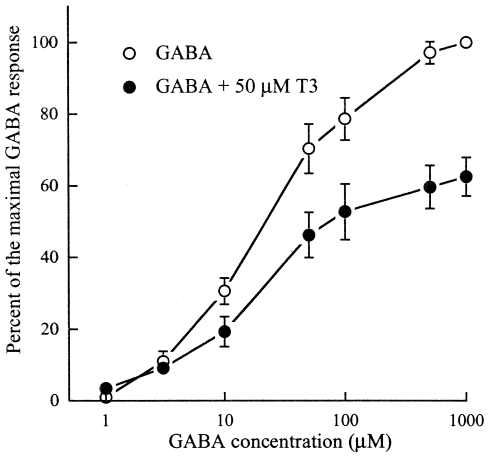

Fig. 2. GABA concentration–response curves in the presence and absence of 50 μM T3. Oocytes expressing $\alpha_1\beta_2\gamma_{2L}$ were perfused with GABA (1–1000 μM) in the presence and absence of 50 μM T3. GABA EC_{50s} were calculated to be 27 ± 7 μM in the absence and 23 ± 7 μM in the presence of T3. Results are presented as a percent of the response to 1000 μM GABA in the absence of T3 (mean ± S.E.M., $n = 3$).

To determine the effect of T3 on the GABA concentration–response curve, oocytes expressing the $\alpha_1\beta_2\gamma_{2L}$ construct were perfused with various concentrations of GABA (1–1000 μM) in the presence and absence of T3 (50 μM) (Fig. 2). The maximum response to GABA (1000 μM) was reduced to 62 ± 5% of control in the presence of 50 μM T3. The EC_{50} of GABA was 27 ± 7 μM in the absence and 23 ± 7 (mean ± S.E.M.) in the presence of 50 μM T3. T3 therefore decreases the efficacy, but not the potency of GABA.

Unexpectedly, T3 alone induced currents in oocytes expressing the $\alpha_1\beta_2\gamma_{2L}$, $\alpha_6\beta_2\gamma_{2L}$ and $\beta_2\gamma_{2L}$ constructs (Fig. 3). No effect of T3 was observed in uninjected oocytes (not shown). A sharp rise in current was frequently seen upon washout of T3 (Fig. 3A). The reason for this is unknown, but it is commonly seen among agents that directly activate the $GABA_A$ receptor through sites distinct from the GABA-binding site (Robertson, 1989; Orser et al., 1994; Hill-Venning et al., 1997). T3-induced currents were largest in the $\beta_2\gamma_{2L}$ construct. In oocytes expressing $\beta_2\gamma_{2L}$ subunits, the response to T3 (50 μM) was 470 ± 130 nA, while the GABA (100 μM) response was 450 ± 130 nA ($n = 4$). Thus, for the $\beta_2\gamma_{2L}$ construct these concentrations of T3 and GABA yielded peak amplitude responses of similar magnitudes. In contrast, for $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_6\beta_2\gamma_{2L}$ constructs, the GABA responses were 160 and 14-fold larger, respectively, than responses to T3.

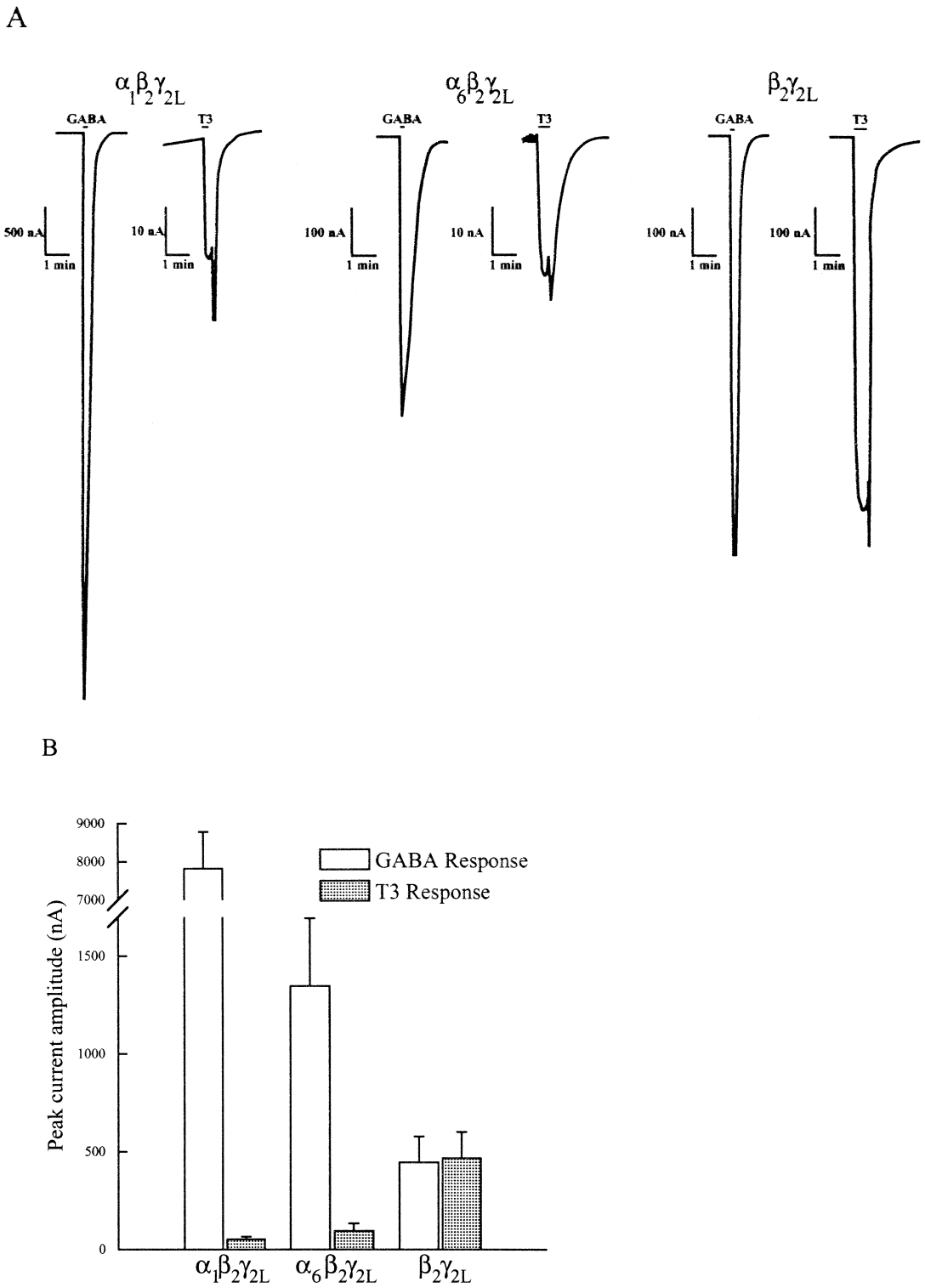

Fig. 3. Direct gating of the $GABA_A$ receptor by T3. (A) Representative tracings showing activation of the $GABA_A$ receptor by GABA or T3 in oocytes expressing $\alpha_1\beta_2\gamma_{2L}$, $\alpha_6\beta_2\gamma_{2L}$ and $\beta_2\gamma_{2L}$ subunits. Oocytes were perfused with GABA (100 μM) until a maximal (nA) response was reached, then perfused with T3 (50 μM) 10 min later. Note the difference in scale between the responses. (B) Average magnitude of the peak current response to GABA (100 μM) and T3 (50 μM) in oocytes expressing $\alpha_1\beta_2\gamma_{2L}$, $\alpha_6\beta_2\gamma_{2L}$ and $\beta_2\gamma_{2L}$ subunits. Oocytes were treated as in 'A'. Results are expressed as mean ± S.E.M., $n = 3–5$.

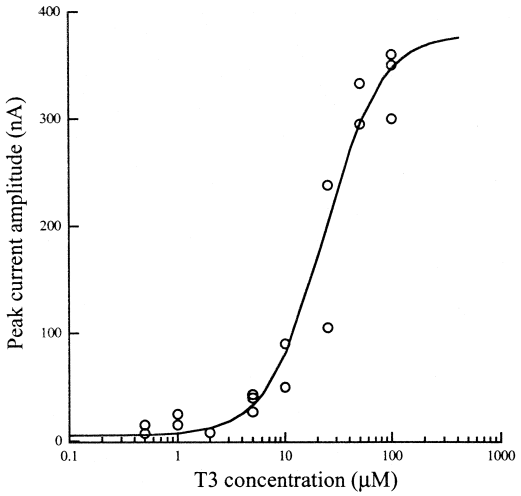

Fig. 4. T3 concentration–response curve in oocytes expressing the $\beta_2\gamma_{2L}$ construct. Oocytes were perfused with 0.5–100 μM T3 until a maximal response was reached. The EC_{50} for T3 in these oocytes was calculated to be 23 ± 5 μM (mean $\pm$ S.E.M.). Data from three oocytes was used to generate this curve. Each point represents the peak current response to an application of T3.

GABA responses in these constructs were 7800 ± 1000 and 1300 ± 300 nA, while responses to T3 (50 μM) in $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_6\beta_2\gamma_{2L}$ constructs were 53 ± 14 and 96 ± 40 nA, (mean $\pm$ S.E.M., $n = 3$–5), respectively. Due to the greater sensitivity of the $\beta_2\gamma_{2L}$ construct to T3, subsequent experiments were performed on the $\beta_2\gamma_{2L}$ construct. For this construct, the T3 EC_{50} was 23 ± 5 μM, with 100 μM T3 exerting a near-maximal effect (Fig. 4).

The reversal potential for both GABA-induced and T3-induced currents in the $\beta_2\gamma_{2L}$ construct was approximately -30 mV (Fig. 5), indicating that the responses to GABA and T3 demonstrate the same ion selectivity. Both

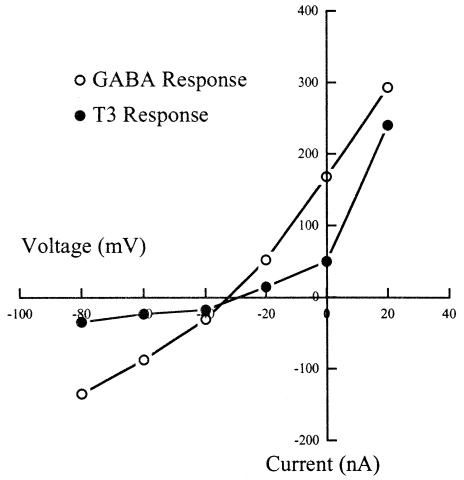

Fig. 5. Current–voltage curve for responses to T3 and GABA in oocytes expressing the $\beta_2\gamma_{2L}$ construct. The oocyte was voltage-clamped at -80 to $+40$ mV, and perfused alternately with T3 (50 μM) and GABA (100 μM) at 5 min intervals. Both the T3 and GABA responses display outward rectification and a reversal potential of approximately -30 mV. Results shown here are from a typical experiment.

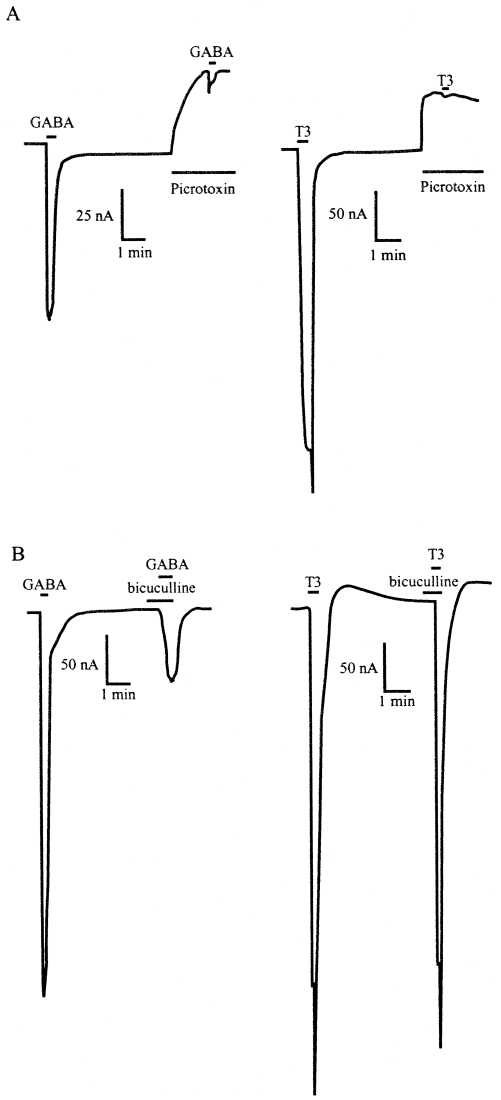

Fig. 6. Effect of picrotoxin and bicuculline on GABA and T3 responses in oocytes expressing the $\beta_2\gamma_{2L}$ construct. (A) Oocytes were perfused with 10 μM picrotoxin for 30–60 s, followed by picrotoxin + T3 (50 μM) or picrotoxin + GABA (100 μM). Picrotoxin blocked both the GABA and T3 responses. (B) Oocytes were perfused with 50 μM bicuculline for 30 s, followed by bicuculline + T3 (50 μM) or bicuculline + GABA (100 μM). Bicuculline inhibited the response to GABA but not to T3. Results shown here are from a typical experiment.

GABA- and T3-induced currents were blocked by the chloride channel blocker picrotoxin (10 μM) (Fig. 6A). Picrotoxin reduced the response of the $\beta_2\gamma_{2L}$ construct to GABA (100 μM) to $7.1 \pm 0.5\%$ of control ($n = 3$), and the response to T3 (50 μM) to $3 \pm 1\%$ of control ($n = 4$, data not shown). The GABA-induced current was reduced by $75 \pm 4\%$ by bicuculline (50 μM), while the T3 current was unaffected by bicuculline.

4. Discussion

T3 inhibits GABA-induced chloride currents in oocytes expressing $\alpha_1\beta_2\gamma_{2L}$ but not $\alpha_6\beta_2\gamma_{2L}$ or $\beta_2\gamma_{2L}$ con-

structs. In addition to the modulatory effect of T3 on GABA-induced chloride current, we now show that in the absence of GABA, micromolar concentrations of T3 can directly activate $GABA_A$ receptor chloride channels. The T3-induced current displays the same reversal potential as the GABA response and both currents exhibit outward rectification. Both the GABA and T3 responses are inhibited by the $GABA_A$ receptor channel blocker picrotoxin, while GABA but not T3-induced currents are blocked by the competitive $GABA_A$ receptor antagonist bicuculline.

Previous data have shown that T3 inhibits GABA responses in $\alpha_1\beta_2\gamma_{2L}$ but not $\alpha_6\beta_2\gamma_{2L}$ constructs (Martin et al., 1996). We sought to determine whether the α_1 subunit confers T3 sensitivity on the receptor or whether the α_6 construct confers insensitivity. To investigate this, T3 was tested on a $\beta_2\gamma_{2L}$ construct. In the absence of an α subunit, T3 does not inhibit GABA-induced chloride currents. Thus, it is the α_1 subunit which allows inhibition by T3, rather than a disinhibitory effect of the α_6 subunit.

Studies of the effect of T3 on the GABA concentration–response curve indicate that, in oocytes expressing the $\alpha_1\beta_2\gamma_{2L}$ construct, the mechanism by which T3 inhibits the GABA response is to reduce the efficacy of GABA. Thus, T3 is not acting as a negative allosteric modulator or as a partial agonist at the GABA-binding site, since inhibition of the GABA response by these two mechanisms would be overcome at high GABA concentrations and result in a decrease in GABA potency. Here, T3 is observed to inhibit responses to maximal concentrations of GABA without increasing the GABA $EC_{50,}$ indicating that T3 inhibits the $GABA_A$ receptor in a noncompetitive manner consistent with a channel-blocking mechanism of action.

In addition to the T3 inhibition of the GABA response in the $\alpha_1\beta_2\gamma_{2L}$ construct, T3 was found to induce a current response in the absence of GABA in $\alpha_1\beta_2\gamma_{2L}$, $\alpha_6\beta_2\gamma_{2L}$ and $\beta_2\gamma_{2L}$ constructs. This effect was concentration-dependent, with an EC_{50} of approximately 23 ± 5 μM for the $\beta_2\gamma_{2L}$ subunit construct. Both GABA and T3 responses were blocked by the chloride channel antagonist picrotoxin, indicating that T3 is acting at the $GABA_A$ receptor to open the chloride channel in the absence of GABA. In oocytes expressing the $\beta_2\gamma_{2L}$ subunit construct, picrotoxin was observed to decrease the baseline clamping current. This indicates that there is some conductance through these channels in the absence of GABA or T3, a phenomenon previously observed in β_1 homomeric channels (Sigel et al., 1989; Krishek et al., 1996). This current was not reduced by bicuculline, a competitive antagonist at the GABA-binding site. Similar to the present results, the direct channel-gating effects of other compounds such as etomidate (Hill-Venning et al., 1997), pentobarbitone (Akaike et al., 1987), loreclezole (Sanna et al., 1996), and allopregnanolone (Puia et al., 1990) are also blocked by picrotoxin. The inability of the competitive $GABA_A$ receptor antagonist bicuculline to block T3-induced currents indicates that T3 does not act at the GABA-binding site on the $GABA_A$ receptor, but acts at an unidentified site on the receptor complex.

A variety of compounds which potentiate $GABA_A$ receptors at low concentrations, directly open GABA-gated chloride channels at higher concentrations. These include propofol (Hara et al., 1993), loreclezole (Sanna et al., 1996), etomidate (Evans and Hill, 1977; Hill-Venning et al., 1997) and pentobarbital (Peters et al., 1988) as well as the neurosteroids allopregnanolone (Puia et al., 1990, 1993), alphalaxone (Barker et al., 1987) and 3α, 21-dihydroxy-5 α-pregnan-20-one (THDOC) (Puia et al., 1990). In comparison to these compounds, the actions of T3 are unique in three respects. First, regarding modulation of the GABA response, T3 *inhibits* GABA responses while the other compounds mentioned above that have direct channel-gating properties *potentiate* GABA responses. Secondly, both the modulatory and the direct effects of T3 occur at micromolar concentrations. For other drugs which exert both modulatory and direct effects, $GABA_A$ receptor modulation occurs at low (nM) concentrations, while the channel-gating effects occur at much higher (μM) concentrations. Lastly, bicuculline inhibits the direct channel-gating effects of etomidate (Evans and Hill, 1977), propofol (Hara et al., 1993), loreclezole (Sanna et al., 1996), alphaxalone (Barker et al., 1987) and pentobarbitone (Akaike et al., 1987; Peters et al., 1988), but not that of T3. These data suggest that direct gating of the channel by T3 occurs by a mechanism different from those of other compounds. The nature of that mechanism remains to be elucidated.

Although the modulatory effects of T3 are not seen in receptors containing the α_6 subunit, the direct channel-gating effect was observed in the α_6-containing construct $\alpha_6\beta_2\gamma_{2L}$, suggesting that the mechanism by which T3 inhibits the GABA response and gates the channel are different. Similar differences in subunit specificities have been observed with other compounds. For example, the GABA-potentiating effect of etomidate is greatly attenuated in the presence of the α_6 receptor, while the direct gabamimetic effect is not (Hill-Venning et al., 1997). Similarly, potentiation of the GABA response by the neurosteroid THDOC is reduced in the presence of the δ subunit, while the channel-gating effect is unaffected (Zhu et al., 1996). Thus, the modulatory actions and direct channel-gating effects of several compounds, including T3, have different subunit specificities.

The direct channel-gating effects of T3 were not noted in an earlier study (Martin et al., 1996). The failure, in that study, to detect the direct T3 effect may be due to the difference in the relative magnitudes of the GABA and T3 responses. For the constructs investigated in our previous study, namely $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_6\beta_2\gamma_{2L}$, we show here that the GABA responses are 160 and 14-fold greater, respectively, than the T3 responses. Thus, a comparatively small T3-induced current may not be detected when measuring GABA responses that are many fold larger.

Nongenomic, neurotransmitter-like mechanisms of thyroid hormone action have been proposed based on the presynaptic localization of T3 (Mason et al., 1993; Dratman and Gordon, 1996). The inhibition of the GABA response by T3 has been observed in both native and recombinant receptors and displays subunit specificity. In this regard, we have shown that the inhibitory action of T3 on the $\alpha_1\beta_2\gamma_{2L}$ but not $\alpha_6\beta_2\gamma_{2L}$ constructs is due to the α_1 subunit conferring sensitivity to the receptor, not the α_6 subunit conferring insensitivity. In addition to exerting modulatory effects on the GABA response, we show here, for the first time, that T3 directly gates the $GABA_A$ receptor chloride channel. As discussed, this effect is strikingly unique when compared to other modulatory compounds which display direct channel-gating effects. In this respect, T3 may be an interesting tool with which to study $GABA_A$ receptor function. The physiological relevance of the direct and indirect effects of T3 on $GABA_A$ receptors in the central nervous system remains to be demonstrated.

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