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RESEARCH PAPER

Phosphorylation of sodium channels mediated by protein kinase-C modulates inhibition by topiramate of tetrodotoxin-sensitive transient sodium current

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Background and purpose: Topiramate is a novel anticonvulsant known to modulate the activity of several ligand- and voltage-gated ion channels in neurons. The mechanism of action of topiramate, at a molecular level, is still unclear, but the phosphorylation state of the channel/receptor seems to be a factor that is able to influence its activity. We investigated the consequences of phosphorylation of the sodium channel on the effect of topiramate on tetrodotoxin (TTX)-sensitive transient Na^+ current (I_{NaT}).
Experimental approach: I_{NaT} was recorded in dissociated neurons of rat sensorimotor cortex using whole-cell patch-clamp configuration.
Key results: We found that topiramate (100 μM) significantly shifted the steady-state I_{NaT} inactivation curve in a hyperpolarized direction. In neurons pre-treated with a PKC-activator, 1-oleoyl-2-acetyl-sn-glycerol (OAG; 2 μM), the net effect of topiramate on steady-state I_{NaT} inactivation was significantly decreased. In addition, OAG also slightly shifted the I_{NaT} activation curve in a hyperpolarized direction, while perfusion with topiramate had no effect on the parameters of I_{NaT} activation.
Conclusions and Implications: These data show that PKC-activation can modulate the effect of topiramate on I_{NaT}. This suggests that channel phosphorylation in physiological or pathological conditions (such as epiliepsy), can alter the action of topiramate on sodium currents.
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Keywords: transient Na^+ current; topiramate; PKC; sodium channel; antiepileptic drug

Abbreviations: ACSF, artificial cerebrospinal fluid; I_{NaT}, transient Na^+ current; OAG, 1-oleoyl-2-acetyl-sn-glycerol

Introduction

It has been demonstrated that a number of traditional and new anti-epileptic drugs significantly inhibit the transient Na^+ current (I_{NaT}; Ragsdale and Avoli, 1998), an effect that can play a primary role in preventing the occurrence or spread of epileptic ictal events. However, the functional properties of voltage-gated Na^+ channels in the brain are subject to modulation by the activation of second messenger systems (Cantrell and Catterall, 2001) by either physiological or pathological (epileptic) events. In particular, protein kinase C (PKC)-mediated channel phosphorylation modifies the properties of I_{NaT} by favoring Na^+ channel inactivation at depolarized membrane potentials (Godoy and Cukierman, 1994; O'Reilly *et al.*, 1997; Franceschetti *et al.*, 2000). This effect is comparable to that of a number of anti-epileptic drugs that principally act by stabilizing the inactivated state of the channel and therefore the concomitant action of PKC activation and anti-epileptic drugs could lead to a cumulative inhibitory effect on I_{NaT}.

Topiramate (2,3:4,5-bis-*O*-(1-methyl-ethylidene)-β-D-fructopyranose sulfamate) is a structurally novel anticonvulsant, currently used for treating several seizure disorders (Bourgeois, 1998; Sander, 1998; Reife *et al.*, 2000; Biton *et al.*, 2005). Electrophysiological studies have revealed several effects of topiramate on ion channels, including negative modulation of voltage-gated Na^+ (Zona *et al.*, 1997; Taverna *et al.*, 1999; Curia *et al.*, 2004) and Ca^{2+} channels (Zhang *et al.*, 2000), blockade of kainate-evoked inward

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current (Gibbs *et al*., 2000; Skradski and White, 2000) and modulation of GABAergic current (White *et al*., 1997; Herrero *et al*., 2002). In addition, topiramate is able to provoke depression of sustained repetitive firing that is mediated by Na^+ currents (DeLorenzo *et al*., 2000; McLean *et al*., 2000).

Although the exact mechanism of action of topiramate at a molecular level is not completely understood, results from previous studies suggest that the phosphorylated state of target receptors/channels can affect the activity of topiramate (Gibbs *et al*., 2000; Angehagen *et al*., 2004; Curia *et al*., 2004).

In the present study, we confirmed the previously reported effect of topiramate (Taverna *et al*., 1999) and an activator of protein kinase C, 1-oleoyl-2-acetyl-sn-glycerol (OAG; Franceschetti *et al*., 2000) on the transient component of the TTX-sensitive sodium current (I_{NaT}) and we investigated whether the phosphorylation state of sodium channels could influence the effect of topiramate.

Methods

Cell preparation

All experimental procedures were carried out in compliance with the European 86/609/UE law and the guidelines of the Ethics Committee of 'C Besta' Institute.

Sprague–Dawley rats aged 10–17 days (Charles River, Italy) were anesthetized with ether and decapitated. Their brains were removed and placed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF), containing (in mM): 124 NaCl, 3.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 10 glucose (pH 7.35 with NaOH). Coronal slices (300 μm thick) were cut from the sensorimotor cortex using a vibratome, treated with 1 mg ml^{-1} of Protease XIV (Sigma, Italy) to digest the extracellular matrix, and then stored in enzyme-free ACSF. Immediately before the recordings, single neurons were dissociated using fire-polished Pasteur pipettes and plated in a Petri dish (Falcon, Becton Dickinson and Company, Franklin Lakes, NJ, USA) coated with Concanavalin A (50 μg ml^{-1}) to allow cell adhesion.

Electrophysiology

Whole-cell patch clamp recordings were performed at room temperature using a 200B amplifier (Molecular Devices, Palo Alto, CA, USA). All data were digitized using a Digidata 1200 interface (sampling frequency: 10 kHz) and pClamp 8.0 (Molecular Devices) and analyzed with Origin 6.0 software (OriginLab, Northampton, MA, USA).

Borosilicate glass electrodes (3–4 MΩ) were filled with a solution containing (in mM): 120 CsF, 2 $MgCl_2$, 10 HEPES, 10 ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*,-tetraacetic acid-CsOH, 2 Na_2ATP, 10 phosphocreatine-diTris, 0.3 Na-GTP, and 20 U ml^{-1} creatine phosphokinase, pH 7.2. As external bath solution, a modified ACSF was used, in which 105 mM choline-Cl partially substituted NaCl, in order to reduce the voltage error owing to the large Na^+ current observed in pyramidal neurons. $CdCl_2$, $NiCl_2$ and TEA-Cl in the external solution (respectively 0.4, 0.3 and 20 mM), and Cs in the intracellular one, were added to block calcium and potassium currents.

OAG (Sigma, Italy) 2 μM and topiramate (RAW Johnson Pharmaceutical Research Institute, Rarity, NJ, USA) 100 μM, were dissolved in ACSF and applied using a micromanifold, whose tip was positioned no more than 100 μm from the soma of the selected neuron. Previous studies show that topiramate acts on sodium channels in a dose-dependent manner (DeLorenzo *et al*., 2000; McLean *et al*., 2000) with an IC_{50} value of 48.9 μM and a saturating concentration of 500 μM (Zona *et al*., 1997). For our experiments, we chose the concentration of 100 μM of topiramate in order to compare our data with the ones previously collected from several laboratories (Zona *et al*., 1997; Taverna *et al*., 1999; Franceschetti *et al*., 2000; Curia *et al*., 2004). In addition, this concentration corresponds to 34 μg ml^{-1}, a value that is just over the upper limit of clinically relevant plasma levels in epileptic patients (Contin *et al*., 2002).

Voltage-clamp recordings were performed on 32 neurons dissociated from somatosensory cortex of Sprague–Dawley rats. Principal cells were identified from interneurons by the larger size and the pyramidal-like shape of the soma, which is preserved after the dissociation process. All the traces presented in the figures represent the transient component of the TTX-sensitive sodium current (I_{NaT}), obtained from off-line digital subtraction of recordings before and after application of TTX.

In the episodic stimulus protocol, used to evaluate the voltage dependence of steady-state inactivation, eight preconditioning pulses, from −90 to −20 mV and lasted 300 ms, preceded the test pulse at −15 mV, which lasted 50 ms. Steady-state inactivation curves were obtained by fitting the data points with a Boltzmann relationship in the form: $I/I_{MAX} = 1/\{1 + \exp[(V_{1/2} - V)/k]\}$, where I/I_{MAX} is the relative current, $V_{1/2}$ the voltage at which half-maximal inactivation is reached and k the slope factor.

The episodic stimulus protocol used to evaluate the voltage dependence of steady-state activation, was composed of nine test pulses (from −60 to −20 mV) lasted 200 ms. The activation curves were obtained by fitting the data points with a Boltzmann equation in the form: $G/G_{MAX} = 1/\{1 + \exp[(V_{1/2} - V)/K]\}$, where G_{MAX} is maximal peak conductance, G peak conductance at each test voltage, $V_{1/2}$ the voltage at which half-maximal activation is reached and k the slope factor. Reversal potential for sodium current was measured experimentally from each neuron.

Data analysis

The data are presented as mean values ± standard error of the mean (s.e.m.) and were statistically analyzed using analysis of variance or Wilcoxon's tests. Values of $P < 0.05$ were taken as showing a significant difference between means.

Results

Voltage dependence of steady-state inactivation

Topiramate (100 μM) inhibited the peaks evoked by more depolarized conditioning pulses (cf. −50 versus −70 mV conditioning pulse in Figure 1a), thus leading to a significant hyperpolarizing shift (9.3 ± 1.2 mV) in the steady-state I_{NaT} inactivation curve (Figure 1d, Table 1).

Pretreatment with OAG (2 μM) had a progressively increasing inhibitory effect on the peak current evoked after depolarizing prepulses positive to −70 mV (Figure 1b–c). Between 5 and 8 min after the start of OAG perfusion, the average value of the steady-state inactivation midpoint shifted in a negative direction by 10.2±0.9 mV compared to control conditions (Table 1). Neurons exposed to OAG were subsequently perfused with OAG together with topiramate ($n=5$). Alternatively, the medium containing OAG was immediately replaced with a medium containing topiramate alone ($n=3$). Under both conditions, the steady-state I_{NaT} inactivation curve further shifted in a hyperpolarizing direction, and the midpoint became 4.2±0.7 mV more negative than the one measured in the presence of OAG alone ($n=8$; Figure 1e, Table 1). We obtained similar results in five more neurons preincubated for 20–30 min with OAG. In these cells, addition of topiramate to OAG shifted the midpoint of the steady-state I_{NaT} inactivation curve from −62.4±2.6 ($k=6.4\pm0.1$) to −69.5±2.7 mV ($k=6.5\pm0.2$) ($P<0.05$).

On the basis of these results, we can conclude that topiramate was still capable of inducing a small hyperpolarizing shift of I_{NaT} inactivation in phosphorylated channels (average: −4.9±0.6 mV), but that this effect is significantly smaller than in non-phosphorylated ones (−9.3±1.2 mV; $P<0.05$) (Table 1).

I_{NaT} activation

Addition of topiramate to neurons incubated in standard ACSF ($n=6$) inhibited I_{NaT} by 20.6±7. 7% ($P<0.05$), without affecting either the voltage-dependence or slope of the activation curve (Figure 2a, c; Table 1).

In the presence of OAG, the maximal peak I_{NaT} amplitude did not change significantly when evoked by step potentials to −25 mV, but was increased when evoked by step potentials to −40 mV (Figure 2b). The activation curve was consistently shifted in a hyperpolarizing direction (3.8±0.7 mV; $P<0.05$; $n=7$) and concurrently became less steep (Figure 2d; Table 1). Addition of topiramate to the perfusion containing OAG inhibited the maximal I_{NaT} amplitude by 19.2±6.1% ($n=7$, $P<0.05$), without further changes in the I_{NaT} activation curve (Figure 2d; Table 1). The extent of this inhibitory effect on I_{NaT} was similar to that found in the neurons perfused with topiramate alone.

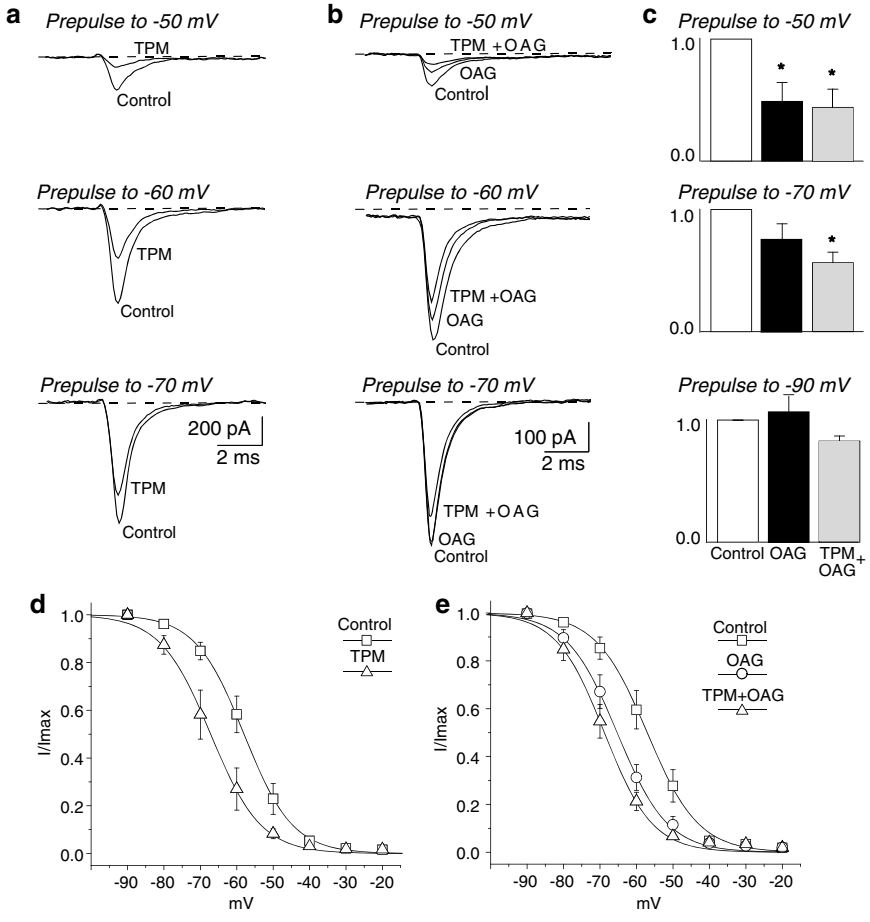

Figure 1 Effects of topiramate (TPM) and OAG on steady-state I_{NaT} inactivation. (**a** and **b**) TTX-subtracted current traces evoked by 50 ms step depolarization to −15 mV after a 300 ms prepulse at different membrane potentials. (**a**) Na^+ currents recorded in a neuron exposed to topiramate 100 μM. (**b**) Na^+ current recorded in a neuron exposed to OAG 2 μM, and then to OAG plus topiramate. (**c**) The normalized peak amplitude of I_{NaT} evoked after conditioning pulses to −90, −70 and −50 mV during perfusion with OAG (black bar) or OAG + topiramate (grey bar) compared with the normalized value of the current peak measured under control conditions (white bar; * = $P<0.05$; $n=8$). (**d** and **e**) Steady-state inactivation curve obtained by plotting the current peaks (normalized to maximal values) against the prepulse potential. The curves show the fit obtained using a Boltzmann function applied to the data points calculated under control conditions and in the presence of topiramate (**d**), and under control conditions, in the presence of OAG and OAG plus topiramate (**e**).

Table 1 I_{NaT} steady-state inactivation parameters (mean values ± s.e.m.)

	Steady-state inactivation			*Activation*		
	n	$V_{1/2}$ *(mV)*	k	n	$V_{1/2}$ *(mV)*	K
Controls	6	−57.9 ± 2.1	5.9 ± 0.3	6	−35.7 ± 0.7	3.1 ± 0.4
TPM		−67.3 ± 2.7*	5.9 ± 0.3		−36.9 ± 0.7	3.3 ± 0.3
Controls	8	−56.1 ± 1.6	6.2 ± 0.4	7	−34.7 ± 0.7	2.9 ± 0.2
OAG		−66.0 ± 1.6**	5.9 ± 0.3		−38.5 ± 1.0*	3.6 ± 0.2*
TPM + OAG		−70.5 ± 1.7*,●	5.7 ± 0.2		−39.4 ± 1.0*	3.9 ± 0.1*

Abbreviations: OAG, 1-oleoyl-2-acetyl-sn-glycerol; TPM, topiramate. * = $P<0.05$; ** = $P<0.01$, in comparison with the values measured under control conditions. ● = $P<0.05$, in comparison with the values measured in the presence of OAG.

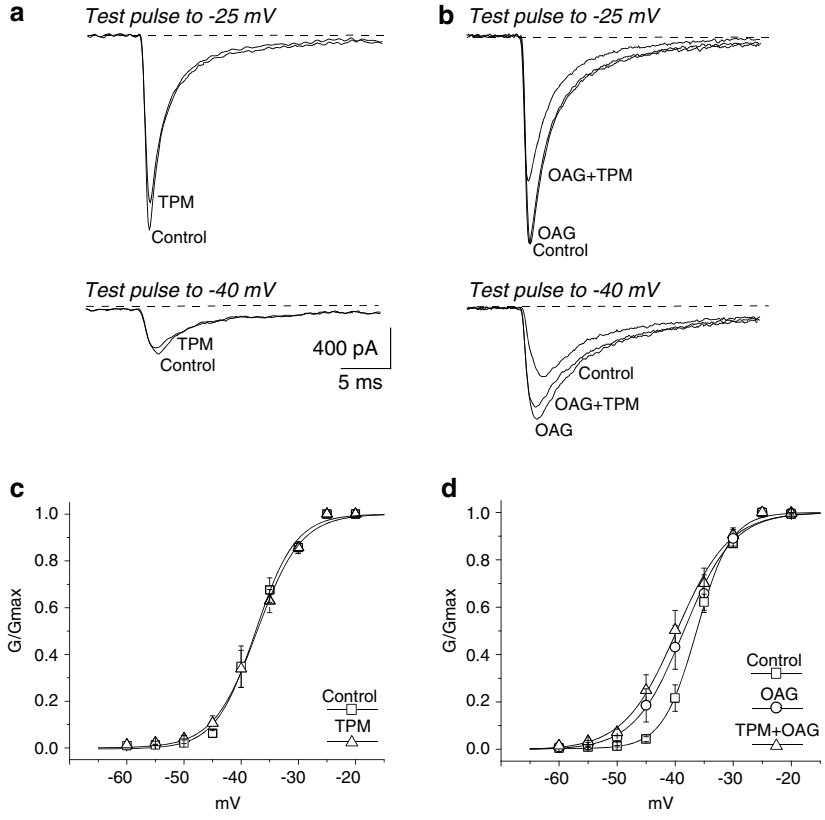

Figure 2 Effects of topiramate (TPM) and OAG on I_{NaT} activation. (**a** and **b**) TTX-subtracted current traces evoked from holding potential (−70 mV) to respectively −40 and −25 mV. (**a**) Na^+ currents recorded in a neuron exposed to topiramate 100 μM. (**b**) Na^+ current recorded in a neuron exposed to OAG 2 μM, and then to OAG plus topiramate. In (**b**), note that the increased amplitude of the current evoked by a depolarizing step to −40 mV in a neuron perfused with OAG is only partially counteracted during perfusion with both OAG and topiramate. (**c** and **d**) Voltage-dependent Na^+ conductance; the curves show the Boltzmann function fit applied to the data points calculated under control conditions and in the presence of topiramate (**c**), and under control conditions, in the presence of OAG and OAG plus topiramate (**d**).

Discussion and conclusions

Our results indicate that the inhibitory effect of topiramate resulting from a hyperpolarizing shift of the steady-state I_{NaT} inactivation curve (Zona *et al.*, 1997; Taverna *et al.*, 1999) is partially prevented by pretreatment with the PKC activator OAG. Moreover, topiramate was unable to counteract the OAG-induced hyperpolarizing shift on I_{NaT} activation.

Both OAG and topiramate individually induced a comparable hyperpolarizing shift on I_{NaT} steady-state inactivation, thus confirming that both experimental manipulations are capable of reducing Na^+-dependent membrane excitability by favouring channel inactivation. PKC-dependent phosphorylation may therefore act as an endogenous system that reduces cell excitability following a sufficiently large depolarization.

However, at the same time, this change seems to limit the inhibitory effect of topiramate, which acts on Na^+ channels by shifting the I_{NaT} inactivation curve toward more hyperpolarized potentials. Chemically different anti-epileptic drugs acting on Na^+ channels seem to bind preferentially

to extracellular channel sites, which became available only in the case of conformational changes leading to inactivation (Kuo, 1998). Our findings suggest that PKC-dependent phosphorylation may act by partially preventing topiramate binding to inactivated channels or by lessening its stabilizing effect on channel inactivation. Shank *et al*. (2000) hypothesized that topiramate binds selectively to protein kinase A-mediated phosphorylation site only when the channels are in the dephosphorylated state. After binding the site, topiramate could exert either a positive or negative allosteric modulation. The variable activity of topiramate could be a result of the variable state of phosphorylation of the channels. In particular Shank *et al*. (2000) suggested an inverse relation between the level of the topiramate effect on the channel conductance and the degree of channel phosphorylation.

A direct excitatory mechanism owing to PKC activation may come from the small but consistent hyperpolarizing shift in the voltage dependence of I_{NaT} activation owing to OAG perfusion (Franceschetti *et al*., 2000). This effect enhances membrane excitability by increasing the availability of Na^+ channels prone to open at negative membrane potentials that are very close to resting levels. Topiramate did not change the activation parameters in the neurons perfused with OAG, or in those perfused with standard ACSF. However, it exerted its inhibitory effect by decreasing the current amplitude. This result is similar to that found by evaluating the persistent fraction of sodium currents, for which activation was also shifted in a hyperpolarizing direction in the presence of OAG (Astman *et al*., 1998; Franceschetti *et al*., 2000), but was left unchanged by topiramate perfusion (Curia *et al*., 2004).

The inhibitory role played by topiramate on I_{NaT} includes a reduction of the peak amplitude that is bigger in comparison with that observed for I_{NaP} (Curia *et al*., 2004). A further inhibitory effect on I_{NaT} is the hyperpolarizing shift of the steady-state inactivation curve. However, because I_{NaP} plays a crucial role in boosting near-threshold depolarizations (Curia *et al*., 2004), even small reductions of its amplitude can significantly modify the excitable properties of neurons. Thus, both I_{NaP} and I_{NaT} can be important targets for TMP action on neuronal hyperexcitability. The concomitant effects on both current fractions suggest that topiramate can act on different functional states of the same channel.

It has been estimated that complete seizure control is achievable in 54–82% of patients with primary generalized epilepsy syndromes. In fact, a substantial group of patients with untreatable epilepsy is present (Faught, 2004). The interaction of PKC activation with the effects of topiramate may be an important mechanism leading to the uneven efficacy of topiramate on Na^+ channels in different patients or at different times during the natural course of an epileptic disorder. Our data suggest that activation of the PKC second messenger pathway, that has been shown to occur in a number of experimental models of epilepsy (Chen *et al*., 1992; Osonoe *et al*., 1994; Akiyama *et al*., 1995), may actually change Na^+-dependent excitability in epileptic patients, thereby altering the sensitivity of Na^+ channels to topiramate.

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Conflict of interest

The authors state no conflict of interest.

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