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Short communication

Felbamate inhibits cloned voltage-dependent Na^+ channels from human and rat brain

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Abstract

The novel antiepileptic and neuroprotective drug felbamate (1 mM) caused a marked inhibition of voltage-dependent Na^+ currents expressed in *Xenopus* oocytes upon injection of the cRNA encoding α-subunits from rat and human brain. This inhibition was present only if felbamate was perfused on the intracellular side of the membrane. In addition, felbamate seems to preferentially bind to and stabilize the inactivated state of the channel, resembling the action of local anesthetics. This study provides an additional mechanism by which felbamate might exert its wide-spectrum anticonvulsant and neuroprotective action.

Keywords: Felbamate; Na^+ channel, voltage-dependent; Anti-epileptic

1. Introduction

Despite the large availability of antiepileptic drugs, there is still a 25% of epileptic patients that are unresponsive to the conventional pharmacological treatment. For this reason a great deal of interest has been devoted to the development of new antiepileptic drugs. Among them, felbamate displays a promising clinical profile since it is effective in the treatment of several uncontrolled epileptic disorders (Upton, 1994), although several toxicities, particularly the occurrence of thrombocytopenia, limit its clinical usefulness.

The molecular mechanism of action of felbamate is still a matter of debate. Several pharmacological actions have been advocated for explaining felbamate effects. These are: (1) an enhancement of γ-aminobutyric acid-(GABA)-mediated inhibitory processes, (2) a reduction of excitatory neurotransmission, by interacting at the strychnine-insensitive glycine binding site on the NMDA receptor, or (3) a modulation of membrane cation conductance (Upton, 1994).

The inhibition of voltage-dependent Na^+ channels is the primary mechanism of action of several traditional antiepileptic drugs such as diphenylhydantoin and carbamazepine (Catterall, 1988; Taylor and Meldrum, 1995). The aim of the present study was to evaluate the possible mechanism of action of felbamate on cloned voltage-dependent Na^+ channels and to characterize its extracellular and/or intracellular site of action.

2. Materials and methods

2.1. Cloning and expression of the cDNAs encoding for the Na^+-channel α-subunits

The cloning of the rat type IIA (Auld et al., 1988) and of the human hB1 (Ahmed et al., 1992) brain voltage-dependent Na^+-channel α-subunits has been already described. cDNAs for both channels were propagated in the plasmid vector pBluescript SK(−) in *Escherichia coli* XL1-blue. cRNAs were in vitro transcribed from these cDNAs by means of a commercially available kit (mMessage Machine, Ambion) and stored in a stock solution (250 ng/μl) at −80°C in 0.1 M KCl.

Xenopus oocytes (stage V–VI) were isolated following the standard procedures and microinjected with 46 nl of

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the respective cRNA stock solution. After 3–10 days from the cRNA microinjection, Na^+ currents expressed in *Xenopus* oocytes were measured by classical electrophysiological techniques.

2.2. Electrophysiology

2.2.1. Two-microelectrode voltage clamp

For two-microelectrode voltage clamp, the oocytes were voltage-clamped with a commercially available amplifier (Dagan 8500, Dagan). Current and voltage electrodes were filled with 3 M KCl, 10 mM HEPES (pH 7.4; ≈ 1 MΩ resistance). The bath solution contained (in mM): 120 NaOH, 2.5 KOH, 122.5 2-(*N*-morpholino)-ethanesulphonic acid) (MES), 2 $MgCl_2$, 10 HEPES, pH 7.3.

2.2.2. Macropatch currents

Macropatch currents were recorded using the giant patch technique (Hilgemann, 1989). We modified the original technique in such a way that no vaseline or other sealant material was used. The diameter of the patch pipette was between 15 and 20 μm (between 0.2 and 0.5 MΩ resistance); the seal resistance was usually > 10 GΩ. The pipette tips were Sylgard coated (Dow-Corning, Midland, MI, USA) to reduce the glass capacitance. The pipette (external) solution contained (in mM): 120 NaCl, 2.5 KCl, 2 $MgCl_2$, 10 HEPES, pH 7.2; the bath (internal) solution contained (in mM): 100 KCl, 10 EGTA, 10 HEPES, pH 7.3). Holding and test potentials applied to the membrane patch are reported as absolute intracellular potentials assuming that the oocyte resting potential was zeroed by the bathing solution.

2.2.3. Single-channel currents

Single-channel currents were recorded in the inside-out configuration of the patch-clamp technique using oocytes dissected free of the vitelline envelope and micropipettes of 2–5 MΩ resistance that were fire-polished and Sylgard coated. The pipette and bath solutions are the same as those utilized in macropatch recordings. Data were low-pass filtered at 2 kHz (−3 db, 4-pole Bessel filter) prior to digitization at 5 kHz. Channels were activated with rectangular test pulses from negative holding potentials and current records were corrected for capacitive and leakage currents by subtracting the smoothed average of records lacking channel activity ('null traces'). Data were analyzed as described (Kirsch et al., 1992). Both whole-cell and

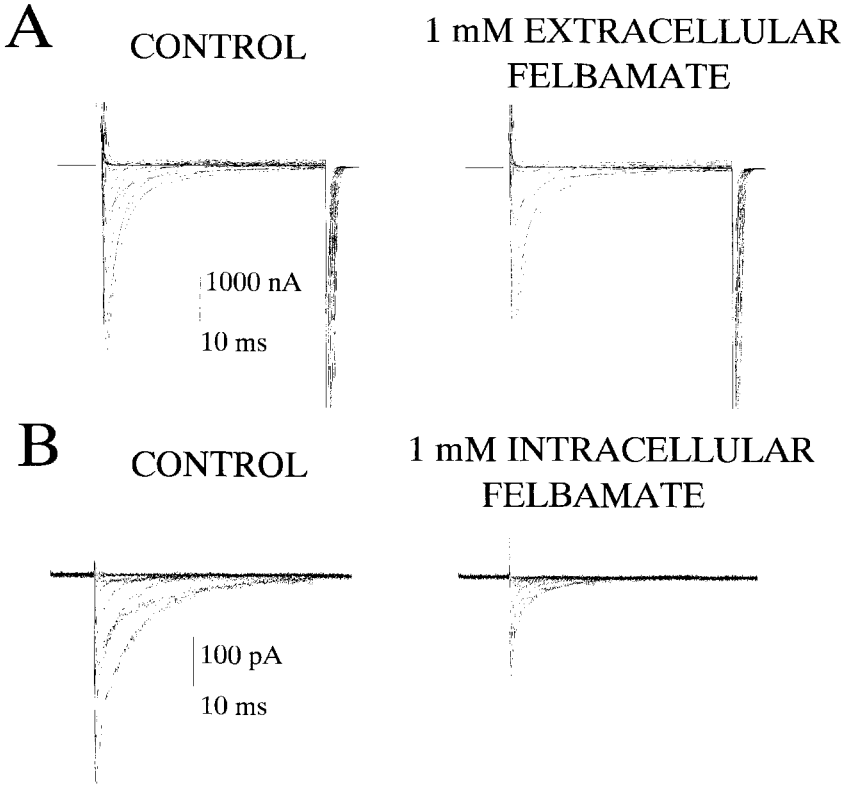

Fig. 1. Effect of external and internal application of felbamate on type IIA rat brain Na^+-channel α-subunits expressed in *Xenopus* oocytes. (A) Rat brain Na^+ channels formed by type IIA α-subunits expressed in *Xenopus* oocytes were activated by depolarizing pulses from −80 to +40 mV in 10 mV steps from a holding potential of −90 mV, both in control conditions and upon extracellular perfusion with 1 mM felbamate. (B) Inside-out macropatches from oocytes expressing the rat brain type IIA Na^+-channel α-subunit. Na^+ channels were activated by depolarizing pulses from −80 to +40 mV in 20 mV steps from a holding potential of −100 mV, both in the absence (left panel) and in the presence (right panel) of 1 mM felbamate.

single-channel experiments were performed at room temperature (22–24°C) in a recording chamber which was continuously superfused at a flow rate of 2 ml/min.

2.3. Drugs and statistics

Felbamate, a kind gift from Schering-Plough (Italy), was dissolved in DMSO at a concentration of 1 M. The final DMSO concentration in the bathing solution never exceeded 0.1%; this DMSO concentration did not affect Na^+-channel function.

Statistical significance between the data was obtained by means of the Student's *t*-test ($P < 0.05$).

3. Results

3.1. Effect of felbamate on rat brain type IIA Na^+ channel α-subunits expressed in Xenopus oocytes

In Fig. 1A are reported the effects of extracellular felbamate on *Xenopus* oocytes expressing the type IIA voltage-dependent Na^+ channel α-subunit from the rat brain. Under this condition, no effect of felbamate was evident, even after oocyte perfusion for more than 15 min. However, when inside-out patches were isolated from oocytes expressing the same type (IIA) of rat brain Na^+ channel α-subunits and the intracellular side of the membrane was perfused with a solution containing 1 mM felbamate, a marked inhibition (approximately 60% of the

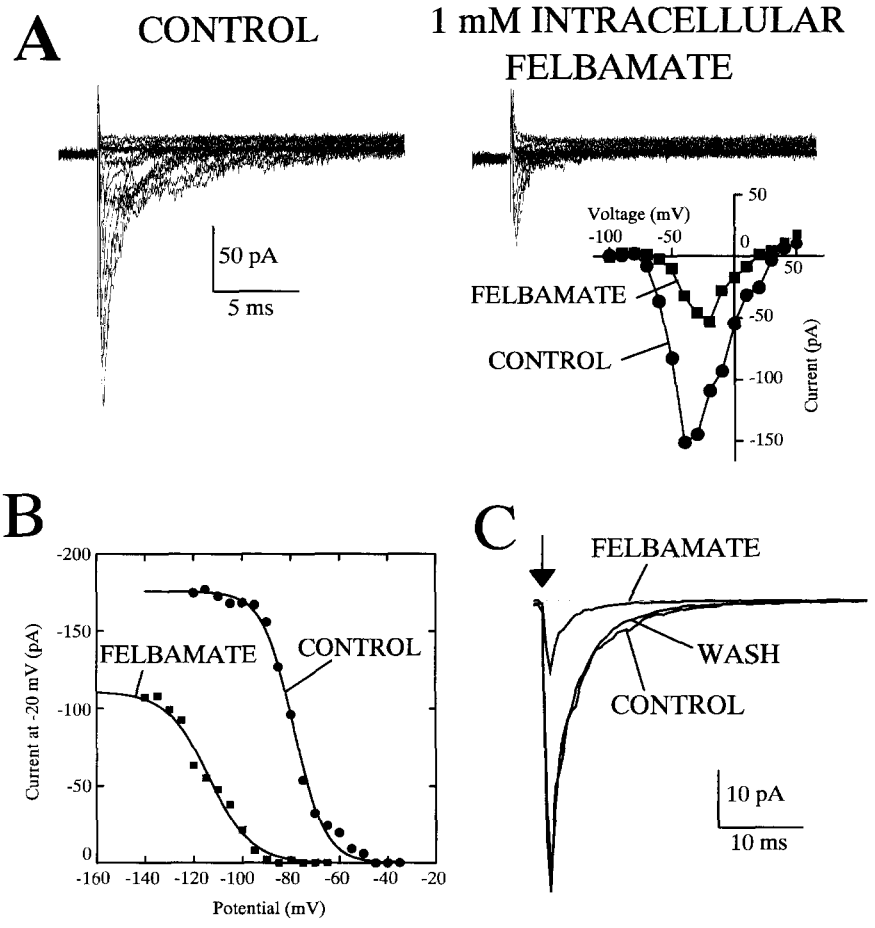

Fig. 2. Effect of external and internal application of felbamate on human brain hB1 Na^+ channels expressed in *Xenopus* oocytes. (A) Inside-out macropatches from oocytes expressing the human brain Na^+-channel α-subunit. Na^+ currents were elicited by depolarizing pulses from −100 mV to +50 mV in 10 mV increments, both in the absence (left panel) and in the presence (right panel) of felbamate. In the insert is also reported the peak current to voltage relationship in the absence (filled circles) and in the presence (filled squares) of 1 mM internal felbamate. (B) Steady-state inactivation of Na^+ currents recorded from oocytes expressing the human brain Na^+-channel α-subunit. The −20 mV test pulse was preceded by a 100 ms conditioning prepulse to the potentials indicated on the abscissa. The peak current elicited by the test pulse at −20 mV is plotted on the ordinate. The solid lines are best fits of the experimental data to the Boltzmann equation described in Section 3. (C) Inward Na^+ currents from oocytes injected with the human brain Na^+-channel α-subunit elicited by a test potential of −10 mV from a holding potential of −120 mV in control condition, after exposure to 1 mM felbamate, and upon drug washout.

peak inward current) of Na^+ currents was evident (Fig. 1B).

3.2. Effect of felbamate on human brain Na^+ channel α-subunits (hB1) expressed in Xenopus oocytes

The recent availability of the cDNA encoding the voltage-dependent Na^+ channel α-subunit from the human brain prompted us to investigate the effects of felbamate on Na^+ currents expressed upon its microinjection into *Xenopus* oocytes. In analogy with the results obtained in the rat clone, also in the human clone the extracellular perfusion of 1 mM felbamate did not modify the macroscopic Na^+ currents elicited by depolarization (data not shown), whereas the exposure of the intracellular side of inside-out patches expressing the human hB1 Na^+ channel α-subunit to the same antiepileptic drug concentration caused a marked inhibition of voltage-dependent Na^+ currents (Fig. 2A). The extent of Na^+ current reduction was similar in both rat and human brain clones (Fig. 1B and Fig. 2A); the peak (−20 mV) inward current inhibition induced by felbamate on hB1 Na^+ currents was 57.8% ± 3.8 ($n = 4$).

3.3. State-dependent blockade of hB1 channels by felbamate

In order to investigate the state dependency of felbamate binding to Na^+ channels, we performed experiments using a double-pulse protocol, in which a −20 mV test pulse was preceded by a 100 ms conditioning prepulse to various potentials. The peak values of the current responses elicited by the −20 mV pulse were expressed as a function of the prepulse voltage and fitted to the following form of the Boltzmann equation: $g\mathrm{Na}_V = 1/1(1 + \exp(ze(V - V_{1/2})/kT))$, where V is the prepulse potential, $V_{1/2}$ is the half-activation potential, z is the effective valence, e the elementary charge constant, k the Boltzmann constant, and T the absolute temperature expressed in Kelvin (Fig. 2B).

Felbamate induced a shift toward more negative potentials of the dependency upon membrane potential of the fraction of inactivated Na^+ channels (steady-state inactivation curve). The fitted values for $V_{1/2}$ were −79 ± 5 mV ($n = 3$) in controls and −114 ± 6 mV ($n = 3$) ($P < 0.05$) in the 1 mM intracellular felbamate group. The fitted values for the effective valence z were 3.95 ± 0.6 ($n = 3$) in controls and 3.05 ± 0.7 ($n = 3$) in the 1 mM intracellular felbamate group ($P > 0.05$).

Despite the limitations imposed by the short (5–15 min) duration of the patches and the fact that the relatively fast Na^+-channel rundown in the inside-out configuration prevented the execution of double-pulse experiments sufficiently long to define the kinetics required for the drug to reach true equilibrium, it is possible to estimate the apparent affinities of felbamate for the resting and inactivated states of the channel, using the formalisms originally developed to characterize the interaction of lidocaine with cardiac Na^+ channels (Bean et al., 1983). The resting state block can be estimated by the percentage of inhibition of the current elicited from negative holding potentials (where most of the channels are in the resting state) and infrequent pulsing. In our double-pulse experiments, from a −120 mV holding potential and using a 0.1 Hz pulsing frequency, we estimated an apparent K_d for the resting state (K_R) of about 2.1 mM. The affinity for the inactivated state (K_I) can be calculated from the shift of the midpoint voltage of the steady-state availability curve (ΔV_h), according to the following equation: $\Delta V_h = k \ln(1 + [\text{felbamate}]/K_R)/(1 + [\text{felbamate}]/K_I)$. Given an estimated k from our experiments of 6.32, in close agreement with the 5.99 value of Bean et al. (1983), and a $\Delta V_h = -35$ mV, we calculated a K_I value of 7 μM.

Furthermore, felbamate binding to the open state could only be partially resolved in the present experiments, since the inactivation kinetics of the macroscopic current were similar in control and felbamate groups. In fact, best fits of the decay of the current traces at −20 mV displayed double-exponential kinetics both in the absence and in the presence of felbamate. The fast and slow time constants were similar in both groups ($P > 0.05$), being, respectively, 0.49 ± 0.13 ms ($n = 4$) and 4.2 ± 1.5 ms ($n = 4$) in the controls and 0.35 ± 0.05 ms and 2.5 ± 0.5 ms ($n = 4$) in the felbamate group. However, the relative amplitude of the two exponentials was clearly different in the two groups; in fact, while the slow time constant accounted for 60.3 ± 4% ($n = 4$) of the total current in the control group, it only accounted for 38.6 ± 5% ($n = 4$) of the total current in the felbamate group ($P < 0.05$). This result seems to suggest that at least part of the Na^+ current fast inactivation process observed after internal perfusion with felbamate is due to drug binding to the open state.

In addition, the results of Fig. 2C indicate that the interaction of felbamate with the Na^+ channel is reversible upon drug removal. After 3–8 min washout (until the rupture of the patch), the recovery of the current was 78 ± 6.5% of the control value before felbamate perfusion ($n = 4$).

4. Discussion

The results of the present study show that felbamate inhibits Na^+ channels expressed upon *Xenopus* oocyte injection with the cRNA encoding both rat and human α-subunits. This inhibition was only exerted when felbamate was applied intracellularly. The lack of an inhibitory action of felbamate when it was applied to the external side of the membrane can be due either to the well-known property of the oocyte membrane to act as a strong barrier against the penetration of several lipophilic drugs or to the oocyte cytoplasmic granules which exert a trapping effect

on lipophilic compounds (Taglialatela et al., 1991). The felbamate concentration effective in reducing Na^+ currents in oocytes in the present study was very close to the concentrations capable of inhibiting the sustained repetitive firing of spinal cord neurons in culture (White et al., 1992) and the duration of CA1 epileptiform bursting caused by Mg^{2+}-free solutions in rat hippocampal slices (Domenici et al., 1994). Furthermore, this concentration of felbamate is similar to that found in the plasma and in the brain of rats after oral or parenteral administration of antiepileptic and neuroprotective doses of the drug (McCabe et al., 1993).

The intracellular felbamate-induced inhibition of macroscopic currents ($I = i \times N \times P_o$) might be the consequence of a reduction of either the single channel conductance (i) or the number of channels available to open (N) or the channel probability of being opened as a function of voltage (P_o). The results obtained in the present study indicated that felbamate does not interfere with the single-channel conductance, but rather reduces the channel availability ($N \times P_o$). In fact, single-channel experiments performed in the rat clone showed that the single-channel amplitude at -30 mV was 1.1–1.2 pA both in control conditions and after felbamate application (data not shown). The fact that the felbamate block of Na^+ channels was relieved by hyperpolarizing prepulses, seems to suggest that the antiepileptic drug preferentially binds to and stabilizes the inactivated state of the channel; using the formalisms originally developed to characterize the interaction of lidocaine with cardiac Na^+ channels (Bean et al., 1983), we calculated the apparent K_d values for both the resting and the inactivated states of the hB1 channels. The existence of an approximately 300-fold difference between the apparent K_d values for felbamate binding to the inactivated versus the resting state of hB1 channels (7 μM vs. 2.1 mM, respectively), supports a tighter binding of the drug to the inactivated state of the channel as the preferential mechanism of blockade of hB1 Na^+ currents. These biophysical properties of felbamate block closely resemble the mechanism of Na^+-channel blockade by local anesthetics. Since at physiological resting potential a significant fraction of Na^+ channels is inactivated, the stabilization of this state by felbamate may effectively reduce neuronal excitability and, by this mean, explain its anticonvulsant activity.

An interaction of felbamate with native Na^+ channels in acutely dissociated rat striatal cells has also been recently shown by Pisani et al. (1995), although in this study it was not possible to identify the Na^+-channel subunit targeted by felbamate.

In conclusion, the results of the present study demonstrate that felbamate inhibits Na^+ currents through voltage-dependent Na^+-channel α-subunit, by acting on the intracellular side of the channel. The biophysical properties of the block recall those of Na^+-channel block by local anesthetics. Therefore, this study provides an additional mechanism by which felbamate might exert its wide-spectrum anticonvulsant and neuroprotective action.

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