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Acute Exposure to 25-Hydroxy-cholesterol Selectively Reduces GABAb and Not GABAa Receptor-Mediated Synaptic Inhibition

K. D. Phelan and H. R. Mahler
Department of Anatomy, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

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Intracellular recording techniques were used to study the effects of the cholesterol oxide, 25-hydroxy-cholesterol (25-OH-Chol), on γ-aminobutyric acid (GABA) receptor-mediated inhibitory postsynaptic potentials (IPSPs) in brain slices of the rat lateral septum. Superfusion of 25-OH-Chol increased the peak amplitude of the GABAa IPSP in more than half of the neurons tested, many of which exhibited a similar increase in the GABAb IPSP. However, some neurons exhibited a gradual decrease in input resistance and a selective reduction or blockade of the GABAb IPSP during prolonged exposure. Cholesterol partly mimicked the effects of 25-OH-Chol. These findings indicate that 25-OH-Chol can selectively reduce or block metabotropic GABAb while sparing ionotropic GABAa receptor-mediated synaptic inhibition. Our results indicate that brain slices can be used to study the effects of short term alterations in cholesterol on the excitability and synaptic integration properties of neurons. © 1997 Academic Press

Cholesterol is an integral molecular constituent of mammalian membranes. It is known to be distributed in a non-uniform fashion among different cellular membranes and to form cholesterol rich domains within individual membranes (1). In the central nervous system, this uneven distribution includes differences in the levels of cholesterol in junctional and non-junctional membranes (2) and may underlie the observed regional differences in membrane fluidity within the brain (3). It has been reported that the level of cholesterol within plasma membranes changes with age, as a result of injury, and during certain neurodegenerative diseases (4-8). The age-dependent changes in the cholesterol content of neuronal membranes are believed to underlie observed decreases in muscarinic and noradrenergic agonist efficacy (9-11). These effects have been reported to be mediated by an alteration in the receptor-effector coupling mechanisms at the level of the receptor-GTP-binding protein (G-protein) interaction. It has been proposed that similar effects may occur for other members of the metabotropic G-protein coupled family of receptors (such as the metabotropic GABAb receptor), but this has not yet been tested. In addition to the effects of changes in cholesterol levels on receptor-effector coupling in neurons, cholesterol and oxidative derivatives of cholesterol have also been reported to alter various non-metabotropic ionic currents and transport functions (e.g., calcium currents, calcium-dependent potassium currents and various ATP-ases) in a variety of cell types [e.g., 12-20). However, little is known about similar effects on central neurons.

The present study represents an effort to characterize the effects of acute alterations of cholesterol homeostasis on neuronal function at the single cell level. Intracellular recording techniques were used to compare the effects of short term application of cholesterol and the cholesterol oxide, 25-OH-Chol, on GABAergic inhibition mediated by activation of the ionotropic GABAa and the G-protein linked metabotropic GABAb receptor. The *in vitro* rat dorsolateral septum slice model was utilized because it is a well characterized system in which neurons consistently exhibit two separate monosynaptically evoked IPSPs mediated by activation of GABAa and GABAb receptors (21).

MATERIALS AND METHODS

Brain slice preparation. Transverse slices of the rat forebrain were prepared from adult Sprague-Dawley rats as previously described (21,22). A total of 20 animals (13 adult male and 7 adult female rats previously used for breeding) were sacrificed to prepare the brain slices. The results from all of the animals were pooled since there was no apparent difference based on sex. Intracellular recordings were obtained from 45 individual dorsolateral septal nucleus neurons in 27 separate brain slices using a standard current clamp recording methodology. The slices were held submerged in the recording chamber and continuously superfused with oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (ACSF) warmed to 30-32° C. The ACSF consisted of (in mM): NaCl 122.8, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 26, and D-glucose 10.

Electrophysiological recording methods. Glass micropipettes were filled with 3M potassium acetate (final resistances 60-90 mΩ).

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Currents were recorded and analyzed on-line using an Axoclamp 2A amplifier connected to a Power Macintosh 7100 computer using Superscope II software (GWI Instruments). Synaptic currents were elicited by applying square wave voltage pulses (0.2 ms duration) to bipolar stimulating electrodes placed locally within the lateral septum. Stimuli were applied at 15 sec intervals using voltages that produced a maximal GABAb IPSP peak amplitude unless otherwise noted. Neurons were routinely held at a membrane potential just below firing threshold during control recordings. The peak amplitudes of the GABAa and GABAb receptor-mediated IPSPs were measured at the control membrane potential after compensating for any drug induced changes in membrane potential by injecting D.C. current through the recording electrode.

Drug preparation and application. Drugs were prepared as stock solutions at a concentration of 2 mg/ml (5 mM) and stored in the freezer. Stock solutions of 25-OH-Chol and cholesterol were dissolved in 100% ethanol. The final concentration of drug used in the experiments was kept at 2 μg/ml (5 μM) in order to limit ethanol exposure to 0.1%. A higher concentration (20 μg/ml) of cholesterol and 25-OH-Chol was tested but did not produce effects different from those described for the lower concentration (data not shown). These concentrations have proven effective in altering cellular function in cultured superior cervical ganglion and PC12 cells (23-25). Results obtained with cholesterol (which precipitated out of solution at 2 μg/ml) were compared to those obtained with a water soluble form of cholesterol (polyoxyethanyl-cholesteryl sebacate). Fresh solutions were prepared daily by dissolving the stock solutions in ACSF. Slices were pretreated by immersing in a static bath of oxygenated ACSF. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Superfusion of the cholesterol oxide, 25-OH-Chol (2 μg/ml), produced a small membrane hyperpolarization of 4-10 mV (5.7 $\pm$ 0.8 mV; mean $\pm$ SEM) that progressively appeared during the 20-60 minute superfusion period in 7/13 cells tested. In these neurons, the spontaneous firing rate either increased (e.g., Fig. 1B) or decreased when compared to pre-drug levels at the control membrane potential. In the majority of cells tested, superfusion of 25-OH-Chol produced an increase in the peak amplitude of the stimulus evoked GABAa IPSP (10-50% increase; 9/13 cells) (e.g., Fig. 1) regardless of whether there was any change in membrane potential. Almost half of these affected cells also exhibited a small increase in the peak amplitude of the GABAb IPSP ($<$20% increase; 4/9 cells) (e.g., Fig. 1). These increases in IPSP amplitudes were accompanied by either a small ($<$10%) increase (n=3) or decrease (n=4) in input resistance or no discernible change (n=2; e.g., Fig. 1C) suggesting that they did not passively occur as a result of changes in input resistance. There was no apparent change in the delay to peak or reversal potential of either the GABAa or GABAb IPSPs (data not shown). The peak amplitude of the initial excitatory postsynaptic potential (EPSP) that preceded the GABAa IPSP in the evoked response (e.g., Fig. 1) was not consistently decreased in those cells that exhibited increased IPSPs. These increases in IPSP amplitude typically occurred within 5-20 minutes of 25-OH-Chol application, though in two cells it slowly appeared during prolonged superfusion. These two neurons also exhibited a concurrent and progressive decrease in the amplitude and/or duration of the GABAb IPSP (Fig. 2A). These effects of 25-OH-Chol on the GABAergic IPSPs did not appear to reverse during a 20 minute washout period (data not shown). Subsequent recordings in the same slices in which neurons exhibited 25-OH-Chol induced changes in GABAergic inhibition indicated the presence of neurons that still possessed large amplitude GABAb IPSPs (data not shown) suggesting that the effects of 25-OH-Chol may be selective for a subpopulation of neurons.

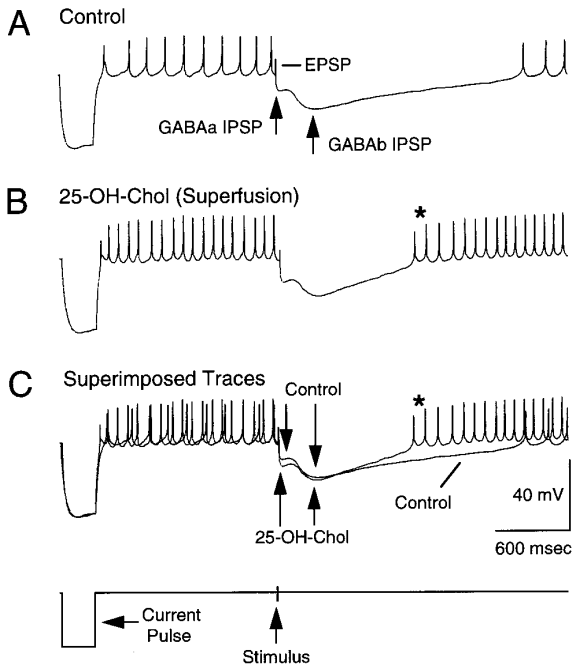

FIG. 1. Intracellular current clamp recordings of the effect of 25-OH-Chol on GABAergic IPSPs in a brain slice preparation of rat dorsolateral septum. (A) A typical response of a neuron to a hyperpolarizing current pulse (0.5 nA, 300 msec) and stimulation of an electrode using the protocol shown at the bottom. An initial EPSP is followed by a fast onset, short duration GABAa IPSP and longer onset, longer duration GABAb IPSP. (B) Superfusion of 25-OH-Chol (2 μg/ml; 20 minutes) produces an increased frequency of spontaneous firing. (C) Superimposition of the traces reveals a 25-OH-Chol induced increase in the peak amplitudes of GABAa and GABAb IPSPs with no change in input resistance as reflected by the overlapping downward voltage deflections on the left. Note the 25-OH-Chol induced decrease in the duration of the GABAb IPSP (*) compared to control. The membrane potential was $-$62 mV.

In contrast to the effects of 25-OH-Chol, superfusion of a similar concentration of the water soluble or insoluble forms of cholesterol (2 μg/ml; 25-120 min) produced no discernible effect in the majority of cells tested (5/7 cells). However, one cell treated with the water insoluble form of cholesterol showed a similar magnitude increase in the peak amplitude of the GABAa and GABAb IPSPs as that induced by 25-OH-Chol (Fig. 3A). Another cell treated with the water soluble form of cholesterol exhib-

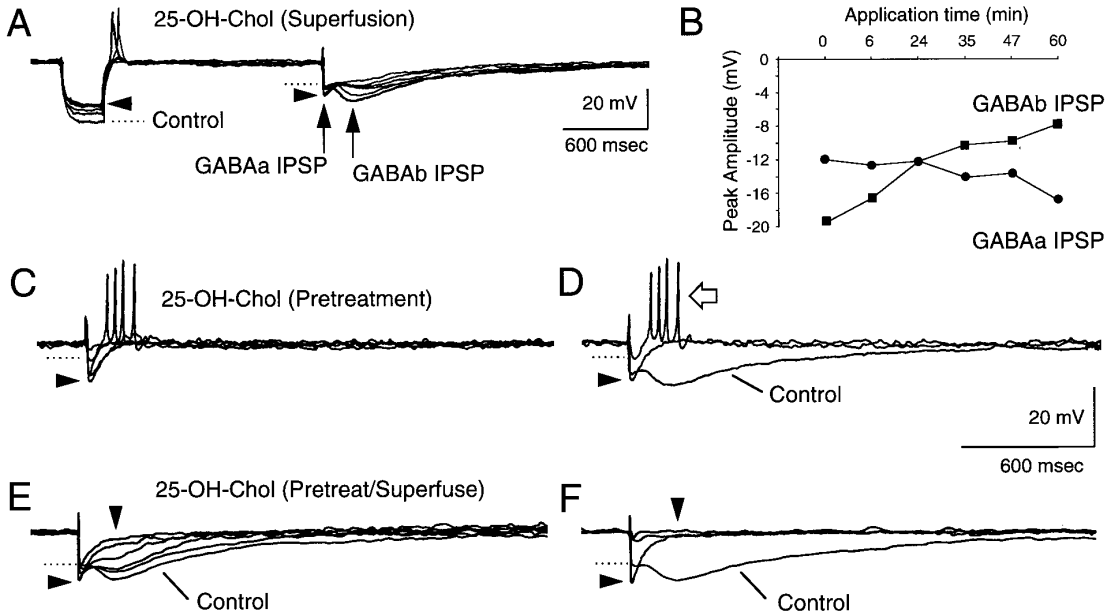

FIG. 2. A comparison of the effects of 25-OH-Chol on input resistance and synaptic inhibition following acute superfusion or pretreatment of slices. (A) Superfusion of 25-OH-Chol (2 μg/ml) produced a time-dependent decrease in the amplitude and duration of the GABAb IPSP, a concomitant increase in the amplitude of the GABAa IPSP, and a ~25% decrease in input resistance. Dotted lines indicate control levels, arrowheads indicate final 25-OH-Chol induced levels. The membrane potential was −60 mV. (B) The peak amplitude of the GABAa and GABAb IPSPs shown in (A) are plotted against time since the start of drug application. Note the ~30% increase in the GABAa IPSP amplitude and the >50% decrease in the GABAb IPSP amplitude. (C) Synaptic potentials elicited in response to a graded series of stimuli in a neuron recorded in a slice pretreated with 25-OH-Chol (2 μg/ml; 2 hrs). Note the absence of a prominent GABAb IPSP and the unmasking of an underlying depolarizing synaptic response which elicits action potentials. The membrane potential was −58 mV. (D) Superimposition of selected traces from the cell shown in (C) and a control trace from the cell shown in (A). Arrowheads in (C) and (D) mark the level of the peak GABAa IPSP amplitude (20 V). The open block arrow marks a trace obtained after applying a higher intensity stimulus (40 V) similar to that used to elicit the maximum GABAb IPSP shown in the control trace. The dotted lines in (C) and (D) mark the reduced level of the GABAa IPSP in this trace. (E) Superfusion of 25-OH-Chol (2 μg/ml; 1 hr) in a neuron that still exhibited a large amplitude GABAb IPSP after a 2 hr pretreatment (control) produced a time-dependent decrease in the amplitude and duration of the GABAb IPSP (▼). The membrane potential was −62 mV. (F) Superimposition of two sets of traces obtained following low intensity and high intensity stimulation prior to (control, dotted lines) and after 25-OH-Chol superfusion (arrowheads) of the cell shown in Fig. 2E. The calibration bar in (D) applies to (C–F).

ited a progressive decline in the peak amplitude and duration of the GABAb IPSP during prolonged superfusion. This was accompanied by a 15% increase in the peak amplitude of the GABAa IPSP despite a significant decrease in input resistance (Fig. 3B).

In order to determine the effect of longer exposures to cholesterol or 25-OH-Chol, we pretreated 11 additional slices with these agents for up to 5 hours prior to recording. In those slices pretreated with 25-OH-Chol (2 μg/ml; 1-4 hrs; n = 7), there was a conspicuous absence of a prominent GABAb receptor-mediated IPSP component to the evoked response in 4/12 cells tested (Fig. 2C). In each of these cells, the amplitude of the GABAa IPSPs steadily increased as the stimulus intensity was increased with no apparent GABAb IPSP component to the response. In two of these cells, the GABAa IPSP reached a maximum peak amplitude, after which further increases in stimulus strength resulted in a progressive decrease in the amplitude of the GABAa IPSP. This decrease was accompanied by the appearance of a late depolarization that elicited a series of action potentials (e.g., Fig. 2C,D). This depolarization presumably represents the unmasking of the underlying EPSP due to the absence of a prominent GABAb IPSP. Similar EPSPs are observed when the GABAb IPSP is either selectively blocked with antagonists or uncoupled from its G-protein (26). The peak of this late depolarization occurred at a time during which the GABAb IPSP would normally have been at or near its peak amplitude (Fig. 2D). The duration of this late depolarization was shorter than the GABAb IPSP and completely returned to baseline at a time at which the GABAb IPSP would have still been relatively large (Fig. 2D) indicating that a GABAb IPSP component is not likely to be masked by the presence of the depolarization.

In order to determine if pretreatment of the slices had presensitized neurons to further exposure to 25-OH-Chol, we superfused 25-OH-Chol (2 μg/ml) onto those neurons which still exhibited a large amplitude GABAb IPSP despite prolonged pretreatment. A small increase in the amplitude of the GABAa IPSP (<20%) occurred in 2/3 cells tested in this manner. One of these

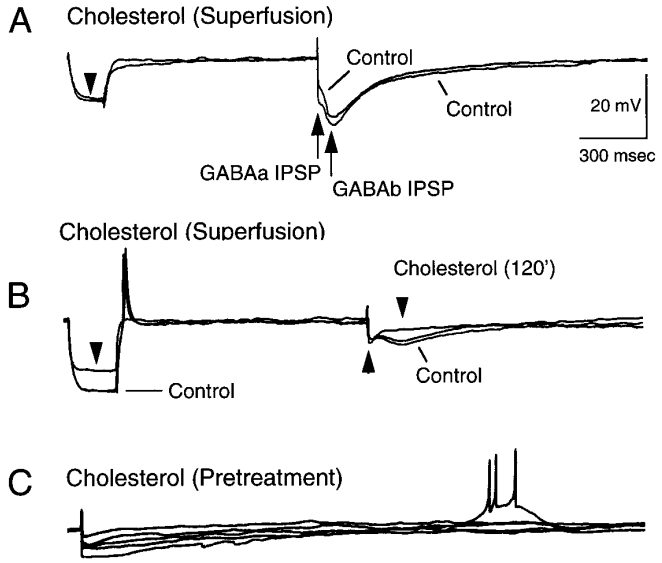

FIG. 3. Effects of cholesterol on GABAergic IPSPs. (A) Superfusion of a water insoluble form of cholesterol (2 μg/ml, 15 min) produced a small increase in the peak amplitude of the GABAa and GABAb IPSPs and a small decrease in input resistance (▼). The membrane potential was −63 mV. (B) Prolonged superfusion of a water soluble form of cholesterol (2 μg/ml, 2 hrs) produced a 30% decrease in input resistance (▼), and a progressive decrease in GABAb IPSP (▼) but an increase in GABAa IPSP amplitudes (▲). The membrane potential was −60 mV. (C) An example of an evoked response in a neuron recorded in a slice pretreated with a water-soluble form of cholesterol (2 μg/ml, 5 hrs). Note the absence of a prominent GABAb IPSP and the presence of a prolonged GABAa IPSP decay compared to Fig. 2C,E. The membrane potential was −62 mV. Calibration bars in (A) apply to (B) and (C).

two cells also exhibited a progressive decrease in the amplitude and duration of the GABAb IPSP (Fig. 2E). Although a prominent GABAb IPSP was not present in this particular cell after a 60 min superfusion, hyperpolarization of the cell membrane toward the reversal potential for the GABAa IPSP revealed a small GABAb component to the response as the GABAa IPSP was reduced in amplitude (data not shown). This same component is evident in Fig. 2F as the low amplitude plateau on the decay phase of the inhibitory synaptic response elicited with the high intensity stimulation in the 25-OH-Chol treated cell. This low amplitude response overlaps with a similar response seen following low intensity stimulation in the control trace (Fig. 2F).

Pretreatment of slices with the water soluble form of cholesterol (2 μg/ml; 3-5 hrs) resulted in only 1/9 cells that exhibited a synaptic profile that did not contain a prominent GABAb IPSP compared to control (Fig. 3C). In this cell, however, it appeared as though a GABAb component to the response was masked by the large amplitude GABAa IPSP, since the decay phase of the inhibitory response was much longer than that seen in cells which clearly lacked a GABAb IPSP (compare Figs. 2C,E and 3C).

DISCUSSION

The present study is the first to demonstrate that acute exposure of neurons in a brain slice preparation to cholesterol or the cholesterol oxide, 25-OH-Chol, can produce significant changes in evoked synaptic responses. Interestingly, although the peak amplitude of the GABAa IPSPs increased in 70% of cells superfused with 25-OH-Chol, a similar of the GABAb IPSP only occurred in 44% of these same neurons (~30% of the total neurons tested) during the relatively short time course studied (0.5-5.0 hrs of exposure). This difference may reflect the fact that some cells exhibited a dramatic decrease in the amplitude and duration of the GABAb IPSP following prolonged exposure which might have offset any concurrent increase. The overall percentage of cells affected following pretreatment of the slices was not much different from that seen following acute superfusion suggesting that the length of exposure may not be a determining factor for whether a cell exhibits changes in GABAergic inhibition. However, it is not known if longer exposures (>5 hours) might increase either the number of cells showing 25-OH-Chol induced changes or the extent to which the GABAa and GABAb IPSPs are affected. Although neurons in the dorsolateral septum are heterogeneous with respect to cell morphology, neurotransmitter content and electrophysiological properties (22, 27-29), it remains to be determined if those cells affected by acute exposure to cholesterol or 25-OH-Chol represent a distinct subpopulation of neurons. It could be, for example, that the selective effects observed here occur in a subpopulation of neurons that express different GABAa and/or GABAb receptor subtypes. Alternatively, the different sensitivity to 25-OH-Chol exposure could simply reflect differences in the accessibility of the drug to cells located deeper within the slice.

The mechanism(s) that underlie the 25-OH-Chol induced increase in the peak amplitudes of the GABAa and GABAb IPSPs are unknown. It is unlikely that these reflect passive changes in response to changes in input resistance since we failed to observe any consistent change in input resistance. This is supported by the fact that the peak amplitude of the GABAa IPSP was seen to increase even in those cells which exhibited a significant decrease in input resistance during 25-OH-Chol superfusion (e.g., Fig. 2A,B). The present study cannot exclude the possibility that these increases resulted from a decrease in the EPSP component to the synaptic response, though this is unlikely since such a decrease was not consistently observed in each cell that exhibited an increase in the GABAa IPSP. In fact, some cells in the pretreated slices exhibited a large amplitude GABAa IPSP and EPSP in the absence of a prominent GABAb IPSP (e.g., Fig. 2C).

One mechanism that could account for the observed increase in the amplitude of both the GABAa and the

GABAb inhibitory responses is the possibility that 25-OH-Chol decreases the re-uptake of GABA from the synaptic cleft since GABA transport function is reportedly dependent on the cholesterol content of the plasma membrane (30). Membrane lipid peroxidation (a process known to produce oxide derivatives of cholesterol) also reportedly decreases GABA uptake into synaptosome preparations (31). The observed increase in IPSP amplitudes could also reflect a 25-OH-Chol induced increase in the presynaptic release of GABA by reducing the efficacy of presynaptic GABAb autoreceptors in a manner similar to its reduction of the postsynaptic GABAb receptor as reported here. Some neurons exhibited an increase in the GABAa IPSP amplitude only as the amplitude and duration of the GABAb receptor-mediated IPSP decreased which might represent a reduction in a shunting effect imposed by the concurrent activation of the ionic conductance changes underlying the GABAa and GABAb IPSPs. Similar magnitude increases in the peak amplitude of GABAa IPSPs are seen following uncoupling of the GABAb receptor from its G-protein by exposure to N-ethyl-maleimide (26). A direct effect of 25-OH-Chol on the GABAa receptor itself is also possible since GABAa receptor-channel function is reportedly modulated by changes in the cholesterol content of the membrane in a regionally specific fashion (32). Although a similar direct modulatory effect of cholesterol on the GABAb receptor has not yet been reported, it is known that alterations in the membrane cholesterol/phospholipid ratio can alter the fluidity and interdigitation of plasma membranes (20). Such changes could affect the three dimensional protein structure of the ionotropic GABAa and/or metabotropic GABAb receptor-effector complexes. Additional studies will be required to determine if one or more of these possibilities underlie the 25-OH-Chol induced increase in GABAergic inhibition.

In some neurons, prolonged exposure to 25-OH-Chol produced a significant reduction in input resistance accompanied by a selective reduction or block of the GABAb but not the GABAa IPSP. This difference could indicate that GABAb IPSPs are more sensitive to changes in resistance than GABAa IPSPs. However, the complete absence of GABAb IPSPs in 25% of the neurons recorded in 25-OH-Chol pretreated slices suggests that the 25-OH-Chol induced change in resistance may not be coupled to the concurrent decrease in GABAb IPSP amplitude. It is more likely that the selective reduction or block of the GABAb IPSP reflects the metabotropic nature of the GABAb receptor-effector coupling mechanism since it is known that as cholesterol accumulates in membranes as a function of age it exerts a deleterious effect on receptor-G-protein interactions (9,10). It will be interesting to determine if the effects reported here are generalizable to GABAb receptors in other regions of the brain or other G-protein linked metabotropic receptors. It is not clear why only a small percentage of GABAb IPSPs were affected by acute exposure to 25-OH-Chol. However, it has recently been shown that estrogen rapidly attenuates a postsynaptic GABAb response in only a subpopulation of hypothalamic neurons (10,33). Interestingly, GABAb receptor expression in the cerebral cortex appears to be regulated by changes in steroids during the estrous cycle (34,35). Since cholesterol is a precursor for steroid synthesis, it is possible that long term alterations in cholesterol homeostasis might also indirectly alter GABAb receptor-mediated inhibition through changes in the levels of steroid synthesis.

The results of the present study revealed differences in the acute effects of exposure to cholesterol and 25-OH-Chol on GABAergic synaptic inhibition. It is known that long term exposure to oxysterols decreases the synthesis of cholesterol (36,37), but the effects observed here occurred within a time frame that is unlikely to involve effects mediated by changes in cholesterol synthesis. It is more likely that the effects reflect the insertion of the cholesterol or 25-OH-Chol into the plasma membrane. Our finding that 25-OH-Chol is more effective in producing changes in GABAergic inhibition than is cholesterol is consistent with previous reports that the biological activity of oxysterols far exceeds that of cholesterol in a wide variety of cells [for review see 38; see also 23).

The functional consequence of chronic alterations in cholesterol homeostasis on GABAergic inhibition remain unknown. A reduction in GABAb receptor-mediated inhibition similar to that shown here may be partly offset by an enhanced GABAa receptor-mediated inhibition. However, it is clear that in the absence of GABAb IPSPs there is a possibility for an increase in synaptic excitation as revealed in the present study. It remains to be determined if this increased excitation contributes to any pathological consequence. Chronic inhibition of cholesterol biosynthesis has been used to produce a rat model of absence epilepsy (39). It will be interesting to determine if there are any significant changes in GABAergic inhibition in hypercholesteremic animals or in other animal models that exhibit defects in cholesterol synthesis, transport or storage. The present results may have implications with regard to the effects of potential alterations in GABAergic inhibition during development given the role that GABAa and GABAb receptors play in early neuron development (40,41). They may also be relevant with regard to reports that changes in diet can alter central neuronal function (10, 42,43), especially since cholesterol oxides are present naturally in some food products (44).

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