**Giant miniature endplate potentials at vertebrate neuromuscular junctions: a review**

Karim A. Alkadhi

# Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, TX, USA

Correspondence:

Dr. Karim A. Alkadhi, Professor

Dept. of Pharmacological and Pharmaceutical Sciences

University of Houston

Houston, TX 77024

kalkadhi@uh.edu

*gMEPP, calcium insensitive, ouabain, vesamicol,* temperature coefficient, TTX, snake venom.

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**Abstract**

An unusually large amplitude spontaneous miniature endplate potential (MEPP) occurs naturally at low frequency at the vertebrate neuromuscular junction. Unlike the normal MEPPs, these giant MEPPs have long duration and long time to peak. More strikingly, gMEPPs seem to be independent of extracellular and intracellular Ca+2.*and* have a greater temperature sensitivity than nMEPPs. They are potentiated by tetrodotoxin (TTX) but inhibited byacetylcholine (ACh) receptorblockers indicating ACh is the neurotransmitter responsible for gMEPPs. The frequency of gMEPPs is greatly increased in muscles weakened by various drugs, toxins or disease conditions suggesting that gMEPPs may be a part of possible neurotrophic mechanism to preserve effective neuromuscular transmission when normal function is compromised.

**Introduction**

Giant miniature endplate potentials (gMEPPs) were routinely excluded as “outliers” in the calculations of quantal contents and amplitude distribution. They appear spontaneously among normal MEPPs (nMEPPs) in untreated neuromuscular junctions (NMJ) at low frequency with unusually high amplitude, slow time to peak and long duration. It was Lilley in 1957 who was the first to qualitatively described the gMEPPs in untreated mammalian neuromuscular junctions (NMJ).That article had stimulated the interest of many investigators who reported that certain drugs, toxins, pathological conditions or procedures could markedly enhance the frequency of gMEPPs in amphibian and mammalian NMJ (e.g. Menrath & Blackman 1970; Heuser, 1974; Jansen & Van Essen, 1976; Pecot-Dechavassine, 1976; Molgo & Thesleff, 1982; Ashford & Wann, 1983; Alkadhi, 1988; 1989).

These gMEPPs have been variously referred to as giant spontaneous potentials, gMEPPs or slow MEPPs (Liley, 1957; Menrath & Blackman 1970; Heuser, 1974; Jansen & Van Essen, 1976; Pecot-Dechavassine, 1976; Molgo & Thesleff, 1982; Ashford & Wann, 1983; Alkadhi, 1987,1988, 1989). At the amphibian and mammalian NMJ, gMEPPs have been reported more commonly after exposure to drugs or toxins. Although various possible mechanisms for the generation of these potentials have been suggested, the actual mechanism remains uncertain*.* What is certain, however, is that these spontaneous potentials are produced by acetylcholine (ACh) released by a processthat is seemingly independent of Ca2+. This is, obviously, different from the strictly Ca2+ dependent normal release process. Although it is well known that removal of extracellular Ca2+ results in a pronounced decrease in nMEPP frequency, the ability of certain drugs to increase gMEPP frequency was not affected by removal of Ca2+ from the bathing solution (Durrant & Marshall. 1980; Molgo & Thesleff, 1982; Alkadhi. 1988,1989). For instance, procedures that increase intracellular Ca2+ concentration including treatment with ouabain, ethanol or Mn2+, markedly enhanced nMEPP frequency but have no significant effect on the emetine- or 4-aminoquinoline-induced increase in gMEPP frequency (Molgo & Thesleff, 1982; Alkadhi, 1989). Moreover, the frequency of gMEPPs in frog muscle increases after repetitive nerve stimulation but only under certain conditions including hypertonic solution (Pecot-Dechavassine & Conteaux, 1972; Van der Kloot & Van der Kloot, 1985), low temperature (10 °C, Heuser, 1974, Sellin et al., 1996), the presence of La3+ (Heuser, 1974) and low pH (Pecot-Dechavassine & Conteaux. 1972).

Treatments with drugs or toxins increase the frequency of the gMEPPs. For instance, the following agents markedly increased the frequency of gMEPPs: exposure to crotoxin from the venom of a South American rattlesnake in frog muscle (Hawgood et al., 1988), notexin from venom of an Australian elapid in rat muscle (Cull-Candy, et al., 1976), black widow spider venom (Smith et., 1977), botulinum toxins (Cull-Candy et al,.1976; Colmeus et al., 1982; Thesleff et al.,1983; Kim et al., 1984), 3,4-diaminopyridine (Durant & Marshall, 1980), 4-aminoquinoline (Molgo & Thesleff, 1982; Thesleff et al., 1983; Lupa & Yu, 1986), emetine and dehydroemetine (Alkadhi, 1987,1988,1989) among others (see table 1). The microtubular function inhibitors, vinblastine and cytochalasin, increased the relative frequency of giant potentials (Turkanis, 1973; Pecot-Dechavassine, 1976; Tabti et al., 1986*).* The increase in the frequency of “large spontaneous potentials” was also reported by Katz & Miledi (1969) after exposure of muscle to hypertonic K+ solution (Molenaar et al., 1987).

gMEPPs in the frog muscle share many characteristics with those recorded at mammalian neuromuscular junction treated with botulinum toxin or 4-aminoquinoline (Molgo & Thesleff 1982; Thesleff et al., 1983; Thesleff & Lupa 1986; Lupa, 1987). Most of the studies often used drastic procedures to induce increases in the frequency of gMEPPs, and only few have studied gMEPPs in untreated mammalian and amphibian preparations (Liley, 1957; Molgo & Thesleff, 1982; Thesleff et al. 1983; Alkadhi, 1988, 1989). Later in this review the details of these procedures are discussed.

***Site of Origin of gMEPPs***

Since denervation stopped both types of MEPPs (Lilley 1957; Molgo and Thesleff, 1982) glial cells may be ruled out as a source of gMEPPs. It has been demonstrated that gMEPPs at the mammalian and amphibian NMJs can be abolished by (+)-tubocurarine (Molgo & Thesleff, 1982; Alkadhi, 1989), therefore, gMEPPs result from release of ACh from nerve terminals and activation of nicotinic ACh receptors (AChRs) on the postjunctional membrane. The facts that gMEPPs totally disappeared when the ACh postjunctional receptors are blocked as well as after denervation of the muscle indicating a prejunctional source (Lilley 1957; Molgo and Thesleff 1982).

The possibility that gMEPPs may have originated because of leakage of cytoplasmic free ACh was ruled out by the finding that 4-AQ did not increase the frequency of gMEPPs after depletion of synaptic vesicles by black widow spider venom (BWSV) ((Molgo and Thesleff, 1982), which presumably causes depletion of vesicular ACh but does not affect free cytoplasmic ACh (Gorio et al. 1978).

***Possible mechanisms for gMEPPs formation***

The failure of TTX, Mg2+ or Mn2+ to affect the frequency of gMEPPs (Molgo & Thesleff, 1982; Thesleff et al. 1983; Alkadhi, 1989), makes it unlikely that gMEPPs are the result of spontaneous opening of large numbers of Na+ or Ca2+ channels in the prejunctional nerve terminals. Various suppositions about mechanism that generates gMEPPs have been introduced over the years. Earlier, working on rat diaphragm, Liley (1957) suggested that gMEPPs consisted of summated nMEPPs, which was also proposed by Pecot-Dechavassine and coworkers (Pecot-Dechavassine & Couteaux I972 a, b; I975; Pecot-Dechavassine 1976). Another suggestion is that gMEPPs are formed by release of ACh from over-sized vesicles formed during synaptic vesicle recycling (Heuser I974). Other groups suggested that gMEPPs are generated because of release of Ca2+. from intracellular stores (Fatt and Katz, 1951; Menrath and Blackman, 1970; Delbono, 2003), or the result of faulty processing of recycled vesicles (Rizzoli and Betz, 2005), which may be accelerated by drugs or toxins. These are unlikely inasmuch as the release in the above-mentioned suppositions would be Ca2+ dependent, whereas release of ACh for gMEPPs is Ca2+ independent.

The possibility that gMEPPs are generated by release of ACh along with a co-transmitter that would modulate ACh action by lengthening the mean open time of AChR-channel complex and delaying the rate at which channels open (Thesleff, 1988a, 1988b). However, this is unlikely because there is no evidence for any significant effect on the AChR of the known co-transmitters including vasoactive intestinal peptide (VIP), n-acetylaspartylglutamate (NAAG) (Walder et al., 2013), ATP and adenosine (Ziganshin et al., 2020) to name a few.

Another possibility that gMEPPs originate when transmitter is released from sites distant from junctional areas of high receptor density. If such release occurred it would be expected to give rise to smaller MEPPs with a slower time to peak, hence a negative correlation would result between their amplitude and time to peak. However, I found a positive correlation between the amplitude and time to peak of gMEPPs suggesting that it is unlikely that the slow time course of these potentials is the result of quantal release from such remote sites. (Alkadhi, 1989).Additionally, neurotransmitter release from sites that are distant from areas of high receptor density does not adequately explain enhancement of gMEPPs frequency by drugs and toxins.

The gMEPPs are also unlikely to be due to discharge of non-quantal pools of cytoplasmic ACh. This is because tetraphenyl borate (TPB) or 2-(4-phenyl-1-piperidineyl)-cyclohexanol (vesamicol), both are known to block non-quantal leakage of ACh, did not block gMEPPs (Anderson et al., 1983; Edwards et al., 1985; Vyskocil, 1985). Instead, these drugs gradually increased the frequency and amplitude of all MEPPs in mammalian muscles treated with botulinum toxin, 4-aminoquinoline (Lupa et al. 1986) or in frog muscle treated with emetine (Alkadhi, 1989). Related to this proposition is the suggestion that the gMEPPs could be due to the discharge of ACh from cisternae of Golgi apparatus (Augustine & Levitan, 1983). Assuming the cisternae are unable to dock into the release sites at the cytoplasmic side of the presynaptic terminal, then this discharge of ACh can only be into the cytoplasmic pools, therefore, the proposition that gMEPPs are formed from cisternae may be ruled out based on the results of vesamicol and TPB experiment as discussed above. Thus, at present time the precise process by which gMEPPs are generated remains uncertain.

**Possible physiological role**

It is unclear why gMEPPs appearatmuscle NMJs and what function they might perform. On examining the various treatments and pathologies reported to show increased frequency of gMEPPs, it becomes readily noticeable that most of these manipulations, including snake venom and some drugs, interfere with NMJ transmission resulting in muscle weakness or paralysis, (e.g. Chang et al., 1988; Vautrin, 1992; Wilson et al., 1995; Pousinha et al., 2015). The frequency of gMEPPs has been shown to increase under various conditions including paralysis with TTX (Gundersen, 1990), nerve terminal sprouting and synapse remodeling (Miledi, 1960; Balice-Gordon, 1997), nerve degeneration and regeneration (Birks et al., 1960; Bennett et al., 1973) and in motor neuron diseases (Birks et al., 1960; Carbonetto, 1977; Weinstein, 1980; Molgó & Thesleff, 1982; Rocha et al., 2013). Moreover, in aged rats, where muscle weakness is evident, the gMEPPs frequency significantly increases (Pousinha et al., 2015). Additionally, in diabetic rats where diabetes-induced pathologies can cause lesions in skeletal muscles, including muscle dystrophy, a marked increase in the frequency of gMEPPs has been reported (Martínez-Sánchez et al., 2023). Emetine, a drug known to interfere with protein synthesis and produces myopathy and cardiopathy, significantly increases the frequency of gMEPPs (Alkadhi, 1988,1989). Therefore, it may be that gMEPPs are generated as part of a neurotrophic mechanism to prevent neuronal death and preserve effective neuromuscular transmission when normal function is compromised. It is worth mentioning here that habitual exercise, which enhances neuromuscular transmission and increases nMEPPs frequency, did not affect gMEPPs frequency (Desaulniers et al, 2001).

**Characteristics of giant miniatures**

At the amphibian and rodent NMJs, gMEPPs appear with nMEPPs at a low frequency at room temperature but they have greater temperature sensitivity than nMEPP (Sellin et al., 1996). The low frequency of gMEPPs seen under normal conditions can increase many folds in the presence of a variety of drugs and toxins. The amplitude of the gMEPPs can be at least twice that of the nMEPPs. In fact, a gMEPP can occasionally be large enough to initiates an action potential at the NMJ (Liley, 1957; Alkadhi, 1989; Gunderson,1990). In addition to the large amplitude of MEPPs is the long time to peak (Fig 1), which can range from 1ms to 10ms, hence the name “slow MEPPs” sometimes used to describe gMEPPs. Interestingly, they occur in the presence of tetrodotoxin (TTX), in Ca2+-free solution or high Ca2+ solution. This calcium insensitivity uniquely distinguishes gMEPPs form the nMEPPs, which are Ca2+ dependent. Therefore, this Ca2+ insensitive spontaneous release is not influenced by nerve impulse (Thesleff, 1986). In the following sections, prominent characteristics of gMEPPs are discussed in greater details.

***Temperature variation:*** The frequency of gMEPPs is more temperature dependent than that of the nMEPPs. At the rodents NMJs, gMEPPs have a high temperature coefficient (Q10 = 12) (Thesleff, 1986; Sellin et al., 1996)*.* The gMEPPs frequency at 30-35°C is drastically decreased when the temperature is reduced to 14-16°C (Heinonen et al., 1982; Thesleff et al. 1990**;** Sellin et al., 1996). At the frog NMJs, the gMEPPs frequency at room temperature (24°C) (Alkadhi, 1989) is similar to that of the mammalian NMJs at 30-35°C (Thesleff, 1986; Sellin et al., 1996)*.*

## ***Effects of important ions and ion channels***

***Sodium channels:*** the involvement of sodium channels of presynaptic nerve terminal in the generation of gMEPPs was ruled out because there was no significant change in gMEPPs frequency or amplitude when rodents or amphibian muscles were bathed in the sodium channel blocker tetrodotoxin (TTX) (Pécot-Dechavassine,1976; Molgó and Thesleff, 1982; Molgó et al.,1982; Ashford & Wann, 1983; Molenaar et al., 1987; Alkadhi 1988; Gundrsen, 1990; Vautrin & Kriebel 1991). In fact, in the presence of TTX, some gMEPPs were even larger than the impulse-evoked EPPs in high Mg2+ and low Ca2+  (Gundrsen, 1990).

**Potassium:** Marked increases in nMEPPs frequency occurred when the extracellular K+ concentration was increased 3-fold because K+ causes depolarization of the presynaptic nerve terminal resulting in Ca2+ influx (Baker et al., 1971; Cull-Candy et al., 1976) without affecting gMEPPs frequency in untreated mammalian (Liley, 1957; Cull-Candy et al., 1976; Molgo & Thesleff, 1982). In frog muscle, increasing K+ by 3 folds did not affect gMEPP frequency in untreated or emetine treated muscle (Alkadhi 1989, (fig 2A).In contrast, Gundersen (1990) reported that increasing K+ concentration enhanced both nMEPPs and gMEPPs frequencies in rat muscle. Frog muscle treated with very high concentration of K+ (42mM)increased gMEPPs frequency (Molenaar et al., 1987). This discrepancy may be due to the prolonged treatment (up to 8 weeks) with TTX reported by Gundersen (1990) or the very high concentration of K+ used by Molenaar et al., (1987).

***Extracellular and intracellular* Ca2+*:***A key identifying characteristic for gMEPPs is its lack of dependence on Ca2+*.* The gMEPPs occur in Ca2+-free solution, high Ca2+ solution, or high Mg2+ low Ca2+ concentrations (Pécot-Dechavassine, 1976; Ashford & Wann, 1983*;* Alkadhi 1988; Gundersen, 1990). This calcium insensitivity uniquely differentiates gMEPPs form nMEPPs, which are strictly Ca2+ dependent. Removal of extracellular Ca2+ markedly diminish nMEPP frequency with no significant effect on that of the gMEPPs (Molgó & Thesleff,1982; Molgó et al., 1982; Alkadhi, 1988). Increasing extracellular Ca2+, which characteristically increased the frequency of nMEPPs, had no significant effect on gMEPP frequency in untreated or drug-treated preparations (Alkadhi, 1989). The presence of ions that block calcium channels including Mg2+ and Mn2+ (fig 2B), have no significant effects on gMEPPs frequency at the frog NMJ (Molgó et al., 1982; Alkadhi, 1988, 1989; Gundrsen, 1990)

Increasing the intracellular concentration of Ca2+ in nerve terminals with drugs and procedures has no significant effect on the frequency of gMEPPs in untreated or drug treated NMJs while profoundly altered the frequency of nMEPPs. Ethanol (fig 2C), ouabain (fig 2D), Mn2+ (fig 1B) and changes in osmolarity markedly increase nMEPPs frequency but have no significant effect on gMEPPs frequency at the rodents and amphibian NMJs (Molgo & Thesleff, 1982; Thesleff et al.,1983; Alkadhi, 1989). By inhibiting the sodium-potassium pump, ouabain consequently increasing intracellular Ca2+ (Baker & Crawford, 1975) and causing a marked increase in nMEPP frequency (Elmqvist & Feldman. 1965). Ethanol, like many other Alcohols, is said to increase intracellular free Ca2+, which enhances nMEPPs frequency and amplitude at the NMJs in the presence or absence of extracellular Ca2+ (Gage. 1965; Quastel et al., 1971). Mn2+ also increases nMEPPs frequency by inducing release of Ca2+ from intracellular stores (Balnave & Gage, 1973).

Osmolarity changes are another method used to influence spontaneous neurotransmitter release. Osmotic pressure of the extracellular fluid strongly influences spontaneous release of neurotransmitter can be demonstrated in the presence or absence of Ca2+. The mechanism of this effect is not well understood. It has been suggested that the effects of Hypotonicity and hypertonicity are due to changing intracellular Ca2+ (Shimoni et al., 1977; Kita et al., 1982). Moreover,Ca2+uptake by mitochondria decreases when osmotic pressure is increased in the medium (Scarpa & Azzone, 1968) leading to increased intracellular free Ca2+. While the frequency of nMEPPs was markedly reduced in hypotonic and increased in hypertonic media, that of the gMEPPs was not significantly affected. When gMEPPs evoking drugs (e.g. emetine or 4-AQ) were added to these media, the drugs produced the usual increase in gMEPP frequency (fig. 3) (Thesleff et al.,1983; Molgo & Thesleff, 1982; Alkadhi, 1989).

***Chloride ion and the effect of pH:*** Exposure of frog muscles to a very high K+ medium caused an increase the frequency of gMEPP,which persisted when the muscles was returned to normal K+ solution***.*** However, because the increase in gMEPPs frequency did not occurin the absence of Cl- it wassuggested that generation of gMEPPswas associated with a Cl--dependent process (Molenaar et al., 1987). To test this assumption, the effect of enhancing Cl- membrane permeability, by keeping muscle in pH 8.2 medium, on the ability of the anti-amebic drug emetine to increase gMEPP frequency was examined. Under this conditionthe frequency of nMEPPs was markedly increased with no significant effect on the frequency of emetine-induced gMEPPs. However, in acidic medium (pH 6.2, which decreases Cl- membrane permeability), emetine failed to increase gMEPP frequency (fig 4). And since pH 6.2 did not affectgMEPPs frequency in untreated preparations, we inferred that the failure of emetine to induce gMEPPs in pH 6.2 may be because at this pH most of the drug (pK1 5.77, pK2 6.64) is ionized. This suggests that the drug must be in neutral state in order to cross the cell membrane to reach an intracellular site (Alkadhi, 1989). We then used 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonate (SITS), a drug known to inhibit Cl- permeability, in frog muscle independently of the pH of the medium (Vaughan & Nok, 1978; Skydsgaard, 1987) and showed that inhibition of Cl- permeability did not affect gMEPP frequency in the untreated or emetine-treated muscles (Alkadhi, 1989). Therefore, it is implausible that an increase in Cl-availability is linked to the generation of gMEPPs. Additional support for the lack of involvement of Cl- in the generation ofgMEPPs, is that emetine is known to decrease the resting conductance of Cl- in rat skeletal muscle (Conte-Camerino & Mitolo-Chieppa, 1981).

**Osmotic pressure of extracellular fluid***:* Altering the osmolarity of the bathing solution causes marked effects on spontaneous release at the NMJ in vitro. Change of osmolarity affects spontaneous release by action on Ca2+ intracellular storage compartments. These actions are seemingly unaffected by the presence or absence of extracellular Ca2+ (Shimoni et al. 1977; Kita et al. 1982).

At the frog NMJs, hypotonicity did not significantly affect gMEPPs frequency in untreated muscles or interfere with emetine-induced marked enhancement of gMEPPs frequency while significantly decreasing nMEPP frequency both in untreated and drugs-treated preparations (fig. 3) (Alkadhi, 1989). Thesleff & colleagues (1983) using mammalian NMJs, reported a decrease of gMEPPs frequency in hypotonic solution, but the hypotonicity did not interfere with 4-AQ induced increase in gMEPPs frequency . This discrepancy may be related to the different species of animals used.

Hypertonic media cause marked increases in gMEPPs frequency in untreated mammalian NMJs without interfering with 4-AQ ability to increase gMEPPs frequency augmentation (Pecot-Dechavassine & Couteaux, 1972a,b; Thesleff et al.,1983). Exposure of frog muscle to hypertonic media generally results in augmentation of both frequency and amplitude of the gMEPPs; such effect on amplitude may be an indication of the increase in muscle fiber input resistance. The impact of hypertonicity on gMEPPs depends both on length of exposure and the level of hypertonicity (Kriebel et al., 1996).

***Summary and Concluding remarks***

The gMEPPs differ from nMEPPs in the following ways: [1] its amplitude is significantly larger than that of nMEPPs (Liley, 1957; Alkadhi 1989). [2] gMEPPs time to peak is highly variable but significantly longer than that of the nMEPPs or sometimes even that of evoked endplate potential (EPP) of a similar amplitude (Liley, 1957; Durant & Marshall, 1980; Thesleff, 1986). [3] Stimulus-evoked EPPs of similar sizes to gMEPPs invariably have shorter times to peak, therefore the ACh responsible for the gMEPPs does not seem to be released by nerve activation (Menrath & Blackman, 1970; Jansen & Van Essen, 1976).[4] The spontaneous release of ACh that evoked the gMEPPs, whether natural or drug-induced, is independent of extracellular Ca2+. [5] It is prominent during NMJs development and when junctional impulse transmission is weakened or inhibited (Thesleff, 1986).

Various treatments markedly increase gMEPP frequency. These treatments may injure the nerve terminals, therefore, the gMEPPS could be a pathological response to injury. Additionally, since gMEPPs are more prominent in impaired muscles and during development it seems plausible to speculate that they play a trophic role in synapse function*.* It is certain that ACh is the neurotransmitter responsible for these spontaneous potentials*.* Strikingly, however, the release of ACh responsible for gMEPPs being calcium-independent indicates that it is released by a different mechanism*.* It is interesting to mention that spontaneous, calcium insensitive potentials have also been reported at inhibitory and excitatory junctions of some invertebrates (Cull-Candy & Miledi, 1982; Finger & Marten, 1986).It is also worth mentioning that similar giant spontaneous miniature potentials (giant mEPSP/mEPSC) have also been reported at synapses of various brain areas of rat and mouse (e.g. Henze et al., 2002; Jang et al., 2006; He et al., 2009).

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Figure1: (A)Time-to-peak distribution of nMEPPs (open bars) and gMEPPs (filed bars) in untreated (Control) and emetine-treated endplates. Data were obtained from eight endplates in 3 untreated muscles and 9 endplates in 4 emetine-treated muscles bathed in Tris Ringer containing tetrodotoxin (0.5μM) and neostigmine (1μM). (B) MEPPs from a typical experiment before (Control) and 20 min after exposure to emetine in Tris Ringer solution. (modified from Alkadhi, 1989).

Figure 2: gMEPPs Frequency in untreated and treated muscle fibers. In each group the frequency was first determined in normal Ringer solution (Control), followed by switching to a drug/ion solution for up to 40m, then after addition of emetine (10 μM). A: K+,7.5 mM; B, Mn2+,10 mM; C. ethanol, 0.5 M; and D: ouabain, 0.2 mM. Each bar is the mean ± S.E.M of 7-19 endplates. (modified from Alkadhi, 1989).

Fig. 3.Comparison of effect of hypotonic bathing solution in the absence and presence of emetine (10μM) on the frequency of nMEPPs (A) and gMEPPs (B) in a typical experiment.The first arrow on the left represents the time of switching from normal to hypotonic medium. The second arrow marks the time when emetine was introduced. Frequency at each point in time was averaged from a 2-3m continuous period of recording in the same fiber throughout. Both experiments were done in the presence of TTX (0.5μM) and neostigmine(1μM) (from Alkadhi, 1989).

Figure 4: Comparison of effect of pH in the presence of emetine (10μM) on the frequency of nMEPPs (A) and gMEPPs (B) in a typical experiment. Frequencies at each point in time were averaged from 2-3m continuous recordings of spontaneous potentials in the presence of TTX (0.5 μM) and neostigmine (1μM) (from Alkadhi, 1989).