Phenobarbital Pharmacological Findings on the Nerve-Muscle Basis

Leandro RUBEM-MAURO 1, Dimas S. ROCHA Jr 1, Caroline C. BARCELOS 2, Gustavo H. COSTA VARCA 1, Newton ANDRÉO-FILHO 1, Silvio BARBERATO-FILHO 1, Yoko OSHIMA-FRANCO 1* & Marta M.D.C. VILA 1

1 Universidade de Sorocaba – Unisco, Curso de Farmácia, Bloco C, Cidade Universitária, Rodovia Raposo Tavares km 92,5 CEP: 18023-000 Sorocaba, São Paulo, Brazil. 2 Departamento de Clínica Médica - Universidade Estadual de Campinas, Unicamp, Campinas, São Paulo, Brazil.

SUMMARY. Phenobarbital and carbamazepine are antiepileptic drugs that act at the nervous central system by different mechanisms of action. In this work we investigated the pharmacological effects of these drugs on mouse phrenic nerve-diaphragm preparations through the myographic technique. Carbamazepine (0.105, 1.05, 2.1 and 4.2 mM, n = 8, 6, 6 and 6, respectively), induced a dose-dependent neuromuscular blockade, under indirect or direct muscle stimulation and the neurotransmission was reestablished after washing. Conversely, phenobarbital caused an unexpected facilitatory effect, under several formulations, such as the acid-extracted commercial tablets (1.05, 2.1 and 4.2 mM, n = 7, 6 and 7, respectively), commercial phenobarbital solution (4.2 mM, n = 7) or its correspondent pure active ingredient (4.2 and 2.1 mM, n = 6 each). Only at a higher concentration the acid-extracted phenobarbital performed a neuromuscular blockade (8.4 mM, n = 10). The different responses between carbamazepine (paralysis) and phenobarbital (facilitatory effect) evidentiated a new effect for phenobarbital until now concealed at the neuromuscular junction and may involve the glutamatergic regulation, since its role as an acetylcholine co-transmitter in motoneurons was already established.

INTRODUCTION

Investigations during the last decade indicate a role for glutamate as a signaling molecule at the neuromuscular junction of vertebrates 1, which role was before attributed only to acetylcholine (ACh) neurotransmitter. Regarding ACh in diaphragms, about 65% is contained in a depletable store in the nerve terminals. The rest of the ACh is contained in a nondepletable store that may correspond to the store that remains in denervated muscles and includes, in addition, ACh in the intramuscular branches of the phrenic nerve. About 4% of the ACh released from the depletable store at rest is secreted as quanta and may comes from the vesicles, while 96% is secreted in a nonquantized form and comes from an extravesicular pool 2.

The physiological role of the ionic nonquantal release has been extensively considered by elsewhere, mainly its role in the formation, maintenance and in the resting membrane potential modulation of synaptic nerve-muscle interaction 3. Nonquantal ACh measure is currently taken using an inhibition by tubocurarine of the ACh receptors of muscles previously treated with anticholinesterases, producing a small hyperpolarization, called the H-effect 4,5, presumably a result of block of the depolarization caused by the ACh released ionically from the nerve terminal. Concerning to the modulation of synaptic nerve-muscle interaction was found that ACh and glutamate are coreleased from synaptosomes of Torpedo electric organ 6, and also demonstrated in rat motor nerve terminals 7. Glutamatergic receptors such as N-methyl-D-aspartate (NMDA) have been identified at the postsynaptic membrane in neuromuscular junction of adult rats 8,9.

Glutamate released from nerve endings probably activates NMDA-receptor mediated...
Ca\textsuperscript{2+} entry into the sarcoplasm followed by activation of nitric oxide (NO) \textsuperscript{8}. Nonquantal ACh acting through M1-cholinergic receptors \textsuperscript{10,11}, activates synthesis of NO to serve as a trophic message from motoneurones that keeps the Cl-transport inactive in the innervated sarcolemma \textsuperscript{12}.

In other words, glutamate modulates the nonquantal release of ACh at rat neuromuscular junction in a multi-step process that includes: the activation of post-synaptic NMDA receptors, stimulation of NO synthesis and its release from muscles whereupon it acts to inhibit secretion of nonquantal ACh from presynaptic motor nerve terminals, in a concentration-dependent manner, as indicated by the decrease in the H-effect. This sequence of events is an example of feedback machinery at which two neurotransmitters, glutamate and ACh, can effectively participate in the physiological regulation of the postsynaptic membrane potential and the sensitivity of its receptors \textsuperscript{13}. These authors also included the presynaptic Schwann cells \textsuperscript{14}, since glutamate carboxypeptidase II (GCP II) was found on its surface and inactivates N-acetylaspartylglutamate (NAAG) to form glutamate and N-acetylaspartate (NAA).

Considering that the evidences of glutamatergic modulation on neuromuscular junction are enough and they were obtained mainly from electrophysiological technique, by nonquantal ACh measure, is also expected that this and other drugs can interfere on neuromuscular response using traditional myographic technique. Thus, we used this one to study two anticonvulsant agents, phenobarbital and carbamazepine, which mechanisms of action are distinct. It is known that phenobarbital acts as a nonselective central nervous system depressant. It promotes binding to inhibitory gama-aminobutyric acid subtype receptors (GABA-A), modulates chloride currents through receptor channels as well as inhibits glutamate induced depolarizations. The carbamazepine mechanism of action is not clear, although several cellular actions have been described including effects over the ion channels, active transport, and general membrane stabilization. The muscle relaxant effect mechanism seems to involve a decrease in the muscle spindles sensitivity to stretches \textsuperscript{15}.

From a clinical point of view, it has been reported that these anticonvulsant agents oral and chronically administered modify the neuromuscular function, both \textit{in vivo} \textsuperscript{16-18} and \textit{in vitro} \textsuperscript{19-21}, although these data have been conflicting with those studies that showed no signs of impairment of the peripheral nerve in patients receiving phenobarbital and carbamazepine \textsuperscript{22}.

In the present study the \textit{in vitro} effects of phenobarbital and carbamazepine were re-investigated on mouse isolated phrenic nerve-diaphragm preparations, since previous studies on rat neuromuscular preparations showed different responses between them \textsuperscript{23,24}.

**MATERIALS & METHODS**

**Drugs**

The following substances were used: phenobarbital 100 mg commercial tablets (União Química, Brazil) and carbamazepine 200 mg commercial tablets (FURP, Brazil) for acid- and basic- extractions, respectively; commercial phenobarbital solution (CPS, 4.2 mM, Gardenal®, Roche, Brazil); pure active ingredient phenobarbital (PAI, imported from India by Phito Fórmulas®, Brazil); monosodium L-glutamate (L-glutamic acid monosodium salt monohydrate, FW 187.13, CAS number 142-47-2, certified by JECFA, Compendium of Food Additive Specifications).

**Experimental subjects**

Male Swiss white mice (26-32 g) were supplied by the Animais de Laboratório (Anilab, Paulinia, Brazil). The animals were housed at 25 ± 3°C on a 12 hours light/dark cycle with \textit{ad libitum} access to food and water. This project (protocol number A100/CEP/2007) was approved by the institutional Ethics Committee from Vale do Paraíba University (UNIVAP), and the experiments were within the guidelines of the Brazilian College for Animal Experimentation. All efforts were made to minimize the animal amount and suffering.

**Acid-extract phenobarbital (AEP) and basic-extract carbamazepine (BEC)**

In order to extract the active ingredient from commercial tablets was adapted the Moraes's method \textsuperscript{25}. Briefly, the phenobarbital and carbamazepine commercial tablets were separately pulverized in a mortar, solubilized in water and the final content was then transferred to the separation funnel. Phenobarbital content was acidified with HCl 10% (pH 1-2, from Ecibra®, Brazil) and three times extracted using 30-50 mL of ether/chloroform mixture (1:2, v/v, from Synth®, Brazil). The obtained acid-extract was filtered with anhydrous sodium thiosulphate (Synth®, Brazil), dried using heating plate and
the outcome was registered. All carbamazepine content in the separation funnel was basified with NaOH 10% solution (pH 8-10, from Nuclear®, Brazil) and the same procedure adopted for phenobarbital was performed. A basic-extract was obtained and the outcome was registered.

**Chemical tests of identification**

The method for obtaining both acid- and basic-extracted phenobarbital and carbamazepine (AEP and BEC, respectively) was furtherly assayed in order to identify the active ingredients extracted from the commercial tablets. A recommended qualitative analysis method was selected 26.

**AEP identification**

The melting point determination (n = 3) using Fisatom (model 430) equipment and identification reactions allowed the purity evaluation of the acid-extracted phenobarbital. Samples (0.4 g) of the obtained salt was solubilized in 10 mL anhydrous ethanol (from Ecibra®, Brazil), filtered, dried and an aliquot of 0.1 g was solubilized in a solution containing 5 mL deionized water and 0.5 mL NaOH (80 g/L, w/v). After, the mixture was filtered and 1 mL of citric acid (90 g/L, w/v, from Ecibra®, Brazil) was added. A white bulky precipitate is visualized for a positive reaction. The methodologies were in accordance to the OMS standards 26.

**BEC identification**

The same methodology adopted for AEP was used. Briefly, 1 mL formaldehyde (from Ecibra®, Brazil) was added to 5 mg of the basic-extracted carbamazepine and a yellow to orange colour is developed for a positive reaction. Another test was performed using 2 mL of nitric acid (1000 g/L, w/v, Ecibra®, Brazil) added to 10 mg of carbamazepine. The mixture is heated for 1 min and an orange colour indicates positive reaction.

**Mouse phrenic nerve-diaphragm muscle (PND) preparation and experimental procedures**

The phrenic nerve-diaphragm was obtained from anesthetized mice using halotane and sacrificed by exsanguinations 27. The diaphragm was removed and mounted under a 5 g tension in a 5 mL organ bath containing aerated Tyrode solution (control) of the following composition (mM): NaCl, 137; KCl, 2.7; CaCl2, 1.8; MgCl2, 0.49; NaH2PO4, 0.42; NaHCO3, 11.9; and glucose, 11.1. After calibration with 95% O2/5% CO2, the solution pH was 7.0. The preparations were stimulated indirectly with supramaximal stimuli (4 x threshold, 0.06 Hz, 0.2 ms) delivered from a stimulator (model ESF-15D, purchased from Vechio FD, Ribeirão Preto, Brazil) to the nerve through bipolar electrodes. Isometric twitch tension was recorded with a force displacement transducer (cat. 7003, Ugo Basile), coupled to a physiograph 2-Channel Recorder Gemini (cat. 7070, Ugo Basile) via a Basic Preamplifier (cat. 7080, Ugo Basile). Preparations were allowed to stabilize for at least 20 min before the addition of the following substances: Tyrode solution (control, n = 7), BEC (0.105, 1.05, 2.1 and 4.2 mM (n = 8, 6, 6 and 6, respectively), AEP (1.05, 2.1, 4.2 and 8.4 mM, n = 7, 6, 7 and 10, respectively), CPS (4.2 mM, n = 7) and PAI (4.2 and 2.1 mM, n = 6 each). Protocols containing similar concentrations of 2.1 mM each (BEC + AEP, in mixture) (n = 4) or by adding BEC (2.1 mM) followed by AEP (4.2 mM) (n = 4) or vice-versa, AEP (4.2 mM) followed by BEC (2.1 mM) (n = 4), were carried out.

**Statistical methods**

Each pharmacological protocol was repeated at least four times. The results were expressed as the mean ± S.E.M., as appropriate. Student’s t-test was used for statistical comparison of the data 28. A value of P<0.05 was considered to indicate significance.

**RESULTS**

**Outcome of acid-extract phenobarbital (AEP) and basic-extract carbamazepine (BEC) from commercial tablets**

The total weight of commercial phenobarbital tablets was 2.94 g. After acid extraction the obtained AEP weighed 1.164 g showing a final outcome of 58.17%. The total weight of commercial carbamazepine tablets was 9.065 g. After basic extraction the obtained BEC weighed 2.5230 g showing a final outcome of 42.05%.

**Chemical identification tests**

The melting range (n = 3) found for both acid- and basic-extracted phenobarbital and carbamazepine were held around 168-171 °C and 179.6-188.6 °C, respectively. According to the specialized literature 29,30 as the correspondent temperatures for phenobarbital and carbamazepine are 173-176 °C and 190-193 °C. The coloured reactions assay indicated positivity for both AEP and BEC.
**Drugs Effect on the isolated preparation (PND)**

Figure 1 shows the basic-extracted carbamazepine (BEC) exposed to the isolated preparation during 60 min, under indirect stimuli. Concentrations of 1.05 (n = 6), 2.1 (n = 6) and 4.2 mM (n = 6) caused an intense and rapid neuromuscular blockade, whereas the lowest concentration tested (0.105 mM, n = 8) showed a slight blockade. All concentrations were significantly different if compared to the Tyrode control (n = 7, *P* < 0.05). The same effect (paralysis) was also observed under direct stimuli (data not shown).

Figure 2A shows the predominant effect of acid-extracted phenobarbital (AEP), where an unexpected facilitatory effect was present, which was also reproduced with other phenobarbital formulations (Fig. 2B) such as Gardenal® (commercial phenobarbital solution, CPS) or its pure active ingredient (PAI). Only at a higher concentration (8.4 mM, n = 10) AEP induced paralysis that was preceded by the same facilitatory effect.

Figure 3A shows the addition of both anticonvulsants (in mixture) to the bath at the same concentration (2.1 mM) causing an immediate blockade (n = 4). When carbamazepine (2.1 mM) was added after phenobarbital facilitation (4.2 mM), a rapid blockade was observed (Fig. 3B, n = 4) however this fact was not observed when phenobarbital (4.2 mM) was added after the carbamazepine blockade (2.1 mM, Fig. 3C, n = 4).

Figure 4 shows the monosodium L-glutamate influence (Glu, 48 or 96 mM) on the isolated preparations. The 48 mM salt addition (n = 4) induced a paralysis that was reversible after washing (Fig. 4A, n = 4). During the installed glutamate-blockade (48 mM) the phenobarbital (AEP, 4.2 mM) addition immediately recovered the twitches (Fig. 4B, n = 4). The facilitatory effect observed for phenobarbital (AEP, 4.2 mM) was reversed by the glutamate (Glu, 48 mM) addition (Fig. 4C, n = 4). However, during the installed phenobarbital-facilitatory effect (AEP, 4.2 mM), the 96 mM monosodium L-glutamate addition immediately avoided the twitches maintenance, only recovered after washing (Fig. 4D, n = 4).

Figure 1. Dose-dependent neuromuscular blockade induced by BEC on mouse phrenic nerve-diaphragm preparation, under indirect stimuli. Each point represents the mean ± S.E.M. *P* < 0.05 compared to Tyrode control in all subsequent points. BEC, basic-extracted carbamazepine.

Figure 2. Dose-dependent facilitatory effect induced by (A) AEP. The same effect was seen to (B) CPS and PAI. Each point represents the mean ± S.E.M. *P* < 0.05 compared to Tyrode control in all subsequent points. AEP, acid-extracted phenobarbital. CPS, commercial solution of Gardenal®. PAI, pure active ingredient of phenobarbital.
Figure 5 shows the working model proposed by Malomouzh et al. with our hypothesis superposed in numeric sequence (1-4) about how exogenous glutamate culminates in neuromuscular blockade, as seen by myographic recordings (Fig. 4A). When phenobarbital is added to the bath (thin arrow) the glutamatergic influence is interrupted resulting in a facilitatory effect (5) as seen by myographic recordings (Fig. 4C, D).

DISCUSSION
Epilepsies are among the most frequent chronic neurological conditions. Patients suffer from spontaneously recurring seizures due to sudden extensive electrical gray matter discharges. Although the current drug therapy allows many patients at least some degree of a satisfying course of the disease, a substantial number of patients remain without adequate seizure control. Reasons are either refractoriness to anticonvulsant drugs or intolerable drug-related side effects. Peripheral neuropathy has long been attributed to be a complication of the antiepileptic drug treatment. The damage can occur by a functional and transient disturbance of the nerve function caused by an exposure to toxic drug dosages or to a structural lesion of the peripheral nerve.

As introduced in this paper, the anticonvulsant drugs therapeutic action at the central nervous system is well-known although its mechanisms of action are not yet fully understood. Can the same effect be expected at the peripheral nervous system? The anatomy between central and peripheral nervous system is different, but the neurotransmission basis is the same. From a pharmacological point of view the responses depend on the inactivation or activation receptors/channels. In this sense, can the central receptors/channels be present or even
be different at peripheral system for eliciting different responses?

For example, the sodium channels are involved in the carbamazepine antiepileptic action possibly through its inactivation and also blocking the contractile response— as demonstrated here— at the neuromuscular junction (NMJ), which in turn, expresses sodium channels in both axolemmal and sarcolemmal membranes.

In opposition, our results showed an unexpected facilitatory effect induced by different phenobarbital formulations (AEP, CPS or PAI) and were visualized by the increase of twitch-tension amplitude reaching around 100% depending on the pharmaceutical preparation (AEP, 4.2 mM). The ionic charge of phenobarbital formulation did not explain this pharmacological facilitatory event since phenobarbital commercial solution (sodium phenobarbital, CPS) also induced the same effect, as presented by its molecular form (AEP and PAI). This phenobarbital-facilitatory effect is limited, at least under in vitro conditions, by excessive addition as greater as 8.4 mM saturating the nutritive solution, which salt concentration is vital for preparation functionality.

Our results presented in the Figure 3 show that at NMJ, carbamazepine acts on the axon whereas phenobarbital on the nervous terminal. Indeed, the axonal transmission is an event that occurs before and culminates in the neurotransmitter release, therefore explaining the phenobarbital inability to recover the induced carbamazepine-blockade (Fig. 3C). Other consideration is related to the recovery of twitches after washing, showing that these anticonvulsants act in a reversible manner.

At the mammalian NMJ, acetylcholine (ACh) is the main neurotransmitter activating nicotinic acetylcholine receptors and mediating the signaling between nerve terminals and muscle fibers, whereas at the invertebrate NMJ glutamate is the major excitatory neurotransmitter, suggesting that mammal skeletal muscles retain the memory of ancestral glutamatergic transmission.

Taking into consideration the ACh primary role in activating NMJ, recent research has suggested a glutamate participation cholinergic transmission modulation, considering glutamate as a possible ACh co-transmitter in motoneurons. The glutamate application in strips of rat diaphragm has been found to contribute to the maintenance of the resting membrane potential and to inhibit the ACh nonquantal release from nerve endings. Receptors from the central neurotransmitters located at the peripheral nervous system have been found such as the peripheral-type benzodiazepine receptor (PBR), a transmembrane protein distinct pharmacologically, structurally and functionally from the central-type benzodiazepine receptor.

Glutamate effects may be mediated by activation of postjunctional NMDA (N-methyl-D-aspartate) receptors. Working model of the physiological interrelations between N-acetylaspartylglutamate (NAAG), NAAG peptidase (abbreviated as GCP for simplicity) and nonquantal acetylcholine (ACh) at the neuromuscular synapse (see Malomouzh et al., 1995), increased by our hypothesis (in numeric sequence 1-5) about how glutamate (GLU) and phenobarbital act on nerve-muscle synapse. 1. Exogenous glutamate activates NMDA receptors (NMDAR), 2. Nitric oxide (NO) is synthesized, 3. NO diffuses from muscle fibers to the cytoplasm of the nerve ending, 4. Attenuation of the ACh nonquantal secretion (NQACh), culminating in paralysis under myographic parameter. Phenobarbital addition (thin arrow) avoids the glutamatergic regulation (1-4 steps) resulting in a facilitatory effect by spontaneous quantal secretion of ACh (SQACh, 5). GLN, glutamine. CaCh, Ca2+ channel. M1AChR, muscarinic M1 receptor. GC, guanylyl cyclase. cGMP, cyclic guanosine monophosphate. mGLUR, metabotropic glutamate receptors. NAA, N-acetylaspartyl. NADPH, nicotinamide adenine dinucleotide phosphate. CaM, calcium-calmodulin. L-ARG, Arginine, L-Isomer.
partate) receptors inducing NO (nitric oxide) sarcoplasmic synthesis and retrograde diffusion from muscle cells 38 to inhibit ACh nonquantal release 13. Nonquantal secretion of ACh has been shown to participate in various processes at the neuromuscular synapse, from synapse assembly in development and reinnervation 3 maintenance 39 and modulation 14,40 to a desensitizing action on the postsynaptic nicotinic receptors that shortens postsynaptic response to quantal mediator 41.

In line with these findings, the nonquantal concept arises from that the most of the ACh released from resting nerve terminals comes from an extravesicular pool and is not quantal in nature 2. In our results, when ACh metabolism is inhibited by monosodium L-glutamate, via nitric oxide, the extravesicular pool is depleted in a dose-dependent manner, because it is well known that ACh is redistributed between the extravesicular and quantal pools 2 and the pharmacological effect produced was a neuromuscular blockade. When the phenobarbital inhibits the glutamatergic influence on NMJ (Fig. 5) it culminates in the observed and prolonged facilitatory effect (> 60 min) observed, although the type of glutamate receptor involved is not clear, since the expression of glutamate receptors at skeletal muscle endplates gave contradictory results 42,43.

In opposition to carbamazepine which seems to act on the axon, phenobarbital and sodium glutamate seems to act in a competitive manner on the nervous terminal. The studied drugs addition sequence to the bath, phenobarbital and monosodium L-glutamate (Glu), clearly evidenced that (Fig. 4) such antagonism is a dose-dependent event.

Our results reinforce the place where the studied substances act, being the carbamazepine on axon, whereas the phenobarbital and the glutamate, at the nervous terminal. The response magnitude (paralysis or facilitatory action) is concentration dependent. These results are promising and new questions arise from transmission, co-transmission concepts and about the therapeutical drugs influence, like anticonvulsants, on distant targets (skeletal muscle) from that considered the main one (brain).

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