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CONCENTRATION-DEPENDENT EFFECTS OF ANTICONVULSANT ENAMINONE METHYL 4-(4′-BROMOPHENYL)AMINOCYCLOHEX-3-EN-6-METHYL-2-OXO-1-OATE ON NEURONAL EXCITABILITY *IN VITRO*

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Abstract—Enaminones are a novel group of compounds some of which possess anticonvulsant activity in *in vivo* animal models of seizures. We recently reported that some enaminones, including methyl 4-(4′-bromophenyl)aminocyclohex-3-en-6-methyl-2-oxo-1-oate, depress glutamate-mediated excitatory synaptic transmission and that this may contribute to their anticonvulsant activity [Kombian SB, Edafiogho IO, Ananthalakshmi KVV (2005) Anticonvulsant enaminones depress excitatory synaptic transmission in the rat brain by enhancing extracellular GABA levels. Br J Pharmacol 145:945–953]. Here we studied the effects of methyl 4-(4′-bromophenyl)aminocyclohex-3-en-6-methyl-2-oxo-1-oate, on the excitability of male rat (Sprague-Dawley) nucleus accumbens and hippocampal cells *in vitro* using whole-cell patch clamp recording techniques. At low, therapeutically relevant concentrations (0.3–10 μM), methyl 4-(4′-bromophenyl)aminocyclohex-3-en-6-methyl-2-oxo-1-oate reversibly suppressed action potential firing rate in a concentration-dependent manner. This action potential suppression was present when $GABA_A$, $GABA_B$ and glutamate receptors were blocked with their antagonists. Furthermore, methyl 4-(4′-bromophenyl)aminocyclohex-3-en-6-methyl-2-oxo-1-oate suppressed tetrodotoxin-sensitive sodium currents in these cells. At concentrations ≥100 μM, it induced inward currents and increased action potential firing frequency. The inward currents were without changes in input resistance and did not reverse polarity between −120 and −40 mV. These currents were independent of extracellular potassium, but were absent when extracellular sodium was replaced by choline and finally, were occluded by pretreatment with ouabain (200 μM). We conclude that methyl 4-(4′-bromophenyl)aminocyclohex-3-en-6-methyl-2-oxo-1-oate directly inhibits action potential firing at therapeutically relevant concentrations by suppressing tetrodotoxin-sensitive sodium currents, while inducing an ouabain-sensitive current at high concentrations to excite neurons. These two actions of methyl 4-(4′-bromophenyl)aminocyclohex-3-en-6-methyl-2-oxo-1-oate on neuronal excitability would have therapeutic implications in future clinical use of enaminones as anticonvulsants in seizure disorders.

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Abbreviations: ACSF, artificial cerebrospinal fluid; AP, action potential; DL-APV, DL-2-amino-5-phosphonovaleric acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; E139, methyl 4-(4′-bromophenyl)aminocyclohex-3-en-6-methyl-2-oxo-1-oate; I–V, current–voltage; $[K^+]_e$, concentration of extracellular potassium; NAc, nucleus accumbens; R_a, access resistance; TEA, tetraethyl ammonium; TTX, tetrodotoxin.

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Epilepsy is a common neurological disorder characterized by recurrent seizures. This condition which afflicts a sizeable number (1–2%) of people today (Browne and Holmes, 2001; McNamara, 1999) is usually managed with different antiepileptic drugs that belong to different chemical classes (Kwan et al., 2001). Currently available drugs however, have limitations such as intolerable side effects, relatively narrow scope, with up to 40% of seizures being resistant to all available drugs (Loscher, 2002; Kwan and Brodie, 2000; Regesta and Tanganelli, 1999). In response to these limitations, the development of newer agents to optimally manage seizures, especially the treatment-resistant ones, has been strongly advocated (Loscher, 2002; Brodie, 2001).

Enaminones are a novel class of synthetic compounds (Edafiogho et al., 1992) some of which have shown anticonvulsant activity comparable to that of some prototype and/or clinically used agents in animal models of seizures (Mulzac and Scott, 1993). Moreover, these compounds produced fewer side effects such as ataxia when compared with the prototypic antiepileptic agents such as carbamazepine, valproate and phenytoin (Mulzac and Scott, 1993). The enaminone pharmacophoric structure, may therefore serve as a unique starting point for the development of anticonvulsant drugs that possess a superior side effect profile as well as a wider margin of safety.

In order to establish the usefulness of enaminones in the management of seizure disorders, pharmacodynamic and pharmacokinetic studies on these compounds have been carried out. Preclinical pharmacokinetic studies reveal that following i.p. injection of an effective anticonvulsant dose, concentrations of 1–10 μg/g tissue could be achieved in the brains of rats within 15 min (Khurana et al., 2003). This indicates that these compounds when given at therapeutically relevant doses reach the target tissue quickly and remain at these levels for up to 3 h (Khurana et al., 2003). The question that then arises is: are these brain levels adequate to alter neuronal function to produce the reported anticonvulsant effects?

In an attempt to answer this question we recently examined the effects of anticonvulsant enaminones on excitatory synaptic transmission in the rat brain (Kombian et al., 2005). We showed that bath application of the anticonvulsant enaminone methyl 4-(4′-bromophenyl)aminocyclohex-3-en-6-methyl-2-oxo-1-oate (E139) at concentrations

of 0.1–10 μM (equivalent to 0.03–3.4 μg/g which is below the 10 μg/g reported for rat brains) caused a depression of evoked, glutamate-mediated excitatory synaptic transmission which could contribute to its *in vivo* anticonvulsant effects. This depression was found to be indirect through GABA, acting on $GABA_B$ receptors. These synaptic effects occurred with little change in the postsynaptic cell conductance although, at higher concentrations, we consistently observed an inward current.

In addition to a role for synaptic inputs in the pathogenesis of seizure disorders (Dingledine et al., 1990; Sloviter, 1994), alteration in postsynaptic neuronal excitability may also underlie these disorders (Catterall, 2000, 2002; McNamara, 1999; Rogawski and Porter, 1990). Indeed, most first generation antiepileptic drugs such as carbamazepine and phenytoin produce their effects via modulation of postsynaptic excitability by interacting with sodium channels (see reviews by Errington et al., 2005; McNamara, 1999; Rogawski and Porter, 1990). The present study therefore was undertaken to examine the effects of E139 on postsynaptic cell responses in order to thoroughly characterize the effects of these compounds on neurons in an effort to define possible mechanisms that may underlie the anticonvulsant effects of enaminones. Cells from two brain regions, the nucleus accumbens (NAc) and the hippocampus are studied here as model CNS neurons to test the hypothesis that anticonvulsant enaminones modulate neuronal membrane properties as part of their anticonvulsant action. Here we report our finding on the effect of therapeutically relevant ($\leq$10 μM) and higher concentrations of E139 on the excitability of neurons recorded in these regions.

EXPERIMENTAL PROCEDURES

Synthesis of E139

The anticonvulsant enaminone E139 was synthesized for this study according to methods reported previously (Edafiogho et al., 1992). Briefly, it was produced by the condensation reaction between a β-diketo intermediate and *p*-bromo aniline; under carefully controlled reaction conditions (see Kombian et al., 2005).

Animal experiments

All the rats used in this study were obtained from the Kuwait University Animal Resource Centre. All experiments were done in accordance with guidelines on humane handling of experimental animals as contained in those established by the Canadian Council on Animal Care. The procedures employed minimized animal suffering and the minimum number of animals necessary to produce the required results was used.

Slice preparation

Parasagittal forebrain slices containing the NAc and coronal hippocampal slices were generated using previously published techniques. Briefly, male Sprague–Dawley rats (75–150 g) were anesthetized with halothane before decapitation. The brain was quickly removed from the rat and placed in ice-cold (4 °C) artificial cerebrospinal fluid (ACSF) that was bubbled with 95% O_2 and 5% CO_2. The composition of the ACSF was (in mM): 126 NaCl; 2.5 KCl; 1.2 NaH_2PO_4; 1.2 $MgCl_2$; 2.4 $CaCl_2$; 18 $NaHCO_3$; 11 glucose, producing a solution with osmolarity of between 310 and 320 mOsm.

Thin slices (350 μm thick) were cut in ice-cold ACSF using a Leica (VT 1000S; Nussloch, Germany) tissue slicer. Coronal hippocampal slices (350 μm) were prepared using the same physiological solution and following the same procedure as above. Slices were incubated in ACSF (bubbled with 95% O_2 and 5% CO_2) at room temperature and allowed to recover for at least 1 h before use.

Electrophysiological recording and data acquisition

One slice was trimmed and transferred into a 500 μl capacity recording chamber and perfused submerged at a flow rate of 2–3 ml/min (29–31 °C) with regular ACSF that was bubbled with 95% O_2 and 5% CO_2. In some experiments, NaCl was replaced by an equimolar concentration of choline chloride. “Blind patch” recordings were done in the conventional whole-cell mode using glass electrodes with tip resistance of 3.5–8.0 MΩ. The internal recording solution had the following composition (in mM): K-gluconate (135), NaCl (8), EGTA (0.2), HEPES (10), Mg-ATP (2) and GTP (0.2). pH and osmolarity were adjusted to 7.3 (with KOH) and 270–280 mOsm, respectively.

For NAc recordings, bipolar tungsten stimulating electrodes were positioned at the prefrontal cortex–accumbens border to evoke synaptic responses where necessary. In the hippocampal studies, whole cell recordings were done in CA1 pyramidal cells and stimulating electrodes were placed in the stratum radiatum to activate commissural and Schaffer collateral fibers.

Recordings were made using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA, USA) in either voltage or current clamp modes. For current recordings, cells were voltage clamped at $-$80 mV or $-$65 mV (holding potential) and input (R_{input}) and access (R_a) resistances of all cells were determined and monitored regularly throughout each experiment by applying a 75 ms, 20 mV hyperpolarizing pulse. All cells reported in this study had R_a of 10–30 MΩ. Data from cells that showed $>$15% changes in R_a during the experiment were excluded from further analysis.

All data were acquired using pClamp Software (Clampex 7 or 8; Axon Instruments, Forster City, USA) at a sampling rate of 2–10 kHz depending on the type of experiment, filtered at 1 kHz, digitized and stored for off-line analysis.

For action potential (AP) recordings, two approaches were employed. 1: Synaptic responses to increasing stimulus intensity (0–4 mA) were evoked until an AP was recorded, superimposed on the EPSP. 2: A series of current steps (from $-$400–600 pA) was applied to each cell (in increments of 100 pA) and the corresponding voltage response captured to generate a current–voltage (I–V) input–output curve. An appropriate range of currents that yielded more than two APs in the positive range was selected as the protocol for that cell and used for further experiments. Throughout these current clamp experiments, the bridge balance was monitored and adjusted when necessary.

Potassium and sodium currents were recorded in voltage clamp mode performed only in NAc cells. For steady-state potassium currents, ramp experiments were done whereby cells held at $-$80 mV, were stepped to $-$120 mV, held there for 1 s to stabilize the membrane, and then ramped slowly to $-$40 mV over 4.8 s and then stepped back to $-$80 mV. Step-activated sodium currents were recorded in the presence of a solution containing tetraethyl ammonium (TEA; 5 mM) and cesium (Cs^+; 1 mM) and either cadmium (Cd^{2+}; 100 μM) or in the absence of calcium (Ca^{2+}; 0 mM). Series resistance was compensated at $\geq$80%. A series of voltage steps ranging from $-$65 to $+$70 mV in 15 mV increments was applied to cells, each step preceded by a pre-pulse to $-$120 mV. Leak subtraction was applied to all currents to obtain the sodium current.