Mefloquine selectively increases asynchronous acetylcholine release from motor nerve terminals


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Abstract

Effectiveness against chloroquine-resistant Plasmodia makes mefloquine a widely used antimalarial drug. However, mefloquine’s neurologic effects offset this therapeutic advantage. Cellular actions which might contribute to the neurologic effects of mefloquine are not understood. Structural similarity to tacrine suggested that mefloquine might alter cholinergic synaptic transmission. Therefore, we examined mefloquine’s effects at a model cholinergic synapse. Triangularis sterni nerve–muscle preparations were isolated from adult mice and examined with sharp electrode current clamp technique. Within 30 min of exposure to 10 μM mefloquine, miniature endplate potentials (mepps) occurred in summat-
ing bursts and their mean frequency increased 10-fold. The threshold concentration for the increase of mean mepp frequency was 0.6 μM me-
floquine. Mefloquine continued to increase mean mepp frequency for preparations bathed in extracellular solution lacking Ca\(^{2+}\). In contrast, mefloquine no longer increased mean mepp frequency for preparations pre-treated with the intracellular Ca\(^{2+}\) buffer BAPTA-AM. Although mefloquine disrupts a thapsigargin-sensitive neuronal Ca\(^{2+}\) store, pre-treatment with thapsigargin did not alter the mefloquine-induced altera-
tions of mepps. Since mefloquine, like oligomycin, inhibits mitochondrial F\(_{0}\)F\(_{1}\)H\(^{+}\)ATP synthase we tested the interaction between these two
chemicals. Like mefloquine, oligomycin induced bursts and increased mean frequency of mepps. Furthermore, pre-treatment with oligomycin precluded the mefloquine-induced alterations of asynchronous transmitter release. These data suggest that mefloquine inhibits ATP production which increases the concentration of Ca\(^{2+}\) within the cytosol of nerve terminals. This elevation of Ca\(^{2+}\) concentration selectively increases
asynchronous transmitter release since 10 μM mefloquine did not alter stimulus-evoked transmitter release.

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1. Introduction

Activity against chloroquine-resistant Plasmodium falciparum makes mefloquine a useful antimalarial drug (Ohnmacht et al., 1971; Canfield and Rozman, 1974; Peters et al., 1977; Schmidt et al., 1978; Lopez-Antunano and Wernsdorfer, 1979). However, neuropharmacologic effects ranging from ataxia and mood changes to convulsions offset this advantage of mefloquine (Weinke et al., 1991; Ruff et al., 1994; Phillips-Howard and ter Kuile, 1995; Pous et al., 1995; van Riemsdijk et al., 1997; Fuller et al., 2002; Wooltorton, 2002). Available data suggest that these neuropharmacologic effects of mefloquine may be due, at least in part, to synaptic actions. For example, the anticholinesterase action of meflo-
quine (Lim and Go, 1985) alters transmission across the neuromuscular junction (McArdle et al., 2005). Since meflo-
quine enters the central nervous system (Baudry et al., 1997) it is likely that mefloquine’s anticholinesterase action will alter transmission across cholinergic synapses within the brain.

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Mefloquine is structurally similar (Fig. 1) to another anticholinesterase, tacrine (Camps et al., 1999). Since tacrine alters asynchronous transmitter release at the neuromuscular junction (Thesleff et al., 1990), mefloquine may also have this effect. In support of this hypothesis, Gribble et al. (2000) reported that mefloquine enhances insulin secretion from pancreatic islet cells. They attributed this action of mefloquine to inhibition of the ATP sensitive K⁺ channel, Kir6.2/SUR1. More recently, Cruikshank et al. (2004) observed that mefloquine activates asynchronous, but not stimulus-evoked, release of glutamate from the rat hippocampus. While the basis of the latter action of mefloquine was not explored, it was associated with inhibition of heterologously expressed connexin gap junction channels, Cx36 and Cx50. Alterations of Ca²⁺ homeostasis within secretory cells could alter exocytosis. Several observations suggest that this mechanism contributes to the effect of mefloquine on neurosecretion. For example, mefloquine inhibits Ca²⁺ uptake by crude microsomes prepared from brain (Lee and Go, 1996). In addition, mefloquine disrupts a thapsigargin-sensitive Ca²⁺ store within the endoplasmic reticulum of cultured rat neurons (Dow et al., 2003). Finally, mefloquine, like oligomycin, inhibits mitochondrial FOF1H₂O₂ (Martin-Galiano et al., 2002). Decreased ATP synthesis might also elevate the concentration of Ca²⁺ within nerve endings and so increase transmitter release.

To examine the contribution of the above mefloquine actions to the regulation of transmitter release, we exposed mouse nerve–muscle preparations to mefloquine. We observed that mefloquine increased mean frequency and induced bursts of summati mepps. These effects were independent of extracellular Ca²⁺. In contrast, the intracellular Ca²⁺ buffer BAPTA-AM precluded these effects suggesting that mefloquine mobilizes an intracellular Ca²⁺ store. Since pre-treatment with thapsigargin did not prevent the mefloquine-induced increase of mean mepp frequency, it appears unlikely that the endoplasmic reticulum is the source of the Ca²⁺ underlying the increase of asynchronous release. In contrast, oligomycin pre-treatment mimicked and precluded the effect of subsequent mefloquine exposure on asynchronous transmitter release. These data suggest that mefloquine, like oligomycin, disrupts mitochondrial ATP production. This leads to an increase of the cytosolic Ca²⁺ concentration and asynchronous transmitter release. Stimulus-evoked transmitter release, on the other hand, is not altered by this action of mefloquine. Thus, mefloquine is a useful tool to explore distinct processes governing asynchronous and stimulus-evoked transmitter release. Our data have appeared in abstract form (McArdle and Sellin, 2003; Hognason et al., 2004).

2. Methods

2.1. Animals and tissue preparation

Adult Swiss–Webster mice were housed in our Institutional Animal Care Facility. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the New Jersey Medical School. Mice were surgically anesthetized with diethyl ether and the Triangularis sterni nerve–muscle preparation dissected (McArdle et al., 1981). The isolated TS preparation was pinned to a Sylgard-lined Plexiglass chamber, placed on the stage of an upright microscope and superfused (1 ml/min) with a physiological solution containing (mM): NaCl (135), KCl (5), MgCl₂ (1), NaHCO₃ (15), Na₂HPO₄ (1), CaCl₂ (2), D-glucose (5.5). This physiological solution was bubbled with 95% O₂/5% CO₂ to maintain pH 7.4 at 22–24 °C. Muscle nerves were drawn into glass suction electrodes and stimulated with supra maximal square pulses (1 ms) to elicit endplate potentials (epps).

2.2. In vitro electrophysiology and data analyses

Spontaneous (mepps) and stimulus-evoked epps were recorded with sharp electrodes filled with 3 M KCl (5–10 MΩ) and connected to an Axoclamp-2A amplifier (Axon Instruments, Union City, CA, USA). Amplifier output was digitized (Digidata 1321A, Axon Instruments) at 10,000 samples/s. PCLAMP software (Axon Instruments, version 9.2) saved the digitized data to disc for subsequent off-line analyses.

The effects of mefloquine on stimulus-evoked transmitter release were studied for nerve–muscle preparations treated in two ways to eliminate mechanical activity. The first treatment protocol allowed direct estimate of the mean quantal content of epps in the presence of 2 mM extracellular Ca²⁺. Since transmitter release probability was high, it was essential to prevent mechanical responses to nerve stimulation. The delicate anatomy of the mouse Triangularis sterni nerve–muscle preparation is not compatible with the “crush fiber” technique for arrest of mechanical responses. Therefore, we adapted the “glycerol shock” technique which Gage and Eisenberg (1967) developed for amphibian muscle. Isolated Triangularis sterni nerve–muscle preparations were superfused with an oxygenated physiologic solution containing 3% (v/v) glycerol. After 1 h, the preparation was superfused with glycerol-free physiologic solution for 30–45 min. Initiation of nerve stimulation produced several robust muscle twitches and a brief episode of spontaneous mechanical activity. After cessation of all mechanical activity, mepps and epps were readily recorded. However, epps were often contaminated with muscle action potentials. Such contamination was common for endplates having normal resting potentials (i.e., −65 to −80 mV). Quantal content of epps could not be estimated for these endplates. However, another population of endplates was devoid of action potentials. We attribute this to trauma-induced depolarization created by mechanical activity immediately after wash out of the glycerol solution. Thus, glycerol shock achieves the same effect as controlled crushing of muscle fibers; that is, membrane depolarization which inactivates Na⁺ channels and prevents muscle action potentials. Epps at these endplates had an average amplitude of 10–12 nV both before and after mefloquine. Mepps which were clearly due to single quantal events were averaged. This criterion was especially critical to the analyses of mefloquine-treated preparations. Mefloquine not only increased mepp amplitude due to its anticholinesterase effect (McArdle et al., 2005), but also induced “giants” and bursts of mepps. The ratio of average epp/mepp provided a direct estimate of mean quantal content. Correction of epp amplitude of control and mefloquine-treated preparations for non-linear summation had no effect of the estimate of quantal content.

As a further test of mefloquine’s effect on stimulus-evoked transmitter release we superfused preparations with physiological solution containing 11 mM MgCl₂ and 1 mM CaCl₂. After 60 min equilibration in this solution,
supra maximal stimuli (1 ms duration, 1 Hz) were applied to the TS nerve via a suction electrode. The evoked epsps had low quantal content and were susceptible to frequent failures of transmission. The percent of nerve stimuli failing to produce an epp was calculated before and after 30–60 min exposure to mefloquine.

2.3. Treatment protocols to study presynaptic actions of mefloquine

Mefloquine was kindly provided by Drs Eva-Maria Gutknecht and Pierre Weber (Hoffmann-La Roche, LTD, Basel, Switzerland). A stock solution (20 mg/ml) of the racemic salt was prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Inc., Saint Louis, MO, USA). Dilution of this stock solution into physiologic solutions produced the concentrations of mefloquine studied. For most of the mepp studies, mefloquine was added to physiological solution containing 2 mM CaCl2. However, several experiments required removal of extracellular Ca2+. To achieve this, the physiological solution was prepared without added CaCl2 and contained 125 μM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA, Sigma-Aldrich, Inc.). This resulted in a “0” [Ca2+]o, physiological solution containing less than 125 nM Ca2+. Preparations were equilibrated for 60 min in “0” [Ca2+]o, before beginning subsequent protocols. In other experiments, it was desirable to buffer intracellular Ca2+. To achieve this, preparations were equilibrated in “0” [Ca2+]o physiological solution containing 10 μM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA-AM, Calbiochem, La Jolla, CA, USA). This membrane permeable Ca2+ buffer enters a wide variety of cells where enzymes hydrolyze the ester bond. BAPTA is thus trapped in the cytoplasm with removal of extracellular BAPTA-AM. Therefore, preparations were equilibrated with the BAPTA-AM solution for 60 min prior to wash with BAPTA-AM free “0” [Ca2+]o solution and onset of recording.

Preparations were also pre-treated with thapsigargin and oligomycin to examine the role of endoplasmic reticular Ca2+ stores and mitochondrial function, respectively, in mefloquine-induced alterations of mepps. Thapsigargin (Calbiochem) was dissolved in DMSO (1 mg/ml). This stock solution was then diluted in either 2 mM or “0” [Ca2+]o physiological solution to give a final concentration of 2 μM thapsigargin. After a pre-equilibration for 60 min in the physiological solution, preparations were equilibrated for 15 min in the thapsigargin-containing solution prior to recording mepps. This treatment irreversibly eliminates endoplasmic reticular Ca2+ storage within motor nerve terminals (Suzuki et al., 2002). Oligomycin (Sigma-Aldrich, Inc.) was dissolved in 100% ethanol (1 mg/100 μl). This stock solution was diluted into “0” [Ca2+]o physiological solution to give a final concentration of 8 μg/ml oligomycin. After a pre-equilibration of 60 min in “0” [Ca2+]o, preparations were equilibrated for 30 min in oligomycin solution prior to recording mepps.

2.4. Data presentation and statistical analyses

Data were analyzed and plotted with PCLAMP, Sigmaplot (SPSS Inc., Chicago, IL, USA), and GraphPad Prism (GraphPad Software, San Diego, CA, USA) programs. Data are presented as mean ± SEM. Unpaired t-tests compared experimental and control mean values; p < 0.05 indicated a significant difference. The number of muscles and endplates analyzed is indicated along with each mean and SEM.

3. Results

3.1. Mefloquine increases mean mepp frequency and induces bursts of summating mepps

Fig. 2 illustrates the effects of mefloquine on miniature endplate potentials (mepps) of the mouse Triangularis sterni muscle. Within 30 min of adding 10 μM mefloquine to the external bathing solution (2 mM Ca2+), mean mepp frequency increased from a control value of 3.06 ± 0.14 (26 muscles, 236 endplates) to 26.97 ± 2.21/s (6, 78). This nine-fold increase of mean frequency accompanied the appearance of summating bursts of mepps. Concentration–response study (Fig. 3) revealed that 0.6 μM was the threshold mefloquine concentration causing a statistically significant (p < 0.05) increase of mean mepp frequency. Elevating the concentration further increased mepp frequency to a maximum value at 8 μM mefloquine; the mean frequencies recorded during exposure to 8, 10 and 16 μM mefloquine did not differ significantly (p > 0.05). Control studies with the highest level of DMSO applied in the concentration–response study slightly decreased mepp frequency. Since this effect is opposite to that attributed to mefloquine, DMSO did not contribute to and may have blunted the mefloquine-induced increase of mepp frequency.

![Fig. 2. Mefloquine (10 μM) alters mepps of the Triangularis sterni muscle isolated from adult mice. Representative mepps recorded from a control (upper row) endplate and one exposed to 10 μM mefloquine for 30 min (lower row). Four effects of mefloquine are illustrated: (1) increase of mean mepp frequency; (2) appearance of summating bursts of mepps; (3) prolongation of mepp duration; (4) increased incidence of “giant” mepps. The upper and lower records to the right are time expansions of the regions marked with arrows for the traces to the left.](image-url)
3.2. Role of extracellular and intracellular Ca\(^{2+}\) in the mefloquine-induced changes of asynchronous transmitter release

For endplates bathed in extracellular solution lacking Ca\(^{2+}\), 10 \(\mu\)M mefloquine continued to increase mean mepp frequency (Fig. 4). That is, mefloquine increased mean mepp frequency from the control value of 0.65 ± 0.10 (4, 34) to 4.50 ± 1.45/s (4, 25). In contrast, 10 \(\mu\)M mefloquine did not significantly increase \((p > 0.5)\) mepp frequency (Fig. 5) for preparations bathed in “0” [Ca\(_o\)] solutions and pre-treated with the intracellular Ca\(^{2+}\) buffer BAPTA-AM (10 \(\mu\)M for 60 min); mean mepp frequency was 4.74 ± 0.72 (5, 44) and 6.31 ± 1.03/s (5, 42) before and after exposure to 10 \(\mu\)M mefloquine. These observations suggest that the mefloquine-induced increase of mepp frequency requires an elevation of Ca\(^{2+}\) concentration within motor nerve endings.

3.3. Oligomycin, but not thapsigargin, prevents the mefloquine-induced increase of mean mepp frequency

As noted in Section 1, mefloquine mobilizes a thapsigargin-sensitive store of Ca\(^{2+}\) within neural tissue (Dow et al., 2003). Furthermore, a thapsigargin-sensitive Ca\(^{2+}\) store within motor nerve endings regulates transmitter release (Suzuki et al., 2002). Therefore, we pre-treated preparations with 2 \(\mu\)M thapsigargin. Thapsigargin alone added to extracellular solution containing either 2 mM or “0” Ca\(^{2+}\) failed to alter mean mepp frequency recorded at 15 min after this pre-treatment (Fig. 6A,B). For this series of experiments mean mepp frequency prior to exposure to thapsigargin was 0.49 ± 0.04 (7, 45) and 2.83 ± 0.28/s (4, 30) in “0” and 2 mM Ca\(^{2+}\) physiological solution, respectively. At 15–30 min after exposure to thapsigargin the corresponding values were 0.69 ± 0.19 (7, 45) and 2.93 ± 0.32/s (4, 20), respectively. Furthermore, thapsigargin did not prevent the mefloquine-induced increase of mean mepp frequency. That is, at 30 or more min after exposing these thapsigargin-pre-treated preparations to 10 \(\mu\)M mefloquine, mean mepp frequency significantly \((p < 0.05)\) increased to 5.37 ± 1.58 (7, 48) and 13.22 ± 1.44/s (4, 43), respectively, in the absence and presence of 2 mM Ca\(^{2+}\). These data suggest that a thapsigargin-sensitive Ca\(^{2+}\) store within motor nerve endings does not influence basal or mefloquine-activated asynchronous transmitter release.

To test the role of oligomycin-sensitive mitochondrial Ca\(^{2+}\) stores in the mefloquine-induced increase of mepp frequency, preparations were bathed for 30 min in “0” Ca\(^{2+}\) solution containing 8 \(\mu\)g/ml oligomycin. Oligomycin alone significantly increased mean mepp frequency from the control value of 0.66 ± 0.10 (4, 34) to 11.53 ± 1.06/s (5, 59). The pattern
of mepps in the presence of oligomycin closely resembled that observed in the presence of mefloquine alone (Fig. 7). This effect of oligomycin precluded the mefloquine-induced increase of mepp frequency. That is, mean mepp frequency in the presence of 10 µM mefloquine was 15.50 ± 0.49/s (5, 60) after pre-treatment with oligomycin. This mean value was not significantly different (p > 0.05) from that recorded in the presence of oligomycin alone (11.53 ± 1.06/s). In contrast, the lower trace of Fig. 7 shows that oligomycin did not prevent the changes of mepp amplitude and time course due to the anticholinesterase action of mefloquine.

Meffp frequency did not change for preparations exposed to an ethanol concentration equivalent to that of the oligomycin vehicle.

3.4. Mefloquine has minimal effects on stimulus-evoked transmitter release

Fig. 8A illustrates epps recorded from a glycerol-shocked preparation before and after exposure to 10 µM mefloquine. The pronounced prolongation of epp decay is expected because of the anticholinesterase action of mefloquine (Lim and Go, 1985; McArdle et al., 2005). This record also illustrates the failure of mefloquine to alter peak epp amplitude, suggesting a lack of effect on stimulus-evoked release. To test this possibility, we made direct estimates of mean quantal content before and after 10 µM mefloquine (Fig. 8B). Control quantal content was 26 ± 4 (3, 11). At 30–60 min after exposure to mefloquine quantal content was equivalent to control (25 ± 6; 3, 8). This analysis suggests that mefloquine has no effect on stimulus-evoked transmitter release. However, the altered time course, amplitude and frequency of mepps arising from the anticholinesterase action of mefloquine (McArdle et al., 2005) may have reduced the accuracy of the estimate of mean quantal size. Furthermore, the need to select from a sub-population of endplates whose epps were not contaminated with action potentials (see Section 2) may have biased our analyses. Therefore, we also examined mefloquine’s effect on the incidence of failures of stimulus-evoked transmitter release for preparations bathed in physiological solution containing 11 mM Mg2+ and 1 mM Ca2+. To minimize inclusion of mepps in counts of stimulus-evoked epps, we excluded all synaptic potentials occurring outside the 4–10 ms time interval after the stimulus artifact. For control preparations, the ratio of failures to the total number of stimuli (1 Hz for 60 s) was 0.47 ± 0.03 (46 nerve terminals in 5 muscles). The control failure ratio values were normally distributed (Fig. 9). At 30–60 min after 10 µM mefloquine, the mean ratio of failures declined to 0.39 ± 0.04 (37 nerve terminals in 5 muscles). Statistical comparison of these two means yielded a p value of 0.08 which suggested that the slight positive effect of mefloquine on stimulus-evoked transmitter release was not statistically significant. However, the frequency distribution of individual failure ratios after mefloquine suggested a bimodal distribution; that is, the failure ratio distribution was similar to control for 78% while 22% exhibited fewer failures of transmitter release in the presence of mefloquine. Thus, under conditions of low transmitter release, mefloquine can increase transmitter release for a small population of nerve endings.

3.5. Heterogeneity of motor nerve endings to the mefloquine-induced increase of asynchronous transmitter release

As expected (McArdle and Albuquerque, 1973), control mepp frequencies were normally distributed. However, at 30–60 min after 10 µM mefloquine the distribution of mepp frequencies did not conform to a simple distribution (Fig. 10). Nevertheless, two populations can be distinguished. A mefloquine-insensitive population accounted for 19% of endplates treated with 10 µM mefloquine; mepp frequency was essentially equivalent to control for this group. A second larger population of mefloquine-sensitive nerve endings accounted for 81% of endplates. While mepp frequency at these endplates was clearly outside of the control distribution, there was great variability between the magnitude of the mepp
frequency increase for these mefloquine-sensitive nerve terminals. Thus, motor nerve endings are heterogeneous in sensitivity to the mefloquine-induced increase of asynchronous transmitter release.

4. Discussion

Like tacrine, mefloquine inhibits acetylcholinesterases (Lim and Go, 1985; McArdle et al., 2005). The present work demonstrates that activation of asynchronous transmitter release at the neuromuscular junction (Theisleff et al., 1990) is another effect common to these structurally related drugs. It is relevant to note that recent estimates suggest that the concentration is 3.8 \( \times 10^{-23} \) M in the serum of humans receiving mefloquine (Simpson et al., 1999; Kollaritsch et al., 2000). Thus, all of the in vitro effects we report occur at mefloquine concentrations which are relevant to human therapy. This study examines three processes of motor nerve endings which could underlie these effects of mefloquine.

The first target tested was influx of Ca\(^{2+}\) into the motor nerve terminal. In the absence of extracellular Ca\(^{2+}\), mefloquine continued to increase mean mepp frequency. Thus, it is unlikely that mefloquine activates voltage-operated Ca\(^{2+}\) channels to increase the concentration of this ion in the nerve terminal cytosol. Mefloquine’s failure to produce an alteration of stimulus-evoked release comparable to that observed for asynchronous release further argues against an action on Ca\(^{2+}\) permeation across the nerve terminal membrane. On the other hand, mefloquine no longer increased mepp frequency for preparations pre-treated with the intracellular Ca\(^{2+}\) buffer BAPTA-AM. This observation suggests that the mefloquine-induced mepp frequency increase is secondary to an increase of Ca\(^{2+}\) concentration within the motor nerve terminal rather than to direct activation of the molecular processes underlying vesicle secretion. It is interesting to note that in the absence of extracellular Ca\(^{2+}\), BAPTA-AM pre-treatment alone increased the mean mepp frequency from 0.65 \( \pm \) 0.10 (4, 34) to 4.74 \( \pm \) 0.72/s (5, 44) (see Figs. 4 and 5). This surprising observation implies a Ca\(^{2+}\)-independent effect of BAPTA-AM on asynchronous transmitter release. Rather than further explore this observation, we performed experiments to identify the source of intracellular Ca\(^{2+}\) which mefloquine utilizes to increase asynchronous transmitter release.

The scientific literature suggests two intracellular Ca\(^{2+}\) stores with which mefloquine might interact. For example, mefloquine depletes a thapsigargin-sensitive Ca\(^{2+}\) store within neurons (Dow et al., 2003). A thapsigargin-sensitive mechanism regulates stimulus-evoked transmitter release at amphibian neuromuscular junctions (Suzuki et al., 2002). These actions made it necessary to test the interaction of thapsigargin and mefloquine. Thapsigargin alone did not significantly alter mean mepp frequency. Since we did not record mepps immediately after exposing nerve terminals to thapsigargin, a transient rise of asynchronous release may have gone undetected. However, pre-treatment with thapsigargin did not prevent the effect of mefloquine applied at a time when the thapsigargin depletion of its target Ca\(^{2+}\) store is complete. This observation suggests that the concentration of Ca\(^{2+}\) regulating asynchronous release and its response to mefloquine is below the chemical sensitivity and/or outside the spatial domain of the thapsigargin-sensitive Ca\(^{2+}\) uptake mechanism.
Fig. 8. Mefloquine does not alter stimulus evoked transmitter release for glyceral shocked *Triangularis sterni* nerve–muscle preparations. (A) Representative endplates potentials recorded before and 40 min after exposure to 10 µM mefloquine. While the anticholinesterase action of mefloquine resulted in a prolongation of the endplate potential, there was no change of peak amplitude. (B) Direct estimates of mean quantal content of endplate potentials (1 Hz, 1 min) suggest that mefloquine does not alter stimulus-evoked transmitter release. Each bar is the mean plus SEM for the number of muscles and endplates indicated in each bar.

Since mefloquine binds to the oligomycin sensitive (FO) complex to inhibit F0F1H+ -ATPase activity (Martin-Galiano et al., 2002), we pre-treated preparations with oligomycin prior to mefloquine application. Similarly to mefloquine alone, after a delay of approximately 30 min oligomycin increased mean frequency and induced bursts of mepps. Oligomycin also prevented the effect of mefloquine on mepp frequency. Our observations suggest that mefloquine and oligomycin act at a common site to alter asynchronous transmitter release. Thus, the effects of mefloquine are best discussed in the context of our knowledge of oligomycin's synaptic actions.

Studies of the role mitochondria play in transmitter release often employ protonophores, like CCCP, to depolarize the mitochondrial membrane. Depolarization uncouples electron transport and initiates loss of mitochondrial Ca2+ stores. In this condition, the F0F1H+ -ATP synthase operates in the reverse mode to hydrolyze ATP. To inhibit this activity and resultant energizing of pumping mechanisms which can restore mitochondrial potential and Ca2+ uptake, oligomycin is often co-applied with CCCP (David et al. 1998; David, 1999; David and Barrett, 2000, 2003; Calupca et al. 2001; Suzuki et al., 2002; Talbot et al., 2003). We excluded CCCP from our study for two reasons. First, oligomycin and mefloquine act at the same site to inhibit F0F1H+ -ATPase. Thus, our experiments focused on the interaction of mefloquine and oligomycin. Secondly, *Triangularis sterni* preparations bathed in “0” [Ca2+] physiological solution containing CCCP were severely depolarized; this made it impossible to acquire stable recordings of mepps. Thus, under our steady state experimental conditions it is probable that oligomycin, as well as mefloquine, simply inhibited mitochondrial ATP production. For

Fig. 9. Mefloquine enhances transmitter release from a small population of motor neurons exposed to physiological solution containing 11 mM Mg2+ and 1 mM Ca2+. The number of nerve stimuli failing to produce an endplate potential was counted during a 1 Hz train of stimuli lasting 1 min. The ratio of failures of release to the number of applied stimuli, “failure rate”, was computed. The histogram drawn with thin lines and fit with a normal distribution presents the failure rate for the control endplates. The mean control ratio was 0.47 ± 0.03 for 46 endplates in 5 muscles. At 30–60 min after exposure to 10 µM mefloquine, the mean failure rate decreased to 0.39 ± 0.04 for 37 endplates in 5 muscles. Though the difference between the failure rate of control and mefloquine preparations was not significant (p = 0.08), 8 of the 37 (22%) endplates exposed to mefloquine had a failure lower than that observed for any of the 46 control endplates.

Fig. 10. Motor nerve endings are heterogeneous in their response to mefloquine-induced increase of spontaneous transmitter release. The distribution of mepp frequencies for 236 endplates in 26 control muscles is shown by the histogram drawn with a grey line; none of these endplates had a mepp frequency greater than 10/s. The control mepp frequencies were fit with a normal distribution. The more darkly drawn bars indicate the distribution of mepp frequencies for 236 endplates in 26 control muscles. Oligomycin also prevented this effect of mefloquine on mepp frequency on average. However, the distribution of individual values varied greatly.
resting preparations, inhibition of ATP synthesis should not cause a detectable release of mitochondrial Ca\(^{2+}\) stores as observed for stimulated preparations treated with CCCP with or without oligomycin (David et al., 1998; David, 1999; David and Barrett, 2000, 2003; Suzuki et al., 2002). However, inhibition of ATP production might increase mepp frequency in other ways. For example, failure to maintain the concentration of the diffusible Ca\(^{2+}\) buffer ATP could increase Ca\(^{2+}\) concentration in the vicinity of releasable vesicles. Also, inhibition of the F\(_{0}\)F\(_{1}\) H\(^{+}\)-ATP synthase would not prevent diffusion of protons down their concentration gradient across the mitochondrial membrane. This would maintain the electrochemical force favoring Ca\(^{2+}\) entry into the mitochondrial matrix. Increasing Ca\(^{2+}\) concentration within the mitochondrial matrix, [Ca\(_{\text{m}}\)], could activate the permeability transition pore to transiently release Ca\(^{2+}\) into the cytosolic compartment. Such pulses of Ca\(^{2+}\) could have two effects. First, they would decrease [Ca\(_{\text{m}}\)] to below the threshold for opening of the permeability transition pore. Secondly, the transient increase of Ca\(^{2+}\) concentration within the vicinity of releasable vesicles could cause a burst of asynchronous transmitter release. Available data do not exclude the possibility that mitochondrial release of Ca\(^{2+}\) produces a highly localized increase of Ca\(^{2+}\) in close proximity to vesicles responsible for mepps. Further experiments are necessary to test the hypothesis that the slow latency to onset of the mepp frequency increase as well as the bursting pattern observed for preparations treated with either mefloquine or oligomycin depends upon inhibition of ATP synthesis and Ca\(^{2+}\) oscillations across the mitochondrial membrane (Melamed-Book and Rahamimoff, 1998).

In contrast to asynchronous transmitter release, stimulus-evoked release for preparations bathed in physiological solution containing 2 mM Ca\(^{2+}\) was resistant to mefloquine. Thus, mefloquine as well as oligomycin may be useful tools for exploring differences between these two forms of transmitter release. Geppert et al. (1994) provided data suggesting that the processes controlling spontaneous and stimulus-evoked transmitter release may differ at the molecular level. More recently, Sara et al. (2005) suggested that different vesicle pools may mediate spontaneous and stimulus-evoked transmitter release. Our working hypothesis is that release of vesicles responsible for mepps is coupled to the metabolic state of the nerve terminal. Study of motor nerve terminal responses to mefloquine and other chemicals acting on mitochondrial function are needed to further our understanding of metabolic control of synaptic transmission (Alnaes and Rahamimoff, 1975).

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