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INTERACTIONS BETWEEN CALCIUM AND BARIUM AT MAMMALIAN MOTOR NERVE TERMINALS

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To my wife, Irma

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CHAPTER I

INTRODUCTION

Investigations of the physiology of neuromuscular transmission have traversed the synaptic cleft twice. Early presynaptic studies described the release of a "vagus substance" from cholinergic nerve terminals, while quantal release of acetylcholine and its postsynaptic effects have been observed and quantified using intracellular recording techniques at the end-plate regions of muscle fibers. Recent investigations have emphasized the nature of quantal release, especially in studies of the effects of various cations at their presynaptic sites of action.

The quantal unit of transmitter release is monitored at the muscle end-plate as a miniature end-plate potential. Since the frequency of miniature end-plate potentials varies with the concentration of calcium, several theories have been proposed to explain the nature of transmitter release. The purpose of this investigation was to examine the interaction between calcium and another divalent cation, barium, at the presynaptic calcium-binding site. Barium was used to define more precisely some of the physicochemical properties of this site, as well as to determine the mechanism whereby acetylcholine is released from motor

nerve terminals.

Review of Investigations of Transmitter Release from Nerve Terminals

Acetylcholine Identified As the Neuromuscular Transmitter

Claude Bernard (1856) reported the first evidence for electrical discontinuity of the neuromuscular junction. Curare blocked transmission of excitation from nerve to muscle without reducing the excitability of either nerve or muscle to direct stimulation. Until Loewi (1921) showed direct evidence for a chemical transmitter, it was generally held that nerve conduction and synaptic transmission involved similar mechanisms of propagation of "activation waves" (reviewed by Dale, 1937). Kühne (1888) had formulated the theory that the muscle end-plate was electrically excited by the arrival of a nerve impulse at the nerve terminals. His experiments involved waves of excitation spreading between muscles overlaying one another and muscle overlaying nerves.

DuBois-Reymond (1877, cited in Dale, 1937) first proposed a chemical transmitter coupling depolarization of the nerve terminal with excitation of the muscle end-plate, and Elliott (1905) suggested that a chemical transmitter was released from sympathetic nerve endings. In 1921 Loewi demonstrated that stimulation of inhibitory nerves to the frog heart liberated a substance which was capable of eliciting an inhibitory effect on a second heart. The inhibitory transmitter released from the vagus nerve was called "vagusstoff" (vagus substance), and its release was confirmed by others (Brinkman and van Dam, 1922; Kahn, 1926; Bain, 1933). Dale (1933), observing that the vagus substance was released from

many types of nerve endings (including motor nerves), proposed the term "cholinergic synapses." The vagus substance was shown to be very similar to, if not identical with, acetylcholine (Chang and Gaddum, 1933), as observation substantiated by the inactivation of the vagus substance by a specific anticholinesterase, eserine (Loewi and Navratil, 1926). In addition, cholinergic transmission was inhibited by cholinesterase and potentiated by acetylation (Loewi, 1921; Stedman and Stedman, 1937). Therefore, by the late 1930's it was known that the coupling between nerve and muscle excitation in cholinergic neuromuscular transmission was mediated by a depolarization-induced release of acetylcholine, which in turn initiated electrical excitation of the muscle.

Transmitter Release Monitored at Muscle End-plates

The end-plate potential. The postsynaptic action of released transmitter was first observed with extracellular electrodes. Göpfert and Schaefer (1937) noted a local electrical potential change at the junctional region of muscle fibers. This third electrical event interposed between nerve impulse and muscle action potential was found to possess electrical properties distinct from an action potential and was called an "end-plate potential" (Schaefer and Haas, 1939; Eccles and O'Connor, 1939; Feng, 1941). Pharmacological studies indicated that the amplitude of the end-plate potential could be raised with acetylcholine or anticholinesterases and lowered with curare (Eccles, Katz and Kuffler, 1942; del Castillo and Katz, 1954e).

With the introduction of glass microelectrodes by Graham and Gerard (1946), finer resolution of the electrophysiology of the response

of the end-plate to released transmitter became possible. Fatt and Katz (1951), were first to record intracellularly at the end-plate region of muscle fibers, and Katz (1958) observed that the end-plate potential was analogous to a generator potential in a sensory neuron. Acetylcholine displaced the membrane potential of the end-plate toward a level intermediate to Na and K equilibrium potentials, indicating that the end-plate membrane became permeable simultaneously to both Na and K (del Castillo and Katz, 1954d; Takeuchi and Takeuchi, 1960). Although the end-plate potential differs from the action potential in being nonregenerative, the end-plate potential electrically stimulates surrounding parts of its muscle fiber to produce a muscle action potential (Katz, 1962).

Therefore, when a nerve impulse reaches the fine nerve terminals, it causes the release of acetylcholine which diffuses to the end-plate region of the muscle membrane. The local electrical circuits caused by ionic permeability changes at the end-plate membrane initiate the subsequent propagated muscle action potential.

Miniature end-plate potentials. The end-plate potential is a temporal summation of a few hundred spontaneous unit-depolarizations, called miniature end-plate potentials or MEPP's (del Castillo and Katz, 1954a; Boyd and Martin, 1956b; Martin, 1955; Katz, 1962). Spontaneous release of transmitter from motor nerve terminals was first suggested by Rosenbleuth and Morison (1937) as an explanation for eserine-induced fibrillation, and later suggested by Feng, Lee, Meng and Wang (1938) and Feldberg (1945). The microphysiology of spontaneous release was described by Fatt and Katz (1950, 1952), recording intracellularly at muscle end-plates. These local potential changes were similar to end-plate poten-

tials in time course, localization and reactions to curare and anticholinesterases, and therefore were called miniature end-plate potentials (MEPP's). Fatt and Katz (1952) observed that MEPP's were subthreshold potential changes of 0.5 mV which recurred intermittently at random intervals with an average frequency of 1/sec.

It remained to be shown that end-plate potentials and miniature end-plate potentials were related. By depressing release of transmitter with high Mg and low Ca solutions, fluctuations in end-plate potential amplitude were shown to involve quantal steps, each step corresponding to a unit identical to a MEPP (del Castillo and Katz, 1954b, c; Katz, 1958). In addition, depolarization of motor nerve terminals resulted in an increase in MEPP frequency, approaching the release of packets of transmitter expected from a nerve action potential (del Castillo and Katz, 1954d; Liley, 1956b). del Castillo and Katz (1954b, c) observed that the local potential change called a MEPP represented the smallest "quantum" of neuromuscular transmitter resulting from the release of a packet of acetylcholine from the nerve terminal. They proposed that transmission of a nerve impulse is produced by increasing the statistical probability of release of many packets of acetylcholine, whose temporal summation is observed at the muscle end-plate as the end-plate potential.

Investigation of the physiology of neuromuscular transmission has progressed from presynaptic studies of the vagus substance released from cholinergic nerves to postsynaptic extracellular and intracellular recordings of end-plate potentials. Observations associating the quantal contents of end-plate potentials with spontaneous MEPP's have resulted in a return to presynaptic study of transmitter release with an emphasis

on defining the underlying subcellular mechanisms. One line of investigation that has been especially productive is the interaction of various divalent cations on transmitter release.

Role of Calcium and Magnesium in Transmitter Release

Requirement of calcium in evoked release. Feng (1936) described the essential role of calcium in neuromuscular transmission, confirming an earlier observation by Locke (1894). If the concentration of Ca in the bathing solution is decreased, the amplitude of end-plate potentials declines, implying a reduction in the number of packets of acetylcholine released from the nerve terminal (del Castillo and Stark, 1952; del Castillo and Katz, 1954d; Liley, 1956b). In the absence of Ca nerve action potentials still invade nerve terminals, so it is some phenomenon in excitation-secretion coupling that requires the presence of Ca (Hubbard and Schmidt, 1963; Katz and Miledi, 1965). Application of Ca to nerve terminals by ionophoresis restores transmitter release within 1-2 msec, indicating a surface action for Ca (Katz and Miledi, 1965, 1967a). Calcium acts at the external surface of the membrane, since internal application of Ca fails to restore transmitter release (Miledi and Slater, 1966). In the rat neuromuscular junction Ca concentrations between 0.10 and 10 mM affect the quantal content of the end-plate potential. Transmitter release from all types of synapses studied thus far display a Ca-dependence, including the more classical types of neurosecretion, such as adrenal gland and neurohypophysis (see Hubbard, 1970 for review). Therefore, it is evident that Ca is required for transmitter release in response to nerve impulses.

Role of calcium in spontaneous release. Fatt and Katz (1952) could observe no consistent relationship between Ca concentration and MEPP frequency in frog neuromuscular junctions. However, subsequent investigations have indicated that at this, as well as at other synapses, MEPP frequency is dependent upon Ca concentration (Mambrini and Benoit, 1964; Boyd and Martin, 1956a; Usherwood, 1963; Hubbard, Jones and Landau, 1968a). Decreasing Ca concentration over the range 10 to 0.1 mM causes a reduction in MEPP frequency, while below 0.1 mM MEPP frequency becomes independent of Ca concentration (Hubbard et al., 1968a). Possibly there is a Ca-independent fraction of spontaneous transmitter release, although Miledi and Thies (1970) have shown an irreducible amount of Ca remains bound to the nerve terminal membrane, even in the presence of a strong Ca-chelating agent, such as EGTA (ethylene glycol bis {-aminoethyl ether} -N,N'-tetraacetic acid).

Action of magnesium on evoked and spontaneous release. While Ca is an essential "co-factor" for release by nerve impulses, Mg serves as an antagonist to release (del Castillo and Engbaek, 1954; Hubbard, Jones and Landau, 1968b). Magnesium antagonism of transmitter release has been established in amphibian and mammalian neuromuscular junctions, sympathetic ganglia, spinal motoneurons and the squid giant synapse (del Castillo and Engbaek, 1954; Jenkinson, 1957; Hubbard et al., 1968b; Blackman, Ginsborg and Ray, 1963; Katz and Miledi, 1963; Takeuchi and Takeuchi, 1962; Katz and Miledi, 1967b). The quantal content of the end-plate potential is reduced in the presence of 0.1 to 12.5 mM Mg by competition with Ca at three or more sites involved in evoked release (Hubbard et al., 1968b).

Mg can accelerate or reduce MEPP frequency depending upon the concentration of Ca (Hubbard, 1961; Hubbard et al., 1968a). In the absence of added Ca, Mg accelerates MEPP frequency although not as effectively as an equimolar amount of Ca. In addition, in the presence of 2 mM Ca addition of Mg up to 6 mM causes a reduction in MEPP frequency, while Mg from 6-12 mM accelerates MEPP frequency. In order to account for the complex activity of Ca and Mg, it has been proposed that these cations interact at two or more sites in their effects on spontaneous transmitter release and release by nerve impulses. In addition, Mg competitively inhibits the effect of Ca on spontaneous release at a common binding site on the nerve terminal membrane (Hubbard et al., 1968a).

Theories of Transmitter Release

Calcium-Channel Theory

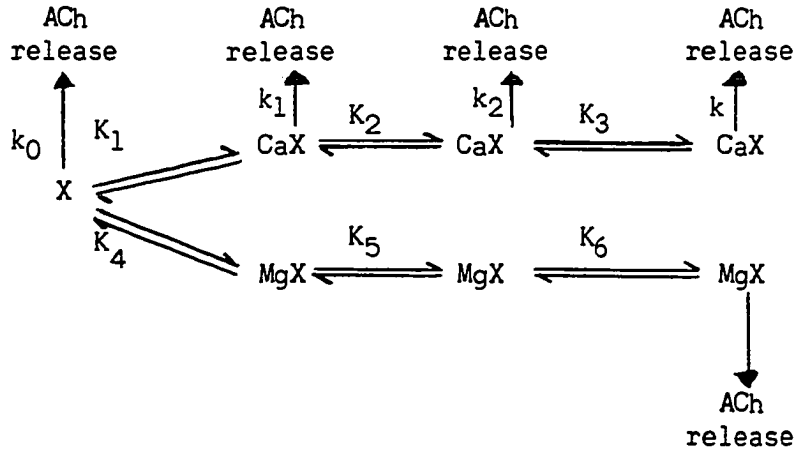
A mechanism explaining the effects of Ca on transmitter release was proposed by del Castillo and Katz (1956) and by Katz (1958). This theory, which has subsequently undergone considerable refinement (Katz and Miledi, 1965, 1967b, 1970), originally proposed that acetylcholine was bound in vesicles, similar to those which Robertson (1956) observed in reptilian nerve terminals. Random thermal motion produces repeated collisions of vesicles with the inner surface of the nerve terminal membrane, but release occurs only when certain "key molecules" on each membrane come into contact. Increased quantal release by a nerve action potential results from an increase of key molecules on the nerve terminal due to membrane depolarization. The change in membrane structure caused by depolarization requires the presence of Ca but is inhibited by Mg. Katz

(1958) described the essential elements required of a release mechanism; it must simultaneously permit acetylcholine to escape from the vesicle and pass through the membrane of the nerve terminal. Transmitter release was proposed to occur by an influx of Ca through special membrane "channels" which are opened by the depolarizing pulse (Katz and Miledi, 1970).

Evaluation of the channel theory. It is unlikely that random thermal motion is primarily responsible for release, because there is an ordering of vesicles near specific regions of the nerve terminal opposite postjunctional folds (Hubbard and Kwanbunbumpen, 1968). However, the more critical part of the theory proposing Ca-influx at the nerve terminal was substantiated by observations in squid axon and crab nerve--Ca moves inward during membrane depolarization (Keynes and Lewis, 1956; Hodgkin and Keynes, 1957). Furthermore, early and late phases of Ca entry have been observed, suggesting that the latency of the second influx may correspond to the Ca influx phase proposed for transmitter release (Baker, Hodgkin and Ridgway, 1970). Therefore, recent experimental evidence indicates that Ca moves from external to internal surface of the membrane, and this influx provides the necessary co-factor for fusion of vesicle and terminal membranes and subsequently for transmitter release. This is consistent with the theory proposed by del Castillo and Katz (1956).

Multiple Ca binding sites. Both spontaneous and evoked transmitter release depend upon a multiple association of Ca^{2+} with some complex, "X". Quantal content of the end-plate potential is related to the third or fourth power of Ca concentration, while MEPP frequency depends upon an association of two or more Ca^{2+} to a binding site (Jenkinson,

1957; Dodge and Rahamimoff, 1967; Hubbard et al., 1968a, b; Katz and Miledi, 1970). Therefore, wherever the Ca receptor site is, as more Ca binds, the probability of transmitter release increases. Hubbard et al. (1968b) proposed the following scheme for transmitter release which could account for this multiple Ca effect.



Here, $K_{1,\ 2 \cdots 6}$ represent dissociation constants, $k_{1,\ 2 \cdots 6}$ represent rate constants; and $k = k_3$ for spontaneous release, but $k = k'_3$ for release by nerve impulses. In this scheme the receptor molecule "X" is capable of releasing transmitter without bound Ca, corresponding to a Ca-independent fraction of release previously described (Hubbard et al., 1968a, b). Spontaneous release is accelerated by the association of 1, 2 or 3 Ca^{2+} while evoked release occurs only by increasing the rate constant k'_3. The inhibitory action of Mg is represented by the very high Mg concentrations (6-12 mM) that are capable of accelerating MEPP frequency (Mg_3X), but cannot substitute for Ca in evoked release (Hubbard et al., 1968a, b). It is possible that Ca and Mg may move through the membrane by some form of channel and produce their effect internally by multiple interaction on the fusion of vesicle and nerve terminal membranes (Blioch, Glagoleva,

Liberman, and Nenashev, 1968). Again, the inability of internally applied Ca to release transmitter would reduce the likelihood of this site of interaction (Miledi and Slater, 1966). Therefore, the multiple interactions of Ca and Mg on releasing sites at the external surface of the nerve terminal membrane seem to indicate a membrane bound carrier of some type, whose diffusion from external to internal membrane surface is facilitated by the binding of Ca and is inhibited by Mg.

Calcium Carrier Theory

The relatively low energy change expected from Ca binding produces a large free energy change resulting in spontaneous release of transmitter. Hubbard (1970) has shown that activation of the Ca_3X complex by a nerve impulse results in a 10^6 fold increase in its rate constant. One explanation for such dramatic energy amplification is that Ca induces an allosteric transition in a membrane bound carrier.

Structural changes in the calcium receptor. The relationship between Ca concentration and quantal content of the end-plate potential is sigmoidal, analogous to substrate-enzyme interactions which produce a conformational change in protein structure (Dodge and Rahamimoff, 1967; Hubbard et al., 1968b; Monod, Wyman and Changeux, 1965). A similar sigmoidal relationship has been observed between Ca concentration and MEPP frequency in amphibian and mammalian neuromuscular junctions (Mambrini and Benoit, 1964; Hubbard et al., 1968a). Changes in membrane structure have been observed during membrane depolarization of squid axons (Cohen, Keynes and Hille, 1968; Carnay and Barry, 1969), indicating the possibility that membrane depolarization may involve a flux of charge carriers from external to internal membrane surface (Hodgkin and Keynes, 1957;

Birks and MacIntosh, 1957; Hubbard, 1970). Since release by nerve impulses and spontaneous MEPP's occur by a common mechanism (Hubbard et al., 1968a, b), it is likely that the association of one or more Ca^{2+} changes the conformation of a membrane bound carrier to permit greater diffusion to the internal surface of the membrane. The hypothetical carrier protein is composed of three or four subunits. While most efficient movement through the membrane occurs as diffusion by the totally associated protein, a less associated protein not bound by Ca is also capable of slight movement in accordance with the theory of Hubbard et al. (1968a). The mono-, di-, tri-, and tetrameric forms of the protein all exist at equilibrium. Binding of Ca on a subunit may cause a shift in the equilibrium toward a conformational state more favorable to the association of the complete protein. Mg may act as a competitive inhibitor with Ca at a common binding site on the subunit.

Katz and Miledi (1966, 1967b) have shown that depolarization with an internal microelectrode at the nerve terminal produces increased release of transmitter, as expected. However, when a strong pulse was applied which actually reversed membrane potential, transmitter release was delayed until the pulse was removed. This was explained by a charged Ca^{2+} or CaX^{+} molecule being moved electrophoretically inward during depolarization and outward when membrane potential was reversed. While a charged carrier is not incompatible with the Ca-carrier hypothesis, it is unlikely that a charged species could diffuse readily through the lipid membrane. Reversed polarization might also prevent the binding of Ca to the membrane carrier, since Ca^{2+} would be expected to move away from the membrane under these circumstances.

In summary, the proposed theory for a membrane bound Ca carrier is consistent with the following three requirements: (1) it explains the sigmoidal relationship between $[Ca^{2+}]$ and transmitter release; (2) it incorporates the requirement for Ca to be effective when applied externally only; and (3) it utilizes the observations of membrane conformational changes during depolarization.

Investigations into the interactions between Ca and Mg have led to a better understanding of the fundamental mechanisms involved in transmitter release. While both divalent cations are endogenous to living systems, the use of wide ranges of concentrations far different from the natural environment could justify Ca and Mg as pharmacological tools. Thus, the *in vivo* environment often must be changed in order to elicit underlying mechanisms, and altering the ionic composition of the bathing solution has been a frequent tool of physiological investigation.

Effects of Other Cations in Transmitter Release

K, H, Ca, Mg, Ba, Sr, La and Th have been shown to increase the frequency of spontaneous MEPP's, while Be, Na and Li tend to decrease this release (Blioch *et al.*, 1968; Hubbard *et al.*, 1968a; Elmqvist and Feldman, 1965; Birks, Burtsyn and Firth, 1968). Of these cations only Ca, Sr and Ba are able to facilitate the accelerated acetylcholine release in response to nerve terminal depolarization (Blioch *et al.*, 1968; Hubbard *et al.*, 1968b; Miledi, 1966; Dodge, Miledi and Rahamimoff, (1969). The disparity observed in the action of some cations on spontaneous release as opposed to evoked release implies more than one site of action, perhaps separate sites for spontaneous and evoked release. Most cations

can act positively at one site, but only Ca, Sr, and Ba can act positively at both. Since transmitter release by nerve impulses has been shown to be an acceleration of spontaneous release (del Castillo and Katz, 1954d; Liley, 1956b), the most fundamental mechanisms would be defined by investigating spontaneous transmitter release. The present study was concerned with further analysis of these basic mechanisms, using two cations, lanthanum and barium.

The Effect of Lanthanum on MEPP Frequency

The addition of 1 mM La^{3+} to solutions in the presence or absence of Ca or Mg causes a pronounced rise in MEPP frequency to rates higher than those observed for mono- or divalent cations (Blioch et al., 1968). While Ca and La compete for a common binding site on some enzymes (Erdös, Debay and Westerman, 1960), it is not known whether such competition occurs at a site on the nerve terminal. A larger cation, such as La^{3+}, might be relatively accessible to the MEPP frequency acceleratory site.

Solubility of trivalent metals. The study of trivalent metals is difficult, because elements capable of being oxidized to the trivalent form have a strong attraction for valence electrons (Kremers, 1955). As a result, any anions in solution will be readily bound by the trivalent cation, which could lead to the formation of an insoluble precipitate if binding is strong enough. When a trivalent salt is placed in aqueous solution, the trivalent cation will readily bind with hydroxyl ions of the ionized water to form an insoluble hydroxide, which precipitates out of solution. Thus, although a known amount of salt can be placed into solution, some of this may precipitate out of solution, reducing the effective cation concentration.

Among the elements capable of being oxidized to the trivalent state, the problem of precipitation varies. Those elements offering the least interference are found in the Lanthanon Series of the Periodic Chart of the elements. Initially La was chosen above all other Lanthanons and all other trivalent metals, because it demonstrates the greatest basicity, making it least likely to bind with hydroxyl ions and form insoluble precipitates.

Rejection of lanthanum as a suitable pharmacological agent. In spite of its relatively high basicity, La still has a tendency to precipitate out of solution, thereby confounding interpretation of results based on free - $[La^3]$. Two methods were available to reduce precipitation out of solution. Firstly, low pH and low La concentrations would serve to decrease the likelihood of precipitation. An acetic acid-Na acetate buffer system was chosen to maintain pH at 6.0, and the maximum allowable La concentration was 5.0 mM, based on precipitation of stock solutions. Secondly, in order to compare the effects of various La and Ca concentrations on MEPP frequency, the free concentrations of these cations in solution had to be at least approximated. Free cation concentrations can be estimated using chelating agents such as EDTA (ethylenediamine-N,N,N',N',-tetraacetic acid) or EGTA (ethylene glycol bis {- aminoethyl ether} -N,N'-tetraacetic acid), which bind metal ions with known affinities (Erdös et al., 1960; Portzehl, Caldwell and Rüegg, 1964; Hubbard et al., 1968a). Based upon the stability constants listed by Portzehl et al. (1964), free ion concentrations of La^{3+}, Ca^{2+}, Mg^{2+} and H^+ in the presence of EGTA were calculated to the second approximation (see Appendix I). The metal ligand EGTA was selected because it binds

Ca and La with greater affinity (10^{11} and 10^{15}), than Mg (10^{5}), providing a more simplified estimation of free ion concentrations. Miledi and Thies (1970) have shown that in the absence of added Ca or Mg residual concentrations of approximately 0.03 mM Ca and 0.01 mM Mg are present in physiological irrigation solutions. Determination of the action of La on MEPP frequency in the absence of added Ca or Mg would be crucial to establishing the basic mechanism of La action. Calculations indicated that in the simplest solution the maximum free-$[La^{3+}]$ possible was 5.78×10^{-10} M, using 5.0 mM La and 10 mM EGTA. Since this La concentration would be approximately 10^{6} times less than the range shown to affect MEPP frequency (10^{-3} to 10^{-4} mM La), the quantitative study of La-Ca interaction on MEPP frequency was not pursued.

The Effects of Barium on MEPP Frequency

While investigation of La-Ca interaction might have indicated relative accessibility of both cations to a frequency acceleratory site, examination of Ba-Ca interactions would indicate the interaction of Ca with another alkaline earth metal. More importantly, MEPP frequency changes due to Ca-Ba interaction would provide data for partially characterizing the field strength of the MEPP frequency acceleratory site.

Early observations of Ba action. There have been two reports concerning the effect of Ba on MEPP frequency. Firstly, Boyd and Martin (1956a) reported a personal communication with del Castillo stating that Ba accelerates MEPP frequency above rates in equimolar Ca at frog neuromuscular junctions. Secondly, Elmqvist and Feldman (1965) observed that in Ca-free solutions Ba accelerated MEPP frequency more than an equimolar amount of Ca. Based upon these observations, a series of experiments

was devised that would examine quantitatively the proposed Ba-induced acceleration of MEPP frequency. Ba does not precipitate readily in aqueous solution, so chelating agents such as EGTA were unnecessary.

The purpose of the investigations described in this report was not merely to catalog the effects of various cations on MEPP frequency, but rather to attempt to characterize the site of CaX binding, whether X is a carrier or an anionic charge within some membrane "channel." Diamond and Wright (1969) proposed a method of analyzing such binding sites based upon the relative binding affinities of the four alkaline earths (Mg, Ca, Sr and Ba). Since Ba has the largest ionic radius of the four alkaline earths and Ca is the most biologically ubiquitous, Ca-Ba interaction studies would be expected to be the most informative of all interactions.

Binding affinities to a common receptor site. The existence of some anionic receptor site for cations is axiomatic. The purpose of investigations reported here was to provide a qualitative description of the field strength of the negative binding site, in order to provide a better understanding for the acceleration of MEPP frequency by cations. In biological systems the negative site is usually due to a partial polarization of oxygen of a phosphate, carboxyl or sulfate group (Diamond and Wright, 1969). Both biological and non-biological cation exchangers have been found to bind divalent cations in discrete affinity patterns, because of a tendency in all systems to maintain fixed charges in only a few of the many possible geometric configurations. While the alkaline earth metals, Mg, Ca, Sr and Ba theoretically have 24 possible permutations (i.e., 4!), with few exceptions only seven sequences of relative binding affinities to cation exchange sites are observed in biological or non-

biological exchangers. The seven sequences described by Sherry and cited in Diamond and Wright (1969) are listed below:

I	Ba > Sr > Ca > Mg
II	Ba > Ca > Sr > Mg
III	Ca > Ba > Sr > Mg
IV	Ca > Ba > Mg > Sr
V	Ca > Mg > Ba > Sr
VI	Ca > Mg > Sr > Ba
VII	Mg > Ca > Sr > Ba

Here group I is the selectivity sequence characteristic of a weak binding site, while group VII represents a strong binding site. Groups II through VI represent transitional sites of intermediate field strength. Assignment of one of these patterns to an observed biological cation exchanger can be further refined by estimating the relative magnitude of cation affinities for a common site and plotting these values with the aid of the selectivity isotherms calculated by Diamond and Wright (1969). Therefore, once the selectivity pattern is determined for the MEPP frequency acceleratory site, the relative field strength of this site can be estimated. In order to be aware of the underlying assumptions in the use of selectivity sequences, the physical chemistry of cation exchange will be examined.

Cation selectivity based on the lowest free energy state.

Eisenman (1962) described the relative binding of cations by biological and non-biological ion-exchangers on the basis of the maintenance of the lowest free energy state in the system. With little error, coulombic

forces can be considered as the major attractive force between the centers of charge of the negative binding site and the cation. This force of electrostatic attraction between opposite point charges is given as:

$$f = \frac{q_1 q_2}{\varepsilon r^2}$$

where q_1 and q_2 are the respective point charges, ε represents the dielectric constant "shielding" the point charges (here water) and r is the distance between the two charges (Barrow, 1966). The "choice" between two adjacent cations made by a particular negative binding site will depend upon the electric field strength of the binding site, the size of the non-hydrated radius of the cation, and the hydration energy of the water surrounding the cation. The two extreme cases of the field strength of a negative binding site will be considered first. When two sites with different free energies are available, a system will be most stable in the lowest free energy state. Assume that two cations are proximal to the negative binding site and are equally susceptible to the electrostatic field of the site. One of the cations has a smaller ionic (non-hydrated) radius than the other. That cation with the smallest ionic radius will permit water molecules to approach more closely to its center of charge. Thus more water will be adsorbed to the smaller cation, providing the cation with a greater hydration energy than the larger cation. Thus, there exists an "antagonism" for the point positive charge between the negative binding site and the water shell. If the negative charge on the binding site has a high charge field strength, then it overcomes attraction of the cation by water, yielding a decrease in free energy as the

cation loses its hydration shell and binds with the anionic site. On the other hand, if the negative binding site has a low field strength, the point negative charge cannot compete with the hydration shell for the small cation. However, if there is another cation with a larger ionic radius (and therefore smaller hydration shell), the negative site can combine with the cation to yield, again, the more stable state of low free energy. These are the two states of negative charge field strengths, while transitional states would have binding properties modified accordingly.

Specific cation affinities of the MEPP acceleratory site. Calcium has been shown to increase MEPP frequency to a greater extent than an equivalent amount of Mg (Hubbard, 1961; Hubbard et al., 1968a). Sr was observed to increase MEPP frequency slightly more than Ca, while Ba had a more pronounced accelerating effect (Boyd and Martin, 1956a; Elmqvist and Feldman, 1965; Blioch et al., 1968; Dodge, Miledi and Rahamimoff, 1969). These data suggest that the receptor acts as a group I receptor, although this assumption may be invalid since it has not been shown that all these cations act at the same receptor site. It may be that some are acting at the MEPP frequency acceleratory site, some may be neutralizing fixed negative charges on the internal surface of the nerve terminal membrane (as suggested by Blioch et al., 1968), while others may be acting merely as chelating agents to the external membrane. In fact, it has been shown that a binding site of similar low field strength is present of the internal surface on the membrane of the squid giant axon (Tasaki, Singer and Takanaka, 1965). Therefore, it is imperative to show that all these cations are acting at the same site, regard-

less of what that site might be.

Applications of partial characterization of the binding site. There have been several other studies concerning the relative binding affinities of various cations for common receptor sites. Hagiwara and Takahashi (1967) have found the following selectivity sequence in substituting for Ca in maintaining the Ca spike of the barnacle muscle fiber membrane; i.e., La, UO_2^{2+} > Zn, Co, Fe > Mn > Ni > Ca > Mg, Sr. Blaustein and Goldman (1968) observed a similar selectivity sequence in substituting for the action of Ca on Na and K conductance curves in squid giant axon; i.e., La, Al, Fe > Ni, Co, Cd, Ba > Ca > Mg.

It is quite possible that this partial characterization of a common binding site for cations on the nerve terminal membrane may be applicable to other membranes as well. In fact, if this binding site for increasing MEPP frequency is a mobile carrier, then similar binding affinities in other membranes, such as squid axon, barnacle muscle and Ca-sensitive secretory cells of the posterior pituitary, could indicate a carrier with similar properties in all these membranes. Thus selectivity patterns serve not only in defining a specific cation receptor site, but also as a means of comparing receptor properties common to many membranes. While partial characterization of a receptor using the kinetics of Ba and Ca action is less direct than a biochemical analysis of the membrane, it does have several advantages. Firstly, a kinetic analysis describes the membrane in its dynamic state, a condition imperative to basic physiological studies of membranes. Secondly, the ambiguity of the various effects of cations on the nerve terminal membrane will be sorted out, so that only one effect, an increase in MEPP frequency, will be considered.

Clarification of this effect will be adhered to rigorously by means of the competitive inhibition of cations at a common binding site.

Procedures for Examining the Effects of Barium on MEPP Frequency

The planned research had three objectives. Firstly, the direct action of Ba on MEPP frequency in the absence of Ca was to be investigated in order to confirm or deny previous reports. Secondly, assuming that Ba induced some change in MEPP frequency, it would be important to determine the time required to reach new stable rates. Thirdly, in order to characterize the MEPP frequency acceleratory site, interactions between Ba and Ca on MEPP frequency were to be investigated.

CHAPTER II

METHODS

Preparation of Tissue

Use of the Rat Hemidiaphragm Muscle

Initial experiments studied the interaction of Ca and Ba at nerve terminals of frog sartorius muscles. A series of preliminary experiments disclosed several difficulties in the use of this system, so the rat hemidiaphragm was used instead.

Multiple puncture experiments. In order to estimate the effects of Ba with time on a population of cells, numerous end-plates must be monitored as rapidly as possible. The frog sartorius muscle does not lend itself to this type of recording. It often took five or more minutes to locate an end-plate in the frog sartorius muscle, too long for adequate estimates of changing MEPP frequencies with time in a population of cells. Also, only marginal regions of the sartorius muscle where there are fewer fibers were sufficiently transluscent to permit location of end-plates. On the other hand, the rat diaphragm is only a few fibers thick, so light passes readily through all regions of the muscle. In addition, end-plates are concentrated along the central region of the hemidiaphragm (Krnjević and Miledi, 1957; Potter, 1970), providing a high probability of finding MEPP's with each insertion of the microelectrode.

Stability of mean MEPP frequency. In 1952 Fatt and Katz observed MEPP's in the frog at mean frequencies varying by a thousand fold (0.1 to 100/sec) in different fibers, making difficult any statistical comparisons of the effect of various solutions. In the cat tenuissimus muscle, as well as rat diaphragm, mean MEPP frequencies usually vary by no more than ten-fold between fibers (Boyd and Martin, 1956a; Hubbard, 1961; Hubbard et al., 1968a; and observations by the author). Thus, the effects of solution changes would be more discernable in rat diaphragms than in frog sartorius muscles.

Calcium-dependent MEPP frequency. In the rat diaphragm MEPP frequency is directly related to Ca concentration of 0.1 to 10 mM (Hubbard, 1961; Hubbard et al., 1968a). This Ca-dependent release has not been consistently observed in the frog. Mambrini and Benoit (1964) observed a Ca-dependent release in the range of 0.25 to 10 mM Ca, but Fatt and Katz (1952) observed no consistent relation. Since a basic assumption in Ca-Ba interaction studies is that varying Ca concentration will affect MEPP frequency, the rat diaphragm was chosen as the more suitable preparation.

Method of Dissection

Rats of the King-Holtzman strain of either sex weighing approximately 200 g were used for all experiments described in this report. This strain supplied by Dr. Allen Stanley has been inbred for twenty-three generations. Since only half of the diaphragm is used, the excised tissue is called a hemidiaphragm.

Rats were anesthetized with ether and placed on their backs on a cork board. Each limb was pinned to the cork, while ether-soaked cotton

maintained anesthesia. With the ventral surface clearly exposed, the abdomen was incised and the portal vein severed. The ensuing hemorrhage permitted the eventual isolation of the diaphragm free from clotted blood. Before the animal died of hemorrhage, the thoracic cavity was opened. The sudden inflow of air at atmospheric pressure collapsed the lungs, facilitating further dissection in the thoracic cavity. On the left side of the rat the chest and abdominal walls were cut from ventral to dorsal surfaces. The paths of the incisions formed arcs on either side of the diaphragm, parallel to its plane. The dissection thus far resembled a section of orange whose apices represent points of dorsal and ventral attachment. The whole diaphragm was then cut in half along its center, from ventral to dorsal surfaces. A final cut separated the hemidiaphragm from the spine. The isolated tissue was immediately placed in a Petri dish of oxygenated Krebs's solution to await further dissection. The dissection required between 4 and 8 min from abdominal incision to immersion in Krebs's solution.

Muscle Chamber

The excised hemidiaphragm was trimmed further, leaving approximately 2 mm of phrenic nerve attached. Stainless steel pins held the muscle to the floor of a Lucite chamber, similar to that described by Krnjević and Mitchell (1961). The volume of the chamber well was 15-20 ml. Dual inflow tubes permitted rapid switching of irrigating solutions, while the solution was maintained at constant level by a suction tube placed diagonally opposite the inflow tube.

Solutions

Composition of Solutions

All solutions were prepared from "Baker Analyzed" or "Fisher Certified" reagents. Distilled water was demineralized with a Barnstead Standard Type ion exchange resin bed. Table 1 lists the composition of solutions of the four series of experiments described in Results. These were modified from the Ringer-Locke solutions described by Liley (1956a) and Krnjević and Mitchell (1960). In order to prevent the precipitation of insoluble carbonates and phosphates from solutions of high divalent cation concentration, $H_2CO_3^-$ was reduced to 12 mM and $H_2PO_4^-$ eliminated from all but some initial experiments (see Table 1, rows I and II). Since Birks et al. (1968) found that reducing external Na increases MEPP frequency, within any series of experiments added Na was constant. The two experiments in which Na was 11% higher (Table 1, row III, last solution) and 19% higher (Table 1, row II) did not change the interpretation of results, since Na was constant within any particular experiment. Hubbard et al. (1968c) have shown that the osmotic pressure of the bathing solution also influences MEPP frequency. Since wide ranges of divalent cations were studied, the osmotic pressure was set at a level calculated for solutions with the highest divalent cation concentration (318 m-osmoles). Osmotic pressures of solutions with lower divalent cation concentrations were compensated with additional glucose on the assumption that 1 mM glucose contributes 1 m-osmole to the osmotic pressure. Since osmotic pressure was constant within any one experiment, the three exceptions (Table 1, rows I, II and III, last solution) did not confound the results. The calculated osmotic pressure of solutions listed in

TABLE 1

COMPOSITION OF SOLUTIONS LISTED ACCORDING TO EXPERIMENTAL SERIES

Experimental Series	Na (mM)	K (mM)	Ca (mM)	Ba (mM)	HCO_3 (mM)	Cl (mM)	Glucose (mM)	Osmotic Pressure (m-osmole)
I Initial Experiments	149	5.6	1, 0, 0	0,1.0	23.8	127.2	7.9	308.9
II Ca vs Ba 0.5 mM	160.6	5	0.5, 0	0, 0.5	23.8	143.3	18.5	353.7
Ca vs Ba 1.25 mM	134.5	5	1.25, 0	0, 1.25	12	130	35	317.8
III Constant Ba	134.5	5	1.25	0, 1.25	12	130	35, 31	317.8
	134.5	5	2.50	0, 1.25	12	132.5	26, 29	317.8
	134.5	5	5.0	0, 1.25	12	137.5	24, 20	317.8
	134.5	5	10.0	0, 1.25	12	147.5	8.8, 5.0	317.8
	149	5	1.25, 5.0	0, 1.25	12	144, 154	5, 9	317, 332
IV Constant Ca	134.5	5	1.25	0	12	130	35	317.8
	134.5	5	1.25	0.25	12	130.5	34.2	317.8
	134.5	5	1.25	0.50	12	131.0	33.5	317.8
	134.5	5	1.25	1.25	12	132.5	31.2	317.8
	134.5	5	1.25	2.50	12	135.0	27.5	317.8

Table 1, row III (317.8 m-osmoles) was only slightly higher than the osmotic pressure of each solution measured on a Fiske osmometer (303-317 m-osmoles). Contamination by extraneous Ca in "Ca-free" solutions was probably less than 0.03 mM and even less for Mg (Miledi and Thies, 1970). Solutions were bubbled vigorously with a mixture of 95% oxygen-5% carbon dioxide. The pH of each solution was measured daily on samples taken from the muscle chamber. At 33°C pH ranged from 7.06 to 7.23 in all solutions and was not noticeably affected by the highest divalent cation concentration (pH was 7.13 in 10 mM Ca + 1.25 mM Ba), indicating that neither $[Ca^{++}]$ nor $[Ba^{++}]$ were reduced by the formation of insoluble carbonates.

Methods of Irrigation

One-liter graduated cylinders served as reservoirs for irrigating solutions. Reservoir solutions were maintained at 38-40°C by standing the cylinders in a 20-gallon heated water bath. From the reservoir solutions moved by gravity flow through Tygon tubing insulated with rubber tubing into the muscle chamber. Solutions entered the chamber through either of two tapered glass nozzles, and the rate of flow was controlled with polyethylene stopcocks.

In an initial series of experiments (see Results) rate of inflow was relatively slow (3 ml/min). In subsequent experiments average rate of inflow was 9.8 ml/min (range 6.0 to 14 ml/min). Rate of inflow was constant within any one experiment and was determined by the flow rate needed to maintain the muscle chamber solution at a constant temperature.

Temperature Control

Temperature was monitored with the thermocouple probe of a

Tele-Thermometer (Yellow Springs Instrument Co.) immersed in the chamber directly over the center of the diaphragm. Hubbard et al. (1967) suggested that the basic form of Ca kinetics is maintained between 5° and 34°C but gradually disappears at higher temperatures. Therefore, except for a few low temperature studies, all solutions in the muscle chamber were maintained below 34°C (average temperature was 32.6°, range 30.0 to 33.7) rather than 37-38°C used by Liley (1956a). Some heat was lost from irrigating solutions as they moved through the insulated Tygon tubing from the reservoir to the muscle chamber. To compensate for this heat loss and the heat lost from the chamber surface, water at 38-40°C was circulated through a compartment beneath the muscle chamber. Without this additional heating from below, faster flow rates would have been required to maintain chamber temperature above 30°C. The chamber temperature never rose above 36°C when inflowing solutions were switched (see below).

Introducing New Solutions

At "zero time" the stopcock of one inflow tube was closed and a second opened permitting irrigation with the second solution. To facilitate bath exchange, the second solution was introduced at maximum flow rate (> 15 ml/min), and the chamber was quickly aspirated with the suction tube. This facilitated rapid equilibration of the entire diaphragm with the second solution. The same procedure was used for solution changes in all experiments, so differences in technique could not be responsible for differences in latency before MEPP frequency was affected by new solutions. In order to test the speed of solution washout, a dye-dilution technique was used to determine the amount of time required to

exchange solutions in the muscle chamber. The reservoir was filled with Krebs solution containing methylene blue. As the new solution began to flow in, 3 ml samples were taken from the chamber every 30 sec. Measurement of optical density of the chamber solution indicated that exchange of solutions was 98% complete within the first minute by using this technique. Therefore, in all experiments the maximum inflow rate described above was maintained for the first minute after introduction of a new solution. After one minute inflow rate was reduced (average 9.8 ml/min) to conserve irrigating solution.

The Hemidiaphragm in Control Solutions

Histological changes. In order to eliminate an additional interaction between divalent cations, Mg was not added to any of the solutions used to study Ca-Ba interaction. Hubbard (personal communication) observed that the lack of Mg in bathing solutions produces histological changes in the rat diaphragm muscle. These observations were confirmed in the present experiments. After two hours of irrigation with a solution containing 1.25 mM Ca and no added Mg, muscle fibers began to decrease slightly in length due to an apparent "shriveling." The ordinarily straight longitudinal borders of the fibers began to assume a wavy appearance, often obscuring the fine nerve branches. Shriveling had increased after seven hours of irrigation, but end-plates were located readily, and there was no difficulty in puncturing muscle cell membranes.

Shriveling was more severe with increased concentrations of Ca or Ba and with a 20% higher Na concentration. Increasing osmotic pressure from 317.8 to 342.8 m-osmoles with added glucose did not affect the intensity of the shriveling, and 1.0 mM Mg appeared to retard the histo-

logical changes. In the presence of higher Ca concentrations Ba often appeared to reduce shriveling. The underlying mechanism for the changes in appearance of diaphragm muscles seemed to be inhibited when Ca and another supporting divalent cation (Mg or Ba) were present in solution, but were activated when Ca or Ba were present alone in solution or when the concentration of a monovalent cation (Na) was increased. A regression analysis of MEPP frequencies during these histological changes showed no relation between shriveling and MEPP frequency, so while the histological changes were technically disturbing, the primary objective of MEPP frequency measurement was not impeded.

Spontaneous muscle activity. In order to determine the lower end of the range of Ca concentrations used in the series of experiments described in Results (p. 70), the effects of Ca concentrations from 0.1 to 1.0 mM Ca on spontaneous twitching were tested. Solutions with 0.1 mM Ca and no added Mg invariably produced spontaneous activity of the diaphragm muscle, confirming similar effects on frog sartorius muscle (Bülbring et al., 1956). Diaphragms in solutions with Ca concentrations between 0.37 and 0.72 mM showed inconsistent spontaneous activity. Twitching was absent in 1.0 mM Ca or above with no added Mg.

Feng (1937) and Fatt and Ginsborg (1958) reported asynchronous twitching of muscle fibers in the presence of Ba; this was confirmed in the present observations. The twitching may be caused by instability of the Ba-depolarized nerve trunk, nerve terminal and muscle membranes (Feng, 1937), as well as the effect of Ca lack itself in depolarizing muscle fibers and inducing spontaneous activity (Bülbring et al., 1956). This action of Ba represents both fibrillation (fiber activity) and

fasciculation (motor unit activity) similar to the effects of eserine (Masland and Wigton, 1940; Fatt and Katz, 1952).

Control MEPP frequency. In the present studies MEPP frequencies in 1.25 mM Ca control solutions were higher than expected. Previous reports indicated that in solutions containing 1-2 mM Ca, 1 mM Mg and 150-162 mM Na, MEPP frequency was 1-2/sec on the rat diaphragm (Liley, 1956a; Hubbard et al., 1968a; Hubbard, 1970). Most control solutions described in this report contained 1.25 mM Ca, 0 mM Mg and 134.5 mM Na, and mean MEPP frequency was 3.69/sec. In addition, previous investigators have used exclusively the Wistar strain of rats, while data described in the present report were obtained using the King-Holtzman strain.

In order to explain the difference in control MEPP frequencies, each variation in composition of solutions was examined individually. The addition of 1 mM Mg reduced MEPP frequency 11.5% (8 and 15%), while increasing Na concentration to 150 mM reduced frequencies by 8.5%. Together these solution differences could reduce observed MEPP frequencies by 20% from 3.69 to 2.96/sec. This rate 2.96 MEPP's/sec is still almost twice as high as previously observed rates (1-2/sec). The remaining difference in basal MEPP frequencies may be due to differences in the strains of rats used (Hubbard, personal communication). This is substantiated by the observation that changes in MEPP frequency produced by various concentrations of Ca, Na and Mg are consistent with previous reports (see Results; Hubbard, 1961; Gage and Quastel, 1966; Birks et al., 1968; Hubbard et al., 1968; Hubbard, 1970). Therefore, while some histological changes, spontaneous activity and fast control MEPP frequencies were observed in rat diaphragms, the tissue was sufficiently stable to warrant

its use in studying the effects of various divalent cation concentrations.

Electrical Recording Systems

Recording Electrodes

Glass capillary microelectrodes. Microelectrodes were manufactured from glass capillary tubing 7.5 cm in length with an outer diameter of 1.3 mm and inner diameter of 1.0 mm. The tubing was pulled with a David Kopf Instruments Model 700C vertical pipette puller. After immersing the microelectrodes in a sealed chamber with 3 M KCl solution, the chamber was heated to about 62°C and evacuated, electrodes being filled by repeatedly replacing and evacuating the air from the chamber. In order to minimize baseline noise, only microelectrodes with resistances between 3 and 10 megohms were used (> 0.5 μ, Frank and Becker, 1964). Microelectrode resistance was measured using a 100 mV pulse from a calibrator battery (see Figure 1), and the equations described by Burés et al. (1967). In order to measure MEPP amplitudes, each microelectrode was periodically calibrated with ten to twenty 0.5 mV pulses from the calibrator battery (Figure 1). The microelectrode was placed into cells using a Zeiss Jena micromanipulator, under direct observation at 18 to 80 power through a Nikon stereo zoom microscope.

Silver-silver chloride electrodes. To minimize polarization of electrodes by applied potential changes, Ag:AgCl electrodes were used to complete the circuits from microelectrode to cathode follower and from muscle bath to ground. Since Ag^+ tends to have adverse effects on tissue, the ground Ag:AgCl electrode was connected to the bath through a 0.9% NaCl-agar bridge.

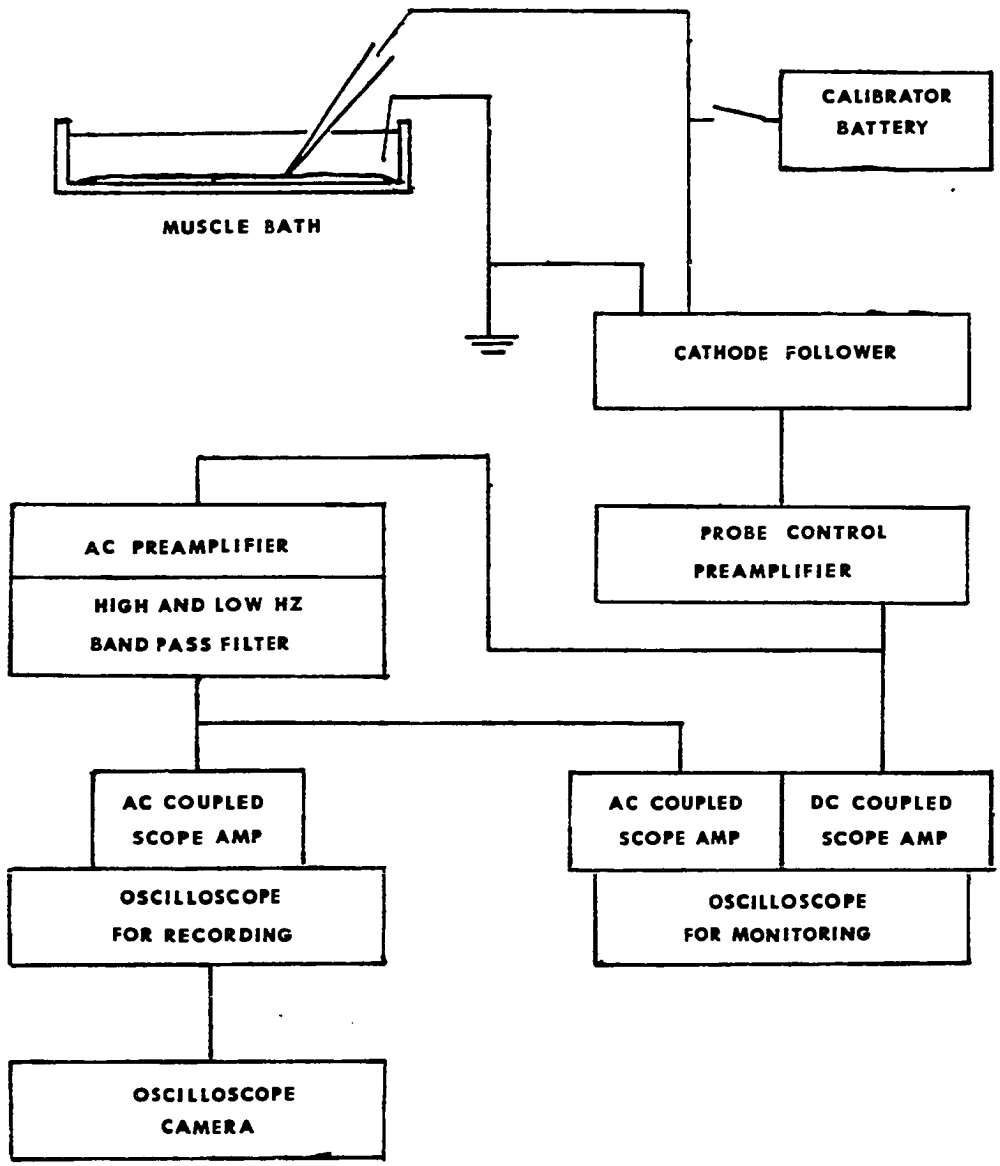


Figure 1. Block diagram of recording apparatus.

Ag:AgCl electrodes were manufactured by a method similar to that described by Burés et al. (1967). Silver wire approximately 15 mm in length and 0.46 mm in diameter was soldered to one end of a braided copper lead, coating the soldered connection with a silicone adhesive. The silver ends of the wire were cleaned with emery paper and briefly dipped in concentrated HNO_3 to produce a pitting of the silver, providing a greater surface area for plating. Both wires were clipped together and served as the anode, while a third silver wire approximately 1 mm in diameter served as the cathode. Anodal and cathodal wires were immersed in 0.1 N HCl saturated with AgCl crystals, and plated by applying current from a 6 volt battery across a 27K ohm resistor. Typically a current density of 0.005 $mAmp/mm^2$ flowed for approximately two hours, or until the AgCl plated silver wires became plum colored. The paired Ag:AgCl wires were shorted together and stored in 0.1 N HCl until used.

Amplification Stages

Potential changes monitored at the microelectrode tip were conducted through the Ag:AgCl wire lead into a Bioelectric Instruments unity gain PFC2 wideband electrometer amplifier with a field effect transistor (similar to a cathode follower), providing a high input impedance (see Figure 1). A probe control preamplifier permitted application of $\pm$ 5 volts to offset DC potentials. To record membrane potential, the signal was direct coupled to one oscilloscope amplifier at relatively low gain (10 mV/cm). However, for recording MEPP's the signal was also AC- (capacity-) coupled to another oscilloscope amplifier at higher gain (0.2 mV/cm). In order to improve the signal-to-noise ratio low and high frequency signals were attenuated by 300Hz and 3K Hz cutoff filters, using

a Grass Instruments Model P15 AC preamplifier between the probe control and oscilloscope AC-coupled amplifiers (Figure 1). While filtering tended to reduce slightly MEPP amplitude and decay time, 60 Hz and amplifier interference were greatly reduced, permitting better discrimination of MEPP's. From the AC-coupled scope amplifiers signals were observed on a monitoring oscilloscope (Tektronix model RM 564) and filmed from a second oscilloscope (Tektronix model RM 565).

Recording Techniques and Data Analysis

Filmed Records of MEPP's

Initial experiments described in Results (Figure 2) were based upon data recorded on a Model LCR light beam oscillograph (Telex-Midwestern Instruments). Because of instrument noise at high gain settings this method of recording was replaced with the use of a continuous recording oscilloscope camera (Model PC-2A, Nihon Kohden Kogy Co.). Most of the data described in Results were obtained from filmed records. MEPP's were recorded on both single frames and continuously moving film, while membrane potentials were photographed only on single frames (compare Figures 4 and 12 in Results). With continuously moving film, membrane potentials were noted but not photographed. In the single-frame method of recording each frame represented an oscilloscope sweep of 1 to 10 sec duration for a total sample time of 10 to 20 sec from each cell. Using this single-frame method of recording, it was often difficult to distinguish MEPP's from one another especially at fast frequencies. In experiments where MEPP frequency was 8/sec or faster, 8% or more MEPP's were uncountable because of the possibility of a single filmed trace represent-

ing 2 or more superimposed MEPP's. This source of error was eliminated by increasing oscilloscope sweep speed and recording 50 traces of 200 msec sweep duration on continuously moving film (total recording time was 10 sec). Therefore, in calculating mean MEPP frequency from each cell MEPP's were summed and averaged from the 50 sweeps to give an average MEPP frequency for 1 second.

Experimental Design and Data Analysis

Sampling patterns. In order to estimate the mean MEPP frequency from a population of muscle fibers, as many widely dispersed areas as possible were sampled. Typically, every 4th to 10th fiber across the muscle was impaled and its end-plate recorded, maintaining sampling patterns as consistently as possible between solutions and experiments. In order to minimize the effects of diffusion time to neuromuscular junctions, sampling was restricted to surface fibers only. This was especially critical in experiments measuring latencies of MEPP frequency changes, whereas preparations equilibrated for 50 min in a given solution displayed no difference in frequency between first and second cell layers. In experiments described in Figures 13 and 19 MEPP frequencies were recorded from 31 fibers in an attempt to provide an unbiased estimate of the true population mean, based upon the Central Limit Theorem. In practice mean MEPP frequencies were based upon 17 to 31 cells due to poor filmed records or inadequate singnal-to-noise ratios.

Calculation of mean MEPP frequency. The binomial distribution gives the probabilities that 0, 1, 2, ...n numbers of sample size n will possess some attribute. Rare events with low probabilities remain relatively skewed in the Binomial distribution whereas a Poisson distribution

of these events tends toward normality (Moroni, 1951; Steel and Torrie, 1960; Snedecor and Cochran, 1967). Since the probability of release of a packet of acetylcholine is low ($p \ll 1$), the occurrence of MEPP's has also been shown to follow a Poisson distribution (Gage and Hubbard, 1965; Katz, 1966). Since for a Poisson distribution the arithmetic mean gives an unbiased estimate of the population mean, the arithmetic mean can be considered the Maximum Likelihood Estimator of a Poisson distribution (based on calculations by Dr. Roy B. Deal, Jr.). Therefore, in order to better estimate the mean MEPP frequency of a population of cells in a given solution, arithmetic means of frequencies were compared.

Gage and Quastel (1966) transformed observed arithmetic means of MEPP frequencies into logarithmic form to compensate for the multiplicative effects of solution changes on MEPP frequency. In addition, statistical tests which assume normality have been used to compare geometric means of MEPP frequencies in different solutions (Gage and Quastel, 1966). Since arithmetic means do not compensate for the effects of solution changes and geometric means represent a biased estimate of a given population mean, neither of these methods is suitable for statistical analyses based on normal distributions. The square root transformation is more suitable for converting a Poisson distribution to a form which can be analyzed statistically (Steel and Torrie, 1960; Snedecor and Cochran, 1967). Analysis of some data described in Results were based on arithmetic, geometric and square root means of MEPP frequencies. Arithmetic means were used for purposes of graphical display requiring plotted estimates of population means of frequency. For the purpose of comparing the present work with previous reports, geometric means were also calculated.

For some statistical analyses arithmetic means were transformed to square roots. Each method of estimating the population mean MEPP frequency is based upon somewhat different assumptions, but each method provides different information about the distribution of MEPP frequencies. Therefore, the data in Results were analyzed by all three methods.

CHAPTER III

RESULTS

This research planned 1) to describe the effects of barium on MEPP frequency, 2) to determine the time necessary to reach new stable levels of frequency in barium, and 3) to describe the interaction between calcium and barium on MEPP frequency.

The Effects of Barium on MEPP Frequency

Random Analyses

The occurrence of transmitter release as MEPP's should always follow the Poisson Law for random events. Solutions containing 0, 9, and 16 mM Ca produce random release of transmitter while accelerating MEPP frequency (Gage and Hubbard, 1965). This is not true for strontium. At low Sr concentrations (1-5 mM) MEPP frequencies followed a Poisson distribution. At higher concentrations (7-10 mM), MEPP frequencies deviated from Poisson's Law (Dodge et al., 1969). In view of this non-random action of Sr at some concentrations, it was imperative to begin the study of the release of transmitter by barium with an analysis for randomness.

In its present application, Poisson's Law describes the probability of observing 0, 1, 2, 3, and more packets of acetylcholine released from a nerve terminal within a given interval of time (e.g., 1 second).

The probability of observing in 1 second the release of a given number of packets, k (where k = 0, 1, 2, 3, etc.) is given by the formula:

$$p(k) = e^{-m} \cdot \frac{m^k}{k!}$$

In this formula for testing random events, p is the probability of observing the release of k packets, m is the mean frequency of release for all samples over the given time interval.

Table 2 indicates the results of three separate tests for randomness of transmitter release in the same preparation. From 446 to 5265 filmed traces of the sweep of an oscilloscope beam were analyzed for the occurrence of 0, 1, 2, 3, and more MEPP's. The effect of solutions containing Ca alone, Ca with Ba, and Ba alone were studied. In all three solutions the observed MEPP frequencies corresponded to the expected MEPP frequencies predicted by the Poisson formula. For example, in 1.25 mM Ca a given interval was expected to have 1 MEPP 1545 times, while experimentally 1562 sample traces showed 1 MEPP. Since the null hypothesis assumes no difference between expected and observed MEPP's, in order to test whether observed MEPP frequencies differ significantly from expected frequencies, the X^2 test was used. A X^2 level of 5% or less would indicate a significant difference between expected and observed numbers of MEPP's. The calculated X^2 percentages listed in Table 2 ranged from 62-76%, showing no significant difference, and these levels fitted well within the X^2 levels found for various concentrations of Ca alone (45-80%, Gage and Hubbard, 1965). These data demonstrate that the random basis of MEPP discharge is not changed by the presence of Ba, whether Ca is present or not.

TABLE 2

TESTS OF GOODNESS OF FIT OF OBSERVED MEPP FREQUENCIES TO FREQUENCIES PREDICTED FROM POISSON'S LAW IN SOLUTIONS CONTAINING CALCIUM ALONE, CALCIUM WITH BARIUM AND BARIUM ALONE

	1.25 mM Ca					
	MEPP's per Interval					X^2
	0	1	2	3	4	
Expected No. of MEPP's	3294	1545	362	57	6	
Observed No. of MEPP's	3285	1562	351	62	5	76%

	1.25 mM Ca + 1.25 mM Ba								
	MEPP's per Interval								X^2
	0	1	2	3	4	5	6	7	
Expected No. of MEPP's	500	762	578	294	112	34	9	2	
Observed No. of MEPP's	477	777	601	298	99	32	9	1	62%

	1.25 mM Ba										
	MEPP's per Interval										X^2
	0	1	2	3	4	5	6	7	8	9	
Expected No. of MEPP's	26	74	105	100	71	40	19	8	3	1	
Observed No. of MEPP's	33	78	101	94	63	39	21	11	4	2	62%

Initial Observations

In order to compare most directly the effects of calcium and barium on MEPP frequency, equimolar amounts of each cation were studied (1.0 mM). The composition of each solution is listed in Table 1, row I (page 27). In either solution the only divalent cation present was Ca alone or Ba alone. Figure 2 illustrates the change in frequency with time in three preliminary experiments. Upon the introduction of 1.0 mM Ba, MEPP frequency recorded at a single end-plate increased (open triangles). This observation was consistent with previous reports of the accelerating action of barium. After 30 min in 1.0 mM Ba the electrode was displaced from the cell. In a second experiment a number of cells were sampled and recorded from 10 to 30 seconds each. Sampling was possible over a much longer period (90 min) by using such a population of cells. During the first 30 minutes, the multiple puncture experiment reproduced the rise in MEPP frequency observed at the single end-plate. However, after 30 minutes, there was a decline in MEPP frequency toward the control rate. In order to compress the two apparent phases of barium action, the inflow rate of solutions was doubled (6 ml per min or greater). The open circles of Figure 2 represent such an experiment. The initial increase in MEPP frequency occurs much earlier than with slower flow (about 3 ml per min) and is rapidly followed by a decrease in frequencies toward and below control rates, suggesting that Ba had a biphasic effect on MEPP frequency, with an initial increase followed by a gradual decrease from control rates.

Multiple Puncture Experiments

Immediately after the introduction of a Ca-free Ba solution, the

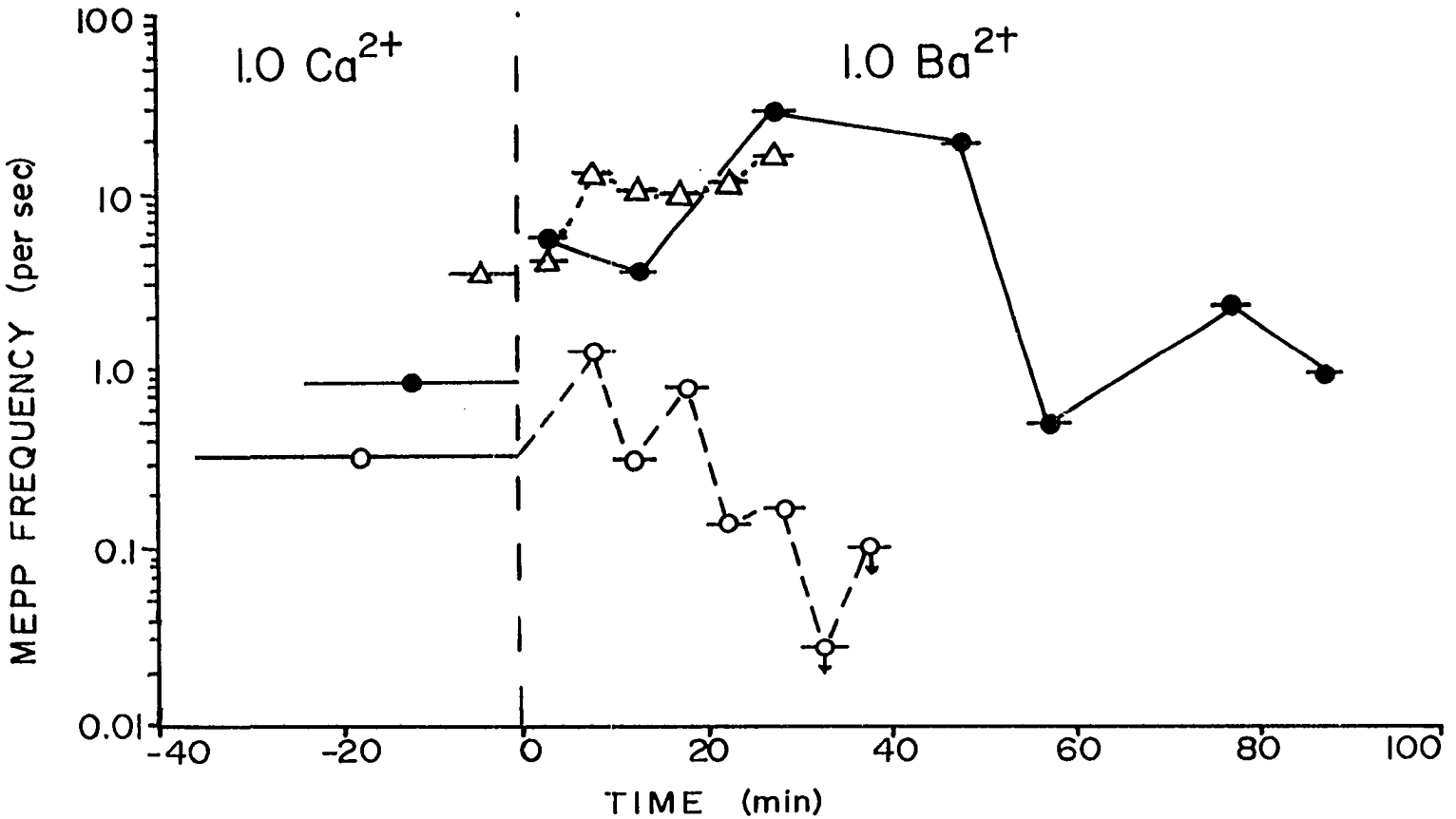


Figure 2. Comparison of the effects of 1.0 mM Ca with 1.0 mM Ba on MEPP frequency. Ordinate: MEPP frequency (per sec, log scale). Abscissa: time (min). Vertical dashed line indicates solution change. Open triangles (△) data based on single end-plate, using slow inflow rate (3 ml/min). Closed circles (●) represent multiple puncture experiment, same flow rate. Open circles (○) represent multiple puncture experiment, faster flow rate (6 ml/min). Temperature was 24-30°C. Arrows (↓) indicate true value is below that shown. Horizontal bars indicate measurement interval, usually 5 minutes.

diaphragm muscle began to fibrillate in almost all experiments. Such behavior has been observed previously (Feng, 1937b; Fatt and Ginsberg, 1958). Fibrillation continued for as little as 10 minutes in some cases to as long as two hours in others. It was impossible to maintain an electrode in place for more than a few seconds during this period. In an attempt to reduce this side effect, the concentration of Ba was halved. Thus MEPP frequency in 0.5 mM Ca was compared with that in 0.5 mM Ba in order to maintain equimolar concentrations of divalent cations. In spite of the reduced barium concentration, the muscle still fibrillated somewhat upon the introduction of Ba. Rather than reduce the barium concentration further, single cell recording was abandoned and multiple puncture sampling was used exclusively. Instead of sampling from the same end-plate for many minutes, samples of many individual cells were recorded as quickly as possible (see Methods, page 38). The temperature in these experiments was maintained between 30° and 33°C. Inflow rate ranged from 7 to 14 ml per min between experiments but was kept relatively constant within any one experiment. Figure 3 illustrates the results from six such experiments. The interval of time over which MEPP frequency was recorded is indicated below each of the four columns. During the first 20 minutes of exposure to barium, three of six experiments showed an initial rise in MEPP frequency above control rates. During the second 20 minutes (Ba, 21-40 min), all six experiments showed a decline in frequency from control. After 41 minutes in Ba, all six experiments were well below control rates (36-67% of control). When the Ca-control solution was reintroduced, two of five experiments showed a return to control frequencies after 40 minutes. Figure 4 illustrates five representative frames

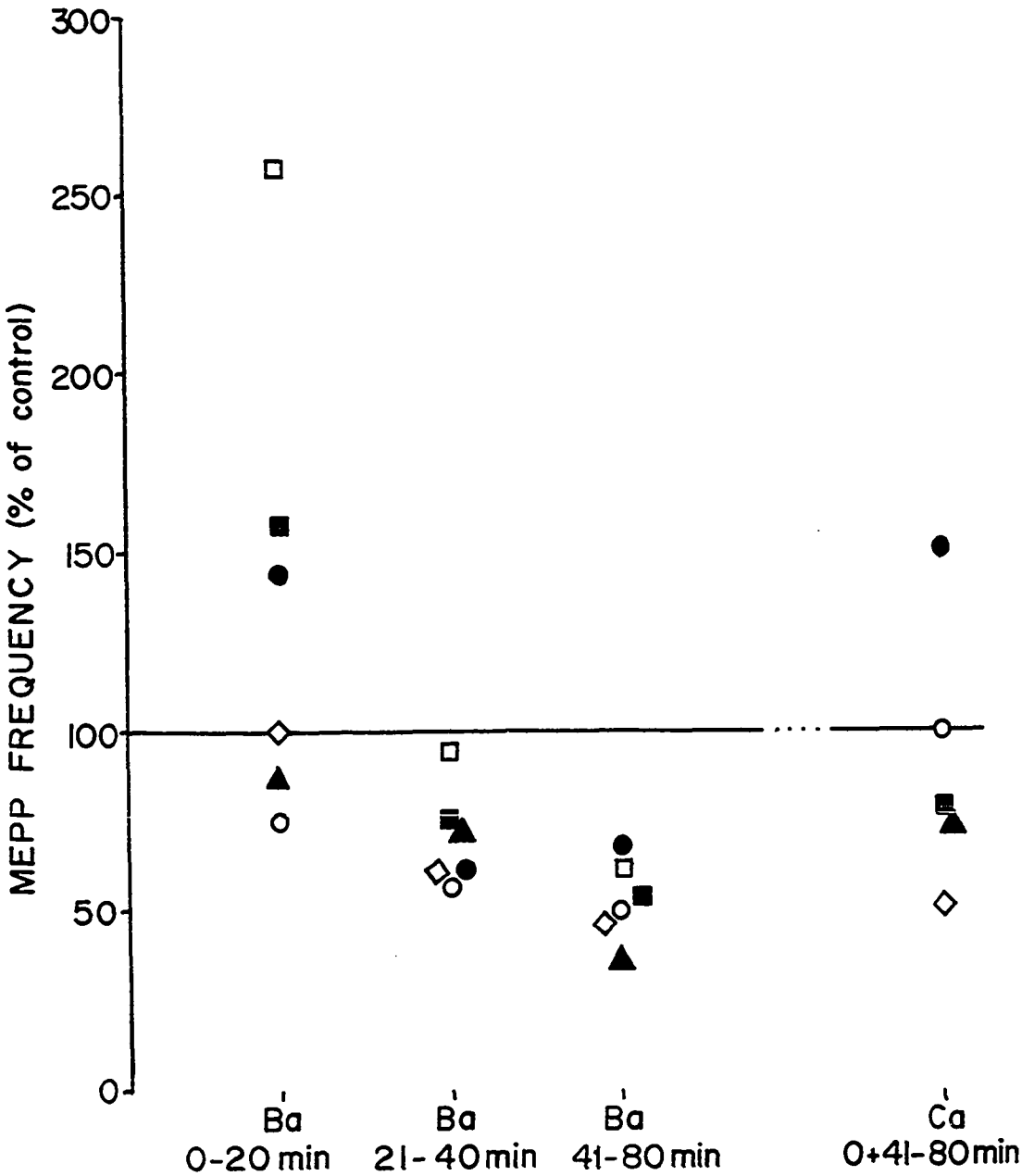


Figure 3. Change of MEPP frequencies by barium. Each symbol indicates a separate experiment. Ordinate: percent of control frequency. Abscissa is arranged in four columns: MEPP frequency during the interval 0 to 20 min after the introduction of Ba, 21-40 min after, 41-80 min after, and 41-80 min after the return to control solution (0.5 mM Ca). Filled triangles (▲) indicate 1.25 mM Ca or Ba. Break in 100% control bar indicates return to control solution. Temperature was 30-33°C

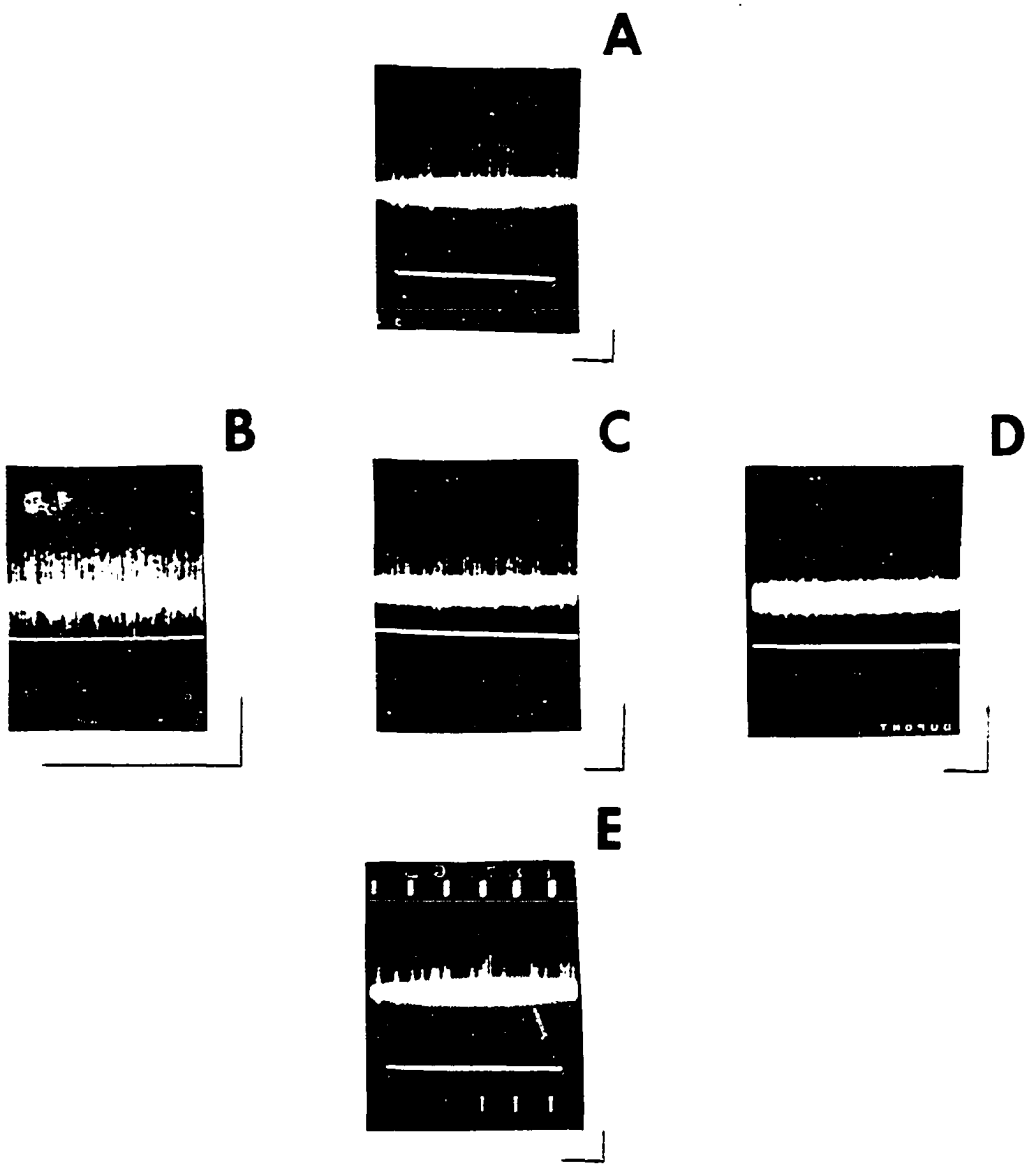


Figure 4. Sample photographs of oscilloscope recordings of MEPP's in barium. Same data as open squares in Figure 3. A. 0.5 mM Ca control, 1.7 MEPP's/sec; B. after 9 min in 0.5 mM Ba, 65 MEPP's/sec; C. after 19 min in Ba, 3.8 MEPP's/sec; D. after 64 min in Ba, 0.9 MEPP's/sec; E. 30 min after return to 0.5 mM Ca control, 3.1 MEPP's/sec. Calibrations: vertical 0.5 mV, horizontal 2 sec. Thin white bar indicates membrane potential relative to the top graticule line as zero potential. Vertical calibration of oscilloscope graticule is 10mV/division for membrane potential.

from one of the experiments described in Figure 3. These data confirmed the earlier observations that the action of Ba on MEPP frequency is biphasic. An initial transient increase in frequency is followed by a second phase during which MEPP frequency achieves a reduced and stable rate. This reduced phase of MEPP frequency, which is the more important phase for the purpose of this study, achieved stability after about 40 minutes. Therefore, 40-50 min was considered a sufficient equilibration time to observe the major action of barium at this temperature and flow rate.

Tests of significance of the decrease in MEPP frequency. The means of logarithms of MEPP frequencies (geometric means) tend to fit the normal curve better than the arithmetic mean frequencies in multiple puncture experiments (Gage and Quastel, 1966). However, statistical methods which assume normality cannot be applied to a distribution of geometric means, especially in cases where zero MEPP's were recorded (see Methods, page 39). Therefore, arithmetic means of MEPP frequencies were compared between Ca-control and Ba-containing solutions. Table 3 lists both the arithmetic ($\bar{f}_a$) and geometric ($\bar{f}_g$) means of MEPP frequencies in the six experiments shown in Figure 3. In each case the geometric means is less than the arithmetic mean, because means of logs are skewed toward lower values. The middle three sets of columns in Table 3 indicate mean frequencies in 1) Ca control, 2) Ba after 40 min and 3) 41 min after return to Ca control solutions. The column headed p_1 indicates the level of significance for each experiment based on the t-test of arithmetic means. One experiment compared 1.25 mM Ca with 1.25 mM Ba indicating that increasing the barium concentration did not confound the phenomenon.

MEPP frequency often fails to return to control rates as seen

TABLE 3

TESTS OF SIGNIFICANCE IN THE MEANS OF MEPP FREQUENCIES BETWEEN Ca (CONTROL) AND Ba (p_1) AND Ca (INTERPOLATED) AND Ba (p_2)

Expt. No.	MEPP Frequency in: Ca (control) $\bar{f}_g$	Ca (control) $\bar{f}_a$	Ba (0+41 - 80) $\bar{f}_g$	Ba (0+41 - 80) $\bar{f}_a$	Ca (return) $\bar{f}_g$	Ca (return) $\bar{f}_a$	p_1	p_2
1	2.21	2.32	1.14	1.56	2.18	2.25	< .05	< .05
2	1.76	2.02	1.18	1.38	2.65	3.37	< .05	< .05
3	1.90	2.04	1.16	1.39			< .05	
4	1.61	1.90	0.85	1.08	1.25	1.55	< .05	< .05
5	1.63	1.87	0.77	0.89	0.84	0.99	< .05	< .05
6	4.09	4.10	1.48	2.09	2.89	3.54	< .05	< .05

in three of five experiments in Figure 3. There are two possible explanations for this. First, barium may have slight irreversible effects on MEPP frequency. Second, the four to six hour period during which these experiments were performed could have witnessed a gradual deterioration of the tissue paralleled by a decline in MEPP frequency. A more thorough explanation of the failure of frequencies to return to control rates will be deferred until later. Briefly, the failure to return is due partly to a slight irreversibility in the effects of Ba and, in some cases, partly to gradual changes in frequency over a several hour period. If it were true that the reduced MEPP frequency is due to tissue deterioration, then could this not account for the major action of Ba on MEPP frequency? That is, perhaps the second phase of barium in reducing MEPP frequency is merely a reflection of the deterioration of the tissue.

To counter this argument, the initial control frequency was averaged with the return control frequency, and this rate was used as the experimental control. This method of averaging two control rates was called "interpolation." This tends to force the data to the limits of their significance. The column headed p_2 in Table 3 shows that when MEPP frequencies from the interpolated controls were tested against frequencies in barium, each case showed a difference at the 5% level.

These observations of the major action of barium in decreasing MEPP frequency below control rates contradict the reports of other investigators in the rat (Elmqvist and Feldman, 1965) and in the frog (del Castillo, referred to in Boyd and Martin, 1956). The burden of proof then remains on this investigation, so that every possible source of error must be examined and eliminated.

Depression of MEPP Amplitude in Barium

The representative photographs in Figure 4 illustrate that one of the side effects of barium is a reduction in the amplitude of miniature end-plate potentials. Frames 4B, C and D, representing samples of cells exposed to 0.5 mM Ba, were recorded at double the amplification of cells in control solutions, 4A and E. The effect of barium is especially apparent in frame 4C where MEPP amplitude is approximately half the amplitude in Ca control, frame 4A.

Figure 5 shows results obtained from measuring three parameters during the same experiment, MEPP frequency, MEPP amplitude, and the membrane potential of the muscle cell. This is the same preparation depicted in Figure 4. As already noted, the initial increase in MEPP frequency is followed by a gradual decline toward control rates. Frequency stabilizes below control rates after 30 to 40 minutes of exposure to Ba. A second effect of Ba is shown in Figure 5B. Immediately after the introduction of 0.5 mM Ba, MEPP amplitude decreases to about half of control heights. The most likely explanation of this reduction in amplitude is shown in Figure 5C. An immediate drop in membrane potential occurs in the presence of barium. Both membrane potential and MEPP's were monitored near the end-plate region of the muscle cell. Because the post-synaptic action of Ba on the muscle membrane potential is the cause of the reduction of MEPP amplitude, the MEPP amplitude depends directly upon the membrane potential of the muscle cell near the end-plate region.

Figure 5B shows that as MEPP amplitude decreases it approaches the level of the baseline noise. Some portion of MEPP's could be lost in this noise and not counted. Could the Ba-induced reduction in MEPP

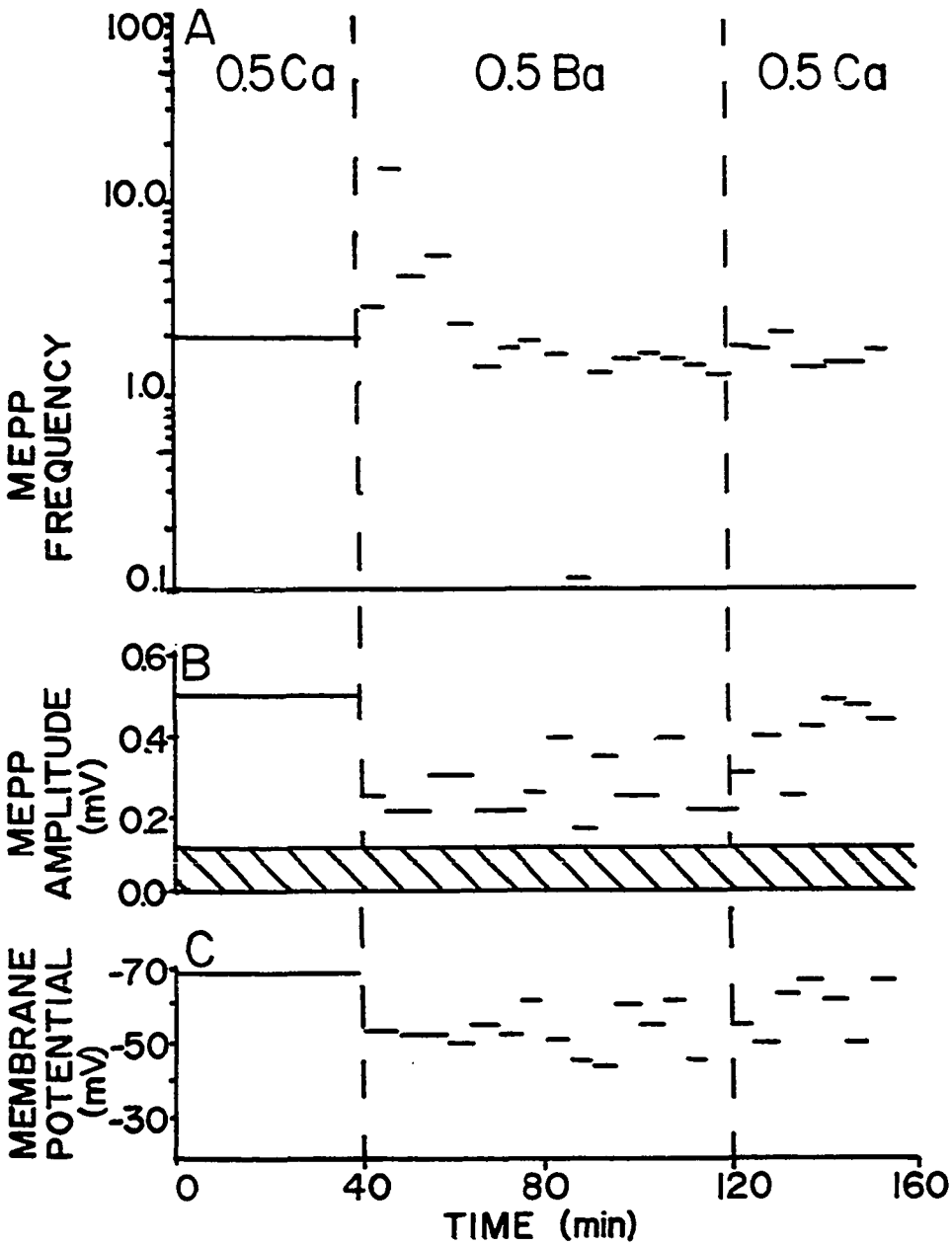


Figure 5. The effects of barium on MEPP frequency, MEPP amplitude and membrane potential in a single diaphragm. Ordinates: A. geometric mean MEPP frequency, logarithmic scale; B. mean MEPP amplitude in mV; C. muscle cell membrane potential in mV. Abscissa is time in minutes. Vertical dashed lines indicate changes in solution. Plotted horizontal lines represent interval of measurement (5 min except for control). Crossmarked bar in B indicates baseline noise.

frequency shown in Figure 5A merely reflect the loss of MEPP's in baseline noise due to the reduction in MEPP amplitude shown in 5B? A method had to be found to improve the signal-to-noise ratio of MEPP's in barium.

Attempts to Increase MEPP Amplitude

Until the distribution of MEPP's in Ba can be shown clearly to be above the noise level, it could be possible that the Ba-induced reduction in MEPP frequency may be secondary to depression of MEPP amplitudes. Three methods of increasing the signal-to-noise ratio were attempted: 1) anticholinesterase drugs, 2) reduction of temperature, and 3) selection of end-plates.

Anticholinesterase drugs. Anticholinesterases, especially physostigmine, facilitate neuromuscular transmission (Brown et al., 1936; Feng and Shen, 1937; Feng, 1937a; Feng, 1937b; Eccles et al., 1942; Fatt and Katz, 1952; Boyd and Martin, 1956a; Riker and Okamoto, 1969). Such facilitation is due to increased MEPP amplitude (Fatt and Katz, 1952; Boyd and Martin, 1956a; Riker and Okamoto, 1969). Since anticholinesterases prevent the destruction of acetylcholine, each packet of acetylcholine produces a greater and longer lasting depolarization of the muscle end-plate membrane. Thereby MEPP amplitude is increased. At 37°C MEPP amplitude in the rat diaphragm is doubled in the presence of 5×10^{-7} g/ml prostigmine, a synthetic anticholinesterase (Boyd and Martin, 1956a).

The method of choice to counteract the depression of MEPP amplitude by barium was the use of prostigmine. Four concentrations of prostigmine were used, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} g/ml on twelve rat diaphragm preparations. Because prostigmine has been shown to have a presynaptic action on MEPP frequency, both Ca-control and Ba-control solu-

tions were prepared with the prostigmine. Solutions were prepared according to the description in Table 1, row II (0.5 mM Ca or Ba). The main problem which plagued these experiments was spontaneous fibrillation of the diaphragm muscle. This effect of prostigmine on muscles has been described previously (Masland and Wigton, 1940; Eccles et al., 1942; Riker and Wescoe, 1946). The spontaneous contraction was especially severe at higher concentrations of prostigmine (10^{-6} to 10^{-8} g/ml), although it was still present in 10^{-9} g/ml. Out of the twelve experimental preparations tested, only 4 showed sufficient stability to permit intracellular recording with a microelectrode. These four experiments were done with the lowest concentration of prostigmine (10^{-9} g/ml). At this concentration MEPP amplitude did not increase. A single experiment using 10^{-9} g/ml physostigmine (eserine), a natural anticholinesterase, was similarly ineffective. Because of the high rate of experimental failure in these experiments and the many confounding side effects of prostigmine, the use of anticholinesterase drugs was abandoned as a means of improving the Ba-depressed MEPP amplitudes.

Reduced temperature. Cholinesterase is an enzyme, and, as with all other enzymes, its activity should be depressed by lowering the temperature. Decreased cholinesterase activity is believed to be the cause of the increased MEPP amplitude observed at low temperature (Boyd and Martin, 1956a). Therefore, low temperature can be used as an alternative "anticholinesterase" agent.

Figure 6 shows the results from three experiments conducted at 19° to 24°C comparing 0.5 mM Ca with 0.5 mM Ba. The initial Ba-induced increase in MEPP frequency observed at higher temperatures (Figure 3) is

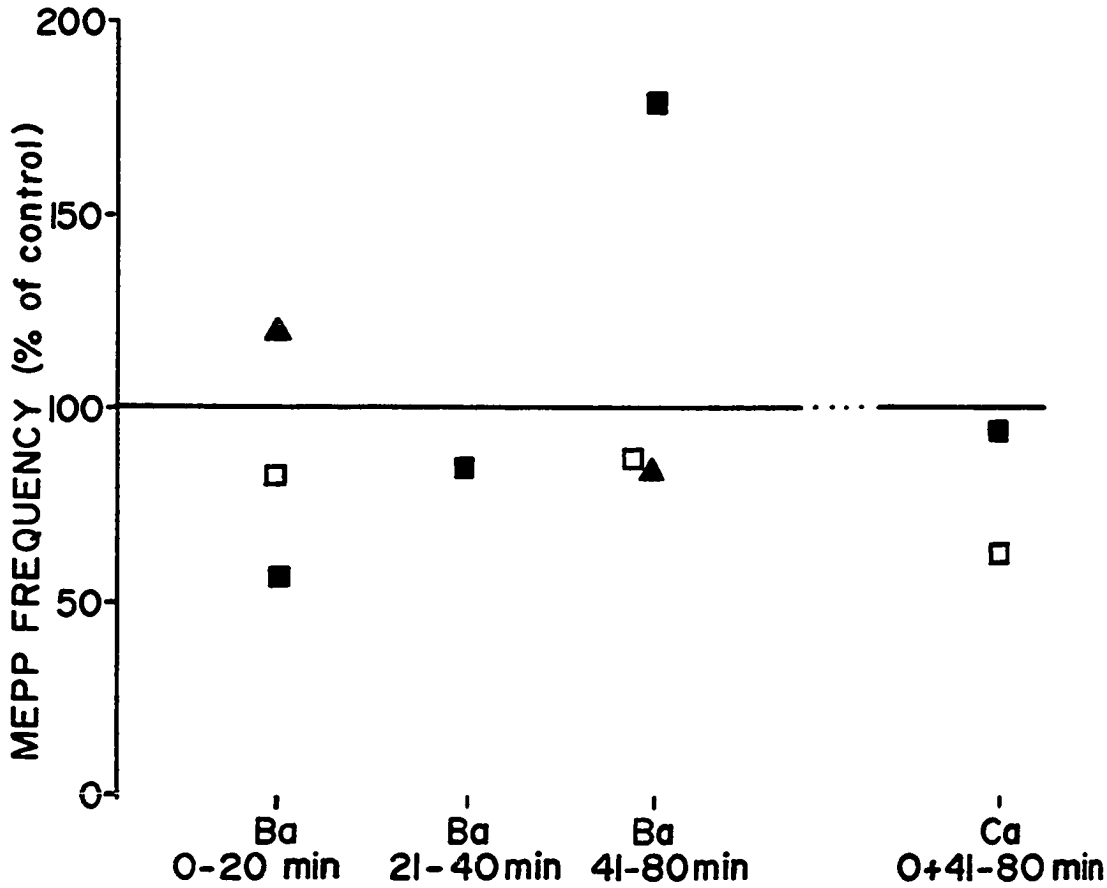


Figure 6. The effect of low temperature on the Ba-induced depression of MEPP frequency. Ordinate and abscissa are the same as in Figure 3. Each symbol represents a separate experiment. Temperature was 19-24°C. Solutions contained 0.5 mM Ca or Ba.

either less pronounced or missing. The reduction is seen during the 21-40 min period of exposure to Ba and stabilizes after 41 minutes to 78 and 84% of control rates (two experiments). In a third experiment represented by filled squares, there was another increase in MEPP frequency after 40 minutes. This event will be discussed later (p. 67). Figure 3 showed that at higher temperatures (30-33°C) the same amount of Ba reduced MEPP frequencies to 56% of control rates (mean of five experiments). The data indicate that at low temperatures, barium has a less pronounced effect on the initial rise in MEPP frequency and its subsequent decline below control rates. Thus low temperature, while improving slightly MEPP amplitude, tended to reduce both phases of the effect of Ba on MEPP frequency. While it may have been valuable to investigate further the temperature dependent actions of barium, this seemed to deviate from the main objective of this study, and further experiments at low temperature were not pursued.

Selection of end-plates - a workable method. A method for improving the signal-to-noise ratio of MEPP's in Ba remained to be found. Only when the mean MEPP amplitude is clearly away from the baseline noise of the oscilloscope sweep can it be firmly stated that Ba decreases MEPP frequency.

In reviewing photographic records of the experiments described in Figure 3, it was apparent that the mean MEPP amplitude in barium varied from cell to cell. The mean height of MEPP's compared with baseline noise could be classified subjectively into three categories. Some cells displayed extremely poor signal-to-noise ratios, and it was quite difficult to distinguish an individual MEPP from baseline noise. A second

class of cells showed a somewhat better distinction of MEPP's from noise; however, it was apparent that some MEPP's were still lost in the noise. A third group of Ba treated cells in each experiment showed a clear distribution of MEPP's well above the noise level.

From these three classes of cells, two test groups were formed. An "accepted" group consisted only of cells with a clear distribution of MEPP's well above baseline noise. A "rejected" group of cells encompassed cells with many MEPP's lost in the noise, as well as cells which showed only some MEPP's lost.

A comparison of "accepted" and "rejected" cells from experiments after 40 minutes exposure to Ba is shown in Table 4A. In experiments marked a, b, c, and d MEPP frequency in each case is higher in the accepted cells than in the rejected cells. Both high and low temperature ranges were considered. Two explanations arise for the higher frequency in accepted cells. Accepted cells may always show a higher frequency because few if any MEPP's are lost in baseline noise, whereas many are lost in rejected cells. This is the most probable explanation. On the other hand, it is possible that accepted cells which display a higher MEPP amplitude were not drawn from the same population as rejected cells. Population differences would arise from an inadequate diffusion of Ba to the neuromuscular junctions. Thus higher MEPP amplitudes and greater MEPP frequencies in these cells might reflect a poorly exchanging compartment such as a sub-surface fiber. However, only surface fibers were monitored in these experiments (see Methods). If the microelectrode moved down to the second cell layer, the cell was not sampled. In addition, the selection of filmed records of accepted and rejected cells was not made until

TABLE 4

A. COMPARISON OF MEPP FREQUENCIES AND MEMBRANE POTENTIALS OF ACCEPTED AND REJECTED CELLS AFTER 40 MINUTES IN BARIUM

Expt.	$[Ca^{2+}]$ or $[Ba^{2+}]$	Temp. Range °C	MEPP Frequency in Barium		Membrane Potential (mV)	
			Accepted (n)	Rejected (n)	Accepted	Rejected
a	0.5 mM	30-33	1.43 (10)	1.14 (8)	54.1	50.1
b	0.5	30-33	1.08 (6)	1.02 (23)	48.2	47.7
c	0.5	30-33	0.91 (7)	0.86 (42)	49.3	47.8
d	0.5	19-24	1.62 (29)	1.49 (7)	54.9	52.1
e	1.25	30-33	2.09 (12)		42.0	

B. COMPARISON OF MEPP FREQUENCIES OF ACCEPTED CELLS IN BARIUM WITH CALCIUM CONTROL FREQUENCIES

Expt.	MEPP Frequency			P_1
	Ca (Control) (n)	Ba (Accepted) (n)	Ca (Return) (n)	
a	2.04 (22)	1.43 (10)		< .05
b	1.90 (28)	1.08 (6)	1.55 (14)	= .05
c	1.87 (23)	0.91 (7)	0.99 (23)	< .05
d	4.10 (14)	2.09 (17)	3.54 (21)	< .05

after 41 min exposure to barium. Finally, barium in fact did diffuse to the neuromuscular junctions of accepted cells as shown by reduced membrane potentials (last column of Table 4A). Comparison of accepted cell membrane potentials with those of rejected cells shows no difference in any experiment at the 5% level of significance. In fact herein lies the probable cause for the difference in MEPP amplitude between accepted and rejected cells. In each case, membrane potentials of accepted cells are larger (though not significantly so) than the membrane potentials of rejected cells. MEPP amplitude reflects muscle membrane potential. A normal population of cells has a membrane potential range of 10-15 mV. Therefore, a method which selects cells with higher amplitude MEPP's also selects cells with a normally slightly greater membrane potential. Selection of cells with a postsynaptic bias should not confound the data, since only the presynaptic effects of Ba are being studied.

Table 4A compares MEPP frequencies of accepted cells exposed for 40 min in barium with the initial Ca control frequencies. There was a decrease in MEPP frequency in the experiments shown ($p \leq .05$). The moderate irreversibility of barium is shown by the lack of complete recovery in the "Ca (Return)" column.

Table 4A, Experiment e, lists an accepted MEPP frequency of 2.09 MEPPS/sec after 40 min exposure to 1.25 mM Ba. This experiment was designed so that the only cells sampled and recorded were those showing an "accepted" MEPP amplitude. Table 4B indicates that this experiment also showed a significant Ba-depressed effect on MEPP frequency. In the experiment testing this new cell selection method in 1.25 mM Ba, amplitude of all MEPP's after 40 min were measured and plotted as a histogram

in Figure 7. The mean amplitude of the major distribution of MEPP's is approximately 0.3 mV. These amplitudes represent a population of cells rather than a single cell. Therefore the distribution does not quite fit a normal distribution curve. Nevertheless, it is clear that few if any MEPP's were lost in the baseline noise by this method.

This cell selection method was used for all the Ca-Ba interaction experiments to be described subsequently. These experiments also incorporated a band pass filter in the amplifier which further enhanced the signal-to-noise ratio.

Depression of Membrane Potential in Barium

Membrane potential of muscle cells monitored near the end-plate region decreased in the presence of barium in every case. This membrane depolarizing effect of barium has been reported in barnacle and frog striated muscle, chick cardiac muscle cells, frog peripheral nerve and cockroach giant axons (Hagiwara and Naka, 1964; Sperelakis et al., 1967; Josse et al., 1965; Sperelakis and Lehmkuhl, 1966; Pappano and Sperelakis, 1969; Lorente de Nó and Feng, 1946; Narahashi, 1961).

Figure 8 illustrates the depolarizing effect of 0.5 and 1.25 mM Ba in seven preparations. The drop in membrane potential occurs rapidly (0-20 min period) and changes little during the next 60 min. Muscle membrane potential does not always return to control levels upon return to Ca solution. A gradual deterioration of the tissue may be the first factor contributing to this failure of return to control potential differences. A control experiment indicated that after 4-1/2 hours in Ca solution under similar conditions of temperature and flow rate there was a 5-9% decrease of the mean membrane potential. In the series of experi-

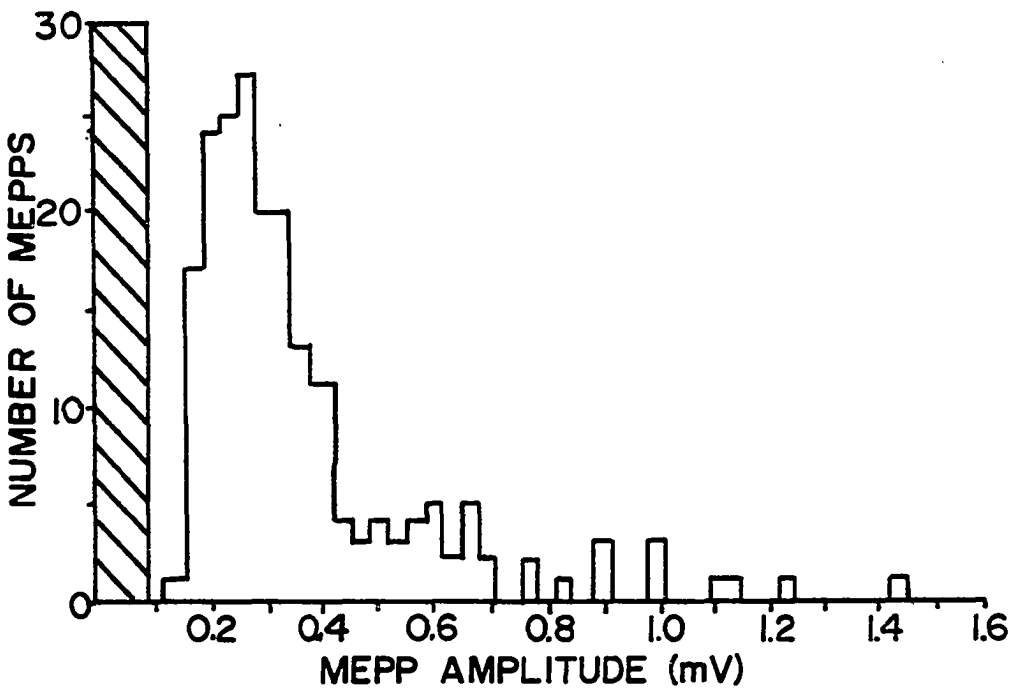


Figure 7. Histogram of MEPP amplitudes from a population of cells after 40 min in 1.25 mM Ba. The first 10 MEPP's recorded from each cell were measured and plotted. Ordinate: number of MEPP's at each amplitude. Abscissa: MEPP amplitude in classes of 0.03 mV. Cross-marked area indicates baseline noise.

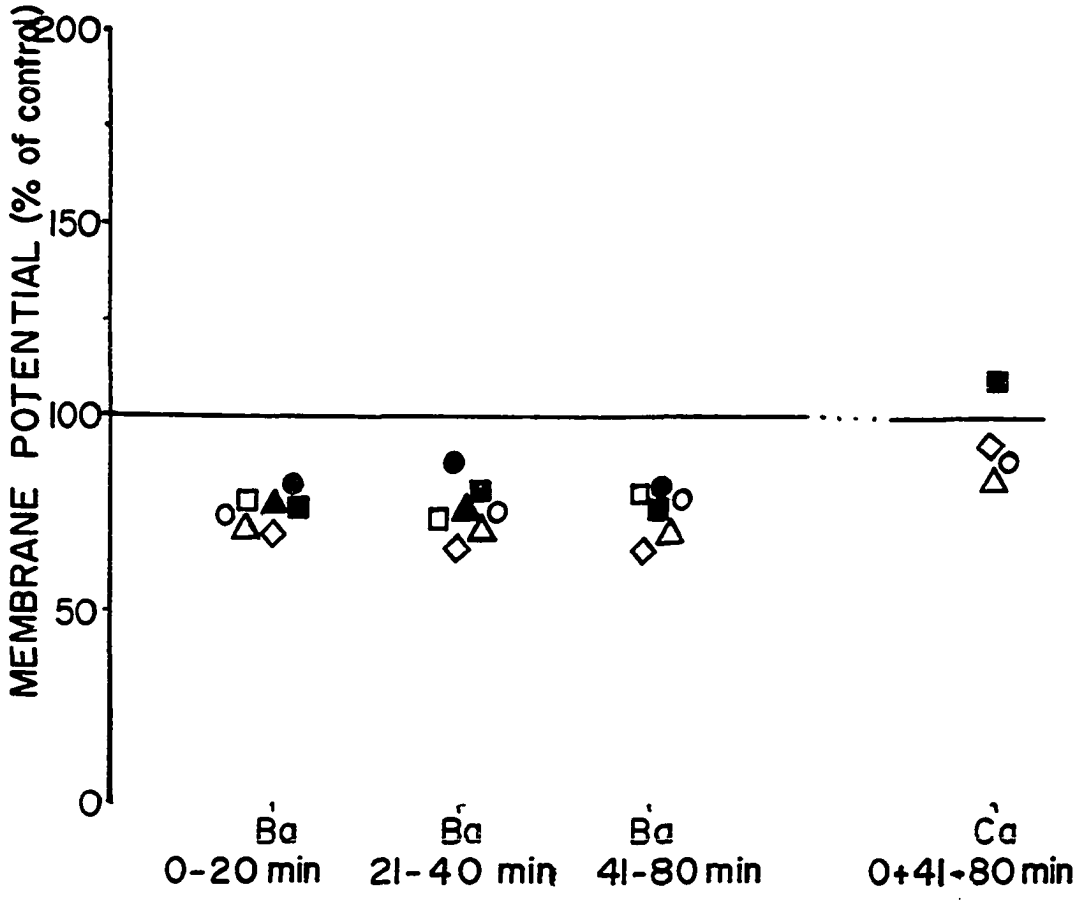


Figure 8. The depolarizing effect of barium on muscle membrane potential. Abscissa: 4 sampling periods, same intervals as in Figure 3. Ordinate: percent of control membrane potential of muscle cells. Each symbol represents a separate experiment. Open diamonds (◇) indicate 1.25 mM Ca or Ba. All others represent 0.5 mM. Open (○) and filled (●) circles indicate experiments at 19-24°C. All others at 30-33°C.

ments under discussion, 5-6 hours was approximately the total experiment time from dissection of the diaphragm until the last sample of data was taken. A second factor may be damage to the muscle membrane arising from repeated puncture by a microelectrode. A third factor, which cannot be resolved, is the possibility of some irreversibility of the membrane potential after exposure to barium.

Figure 8 demonstrates that after exposure to 0.5 mM Ba for 40 min, membrane potential decreased to 73% of control (mean of 3 experiments) at 30-33°C. The mean membrane potential of a preparation treated with 1.25 mM Ba at the same temperature dropped to 68% of control. At 19-24°C, 0.5 mM Ba decreased membrane potential only to 81% of control (mean of two experiments). All these decreases in membrane potential are significant at the 5% level. These experiments suggest that higher temperature and a greater Ba concentration tend to enhance the membrane depolarizing action of barium.

Figure 6 showed that the effect of Ba on the reduction of MEPP frequency was less pronounced at low temperatures. Table 5 lists the differential effects of two temperature ranges on the Ba-induced reductions in MEPP frequency and membrane potential. As mentioned previously, a decrease in temperature produces an increase in MEPP amplitude. Thus low temperature unselected MEPP's are comparable with high temperature "accepted" MEPP's. Table 5 lists the percent of control frequencies in three experiments at 30-33°C and 4 other experiments at 19-24°C. Although the number of experiments is inadequate for an analysis of variance, it is obvious the 0.5 mM Ba is less effective in reducing MEPP frequency at lower temperatures than at higher temperatures. The differential effects

TABLE 5

COMPARISON OF TWO TEMPERATURE RANGES ON MEPP FREQUENCY AND MEMBRANE POTENTIAL 41 TO 80 MINUTES AFTER THE INTRODUCTION OF 0.5 mM BARIUM

MEPP Frequency (Percent of Control)		Membrane Potential (Percent of Control)	
30-33°C (Accepted)	19-24°C	30-33°C	19-24°C
73	85	78	84
66	87	75	78
58	178	65	
	95		

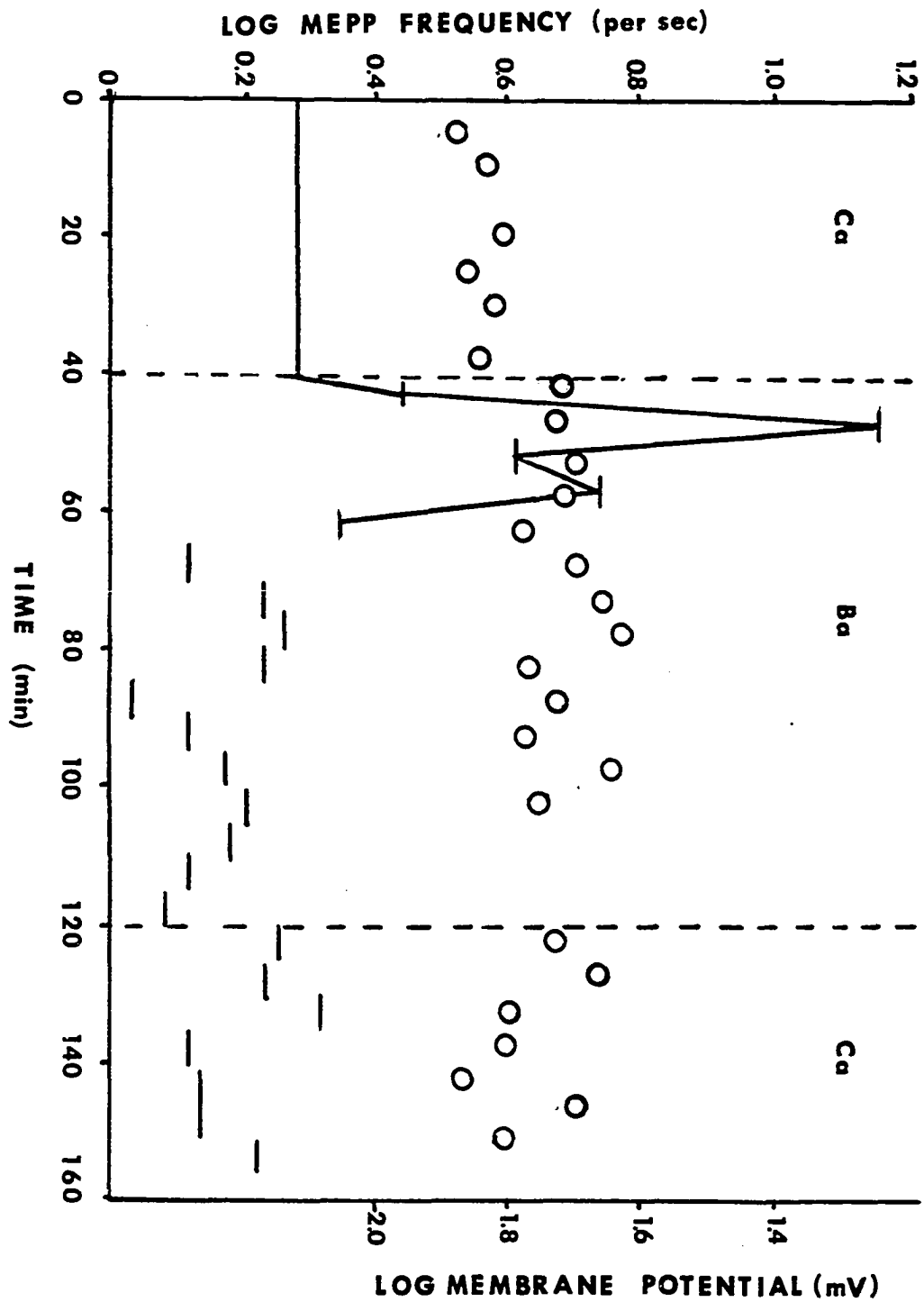
of temperature on membrane potential are also apparent in Table 5. The membrane potential drop at 19-24°C appears less than at 30-33°C, although more experiments need to be performed before any firm conclusions can be drawn from the data. If Ba is less effective at low temperature at two different sites, it is possible that Ba is less ionized and therefore in a lower effective concentration at low temperature. This will be considered further in the Discussion.

The Basis for the Biphasic Action of Barium

It is interesting that the initial action of barium to increase MEPP frequency occurs during the same period (0-20 min) as the membrane depolarizing action of barium. Figure 9 illustrates the membrane potentials and mean MEPP frequencies monitored during one experiment comparing 0.5 mM Ca with 0.5 mM Ba. Within the first 5 min of exposure to barium, the muscle membrane depolarizes (open circles, upward direction) to approximately 78% of control. Simultaneous with the depolarization of the membrane, MEPP frequency increases from 1.8 to 2.8 per sec (horizontal bars), and continues to increase to 14 per sec during the next 5 min. Membrane potential appears to plateau after 5 min. After 10 min, MEPP frequency has declined to 4.1 per sec and continues to drop. Forty minutes after the introduction of Ba, the membrane remains depolarized, and MEPP frequency is well below control rates.

It is likely that the Ba-induced depolarization of the muscle membrane represents a similar phenomenon on the nerve terminal membrane. If this were true, then the rapid depolarization of the nerve terminal membrane by Ba is responsible for a rapid release of transmitter monitored as an increase in MEPP frequency. Ten minutes after this initial

Figure 9. The relationship between the effects of barium on membrane depolarization and MEPP frequency. Data represent a single experiment, also plotted in Figure 5. Left ordinate: log MEPP frequency. Right ordinate: log membrane potential. Note reverse directions of increase in the two ordinate scales. Abscissa: time in minutes. Dashed vertical lines indicate solution changes. Open circles (O) indicate means of 5 minute intervals of membrane potential. Horizontal bars indicate mean of MEPP frequency measured for that interval, usually 5 minutes.


LOG MEPP FREQUENCY (per sec)
0
0.2
0.4
0.6
0.8
1.0
1.2
TIME (min)
0
20
40
60
80
100
120
140
160
Ca
Ba
Ca
2.0
1.8
1.6
1.4
LOG MEMBRANE POTENTIAL (mV)

rise, the membrane potential has stabilized at a new level and MEPP frequency declines. For this study the initial increase in MEPP frequency is merely a side effect of Ba, since Ba is acting at a secondary site. The more important site of Ba action, the potential site of interaction with Ca, is the one which results in a depressed MEPP frequency.

On the basis of these data, we conclude that the increase in MEPP frequency in Ba, which has been observed by others and confirmed here, is a transient phenomenon caused by depolarization of the nerve terminal membrane. This is an indirect action of Ba on a secondary site. However, the direct action of Ba is to decrease MEPP frequency below control rates. Thus our first two objectives have been achieved. Barium produces a decrease in MEPP frequency, and this new rate achieves stability after 40 min. It remains to be shown where Ba acts in decreasing MEPP frequency -- as an inhibitor to Ca at the same or at a different binding site.

Calcium - Barium Interactions

The Period of Equilibration

The results described thus far indicate that the direct action of barium on MEPP frequency is to reduce it below control rates. This reduction of MEPP frequency reaches stability 40 to 50 min after irrigation with barium begins. Consequently, 50 min were allowed for equilibration before sampling commenced in experiments that assumed the effect of Ba on MEPP frequency had reached a steady state.

A long latency rise in MEPP frequency. In general, after 50 min in Ba MEPP frequency is constant at its reduced rate. However, in

Figure 6 there was one experiment in which MEPP frequency rose again to 178% of control after irrigation with Ba for 40 minutes. Figure 10 illustrates the results from four experiments displaying a similar phenomenon. Notice that the initial interval (0-20 min) has been eliminated from this figure, and an additional interval (81-120 min) has been added. Figure 10 shows that in four experiments MEPP frequency rose above control during the 81-120 min period after the usual decline in rate. In one experiment this second rise occurred somewhat earlier, during the 41-80 min period. MEPP frequency returns to slightly less than control rates when Ca is reintroduced. The phenomenon is independent of temperature, since experiments conducted at both temperature ranges display the frequency increase approximately equally. The long-latency rise in MEPP frequency is observed in 0.5 and 1.25 mM Ba.

It is noteworthy that the second increase occurred only once within the first 80 min in Ba. This indicates either that the site of action for Ba which leads to the second rate increase is highly inaccessible to Ba or that the site is readily accessible to Ba, but the series of reactions leading to the frequency increase is limited by a slow rate constant.

Therefore, Ba displays three phases in its effect on MEPP frequency. An immediate transient increase followed by a gradual decrease to a stable level below control. After one hour or longer, MEPP frequency increases once again -- a third effect.

To study and describe the many effects of barium at all its various sites of action would deter the major objective - to determine whether Ca and Ba interact at a common binding site. However, if this

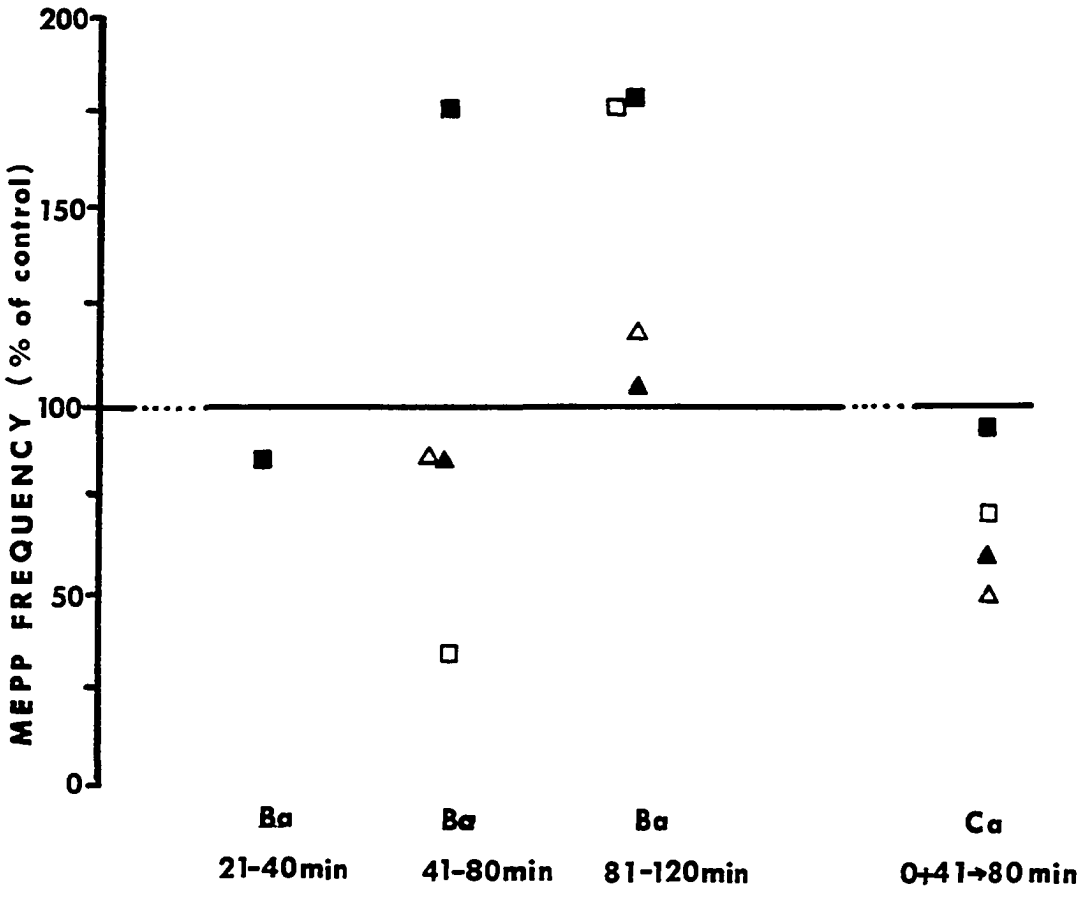


Figure 10. Increase in MEPP frequency during a long latency phase. Ordinate: MEPP frequency, percent of control. Abscissa: four columns representing intervals of time similar to Figure 3. Note that the first interval (0-20 min) has been eliminated (dotted portion of control bar), and another interval has been added (81-120 min). Filled symbols represent experiments at 20°C; open symbols, at 30-33°C. Open squares (□) represent 1.25 mM Ba. All others with 0.5 mM Ba.

third phase of Ba action were to occur during the presumed equilibration period, it could easily confound any data obtained during this period.

Regression analysis of MEPP frequencies during the equilibration period. The third phase of the Ba-induced changes in MEPP frequency was observed when Ba was the only divalent cation in the bathing solution. In the design of experiments testing Ca-Ba interaction, Ca was always present with Ba as a second divalent cation (see Table 1, rows III, IV).

It was imperative therefore to study the changes in MEPP frequency during the interval 50-120 min after the introduction of Ba with Ca. Three experiments containing 1.25 mM Ba and 1.25 mM Ca were conducted. A regression analysis of variance of the changes in MEPP frequency during the 50-120 min interval showed that there was no significant change in MEPP frequency during this time ($p > .01$, slope [b] = .002 MEPP's/min). Based upon the regression analysis of these 3 experiments, it can be stated that after the 50 min equilibration period in Ba with Ca MEPP frequency is truly at a steady state. The causes for the elimination of the third phase by adding Ca to Ba as a supporting cation would be interesting to investigate. However, since this was not germane to the present study, further experimentation in this direction was not attempted.

Experiments to Study Calcium - Barium Interaction

Design of the experiments. It has been shown that MEPP frequency depends upon the concentration of Ca to concentrations as high as 10 mM Ca (Hubbard et al., 1968a). The present results have shown that when Ca is the only divalent cation in solution, a minimum of 0.5 to 1.0 mM is necessary in order to prevent spontaneous contraction (see Methods).

Very seldom was spontaneous contraction observed in solutions with 1.0 mM Ca. Therefore, the range of Ca concentrations was limited to 1.0 to 10 mM Ca. It has been found that MEPP frequency changes with the log of Ca concentration (Hubbard et al., 1968a). Therefore, in selecting Ca concentrations from 1.0 to 10 mM, a change of concentration by a constant multiple is most desirable. The four Ca concentrations chosen were 1.25, 2.50, 5.0, and 10 mM Ca. To test Ca-Ba interaction, a single concentration of Ba was chosen, 1.25 mM. By observing MEPP frequency in various concentrations of Ca with and without Ba, it should be possible to determine whether Ba competes with Ca for a common binding site on the nerve terminal membrane.

Figure 11 illustrates an experiment recording MEPP frequency in the presence of 10 mM Ca with and without 1.25 mM Ba. Note that before and after each experimental variable (10 Ca or 10 Ca + Ba), MEPP frequency was measured in 1.25 mM Ca. Frequency measured in 1.25 mM Ca served as a control within a given experiment. All experiments used the same control concentration of Ca, and therefore MEPP frequencies over four concentrations of calcium from all experiments could be compared. The first samples were taken after a minimum equilibration period of 50 minutes in the new solution. End-plates were randomly sampled until MEPP's from 31 cells had been recorded (see Methods for a more complete discussion of sampling technique).

Figure 12 shows five representative film records from the experiment shown in Figure 11. Notice that MEPP's are clearly above baseline noise in sample D by using the end-plate selection method.

Variations of MEPP frequency with time. Maintaining a rat

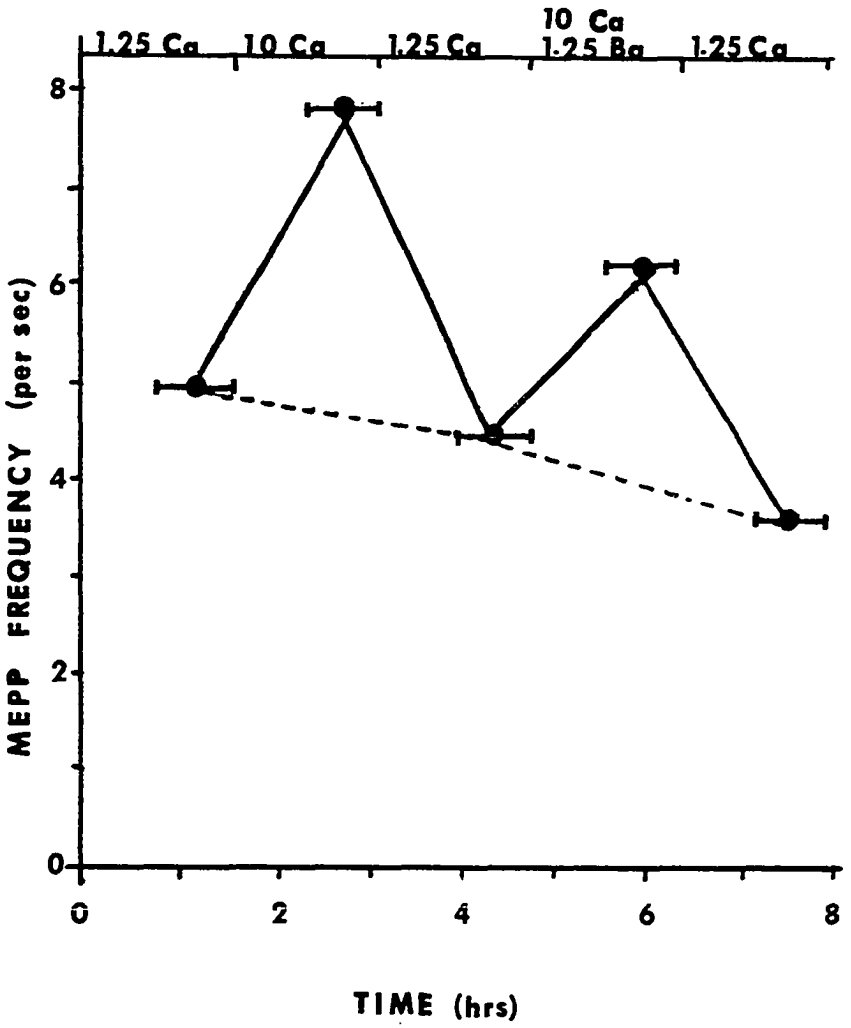


Figure 11. Sample experiment comparing the effects of Ca with and without Ba. Ordinate: MEPP frequency (per sec). Lower Abscissa: time in hours. Upper abscissa: interval of time during irrigation with the indicated solution. Filled circles (●) represent arithmetic means of MEPP frequencies recorded during the period indicated by each horizontal bar. Dashed lines represent assumed linear drift in control frequency, for the purpose of interpolation.

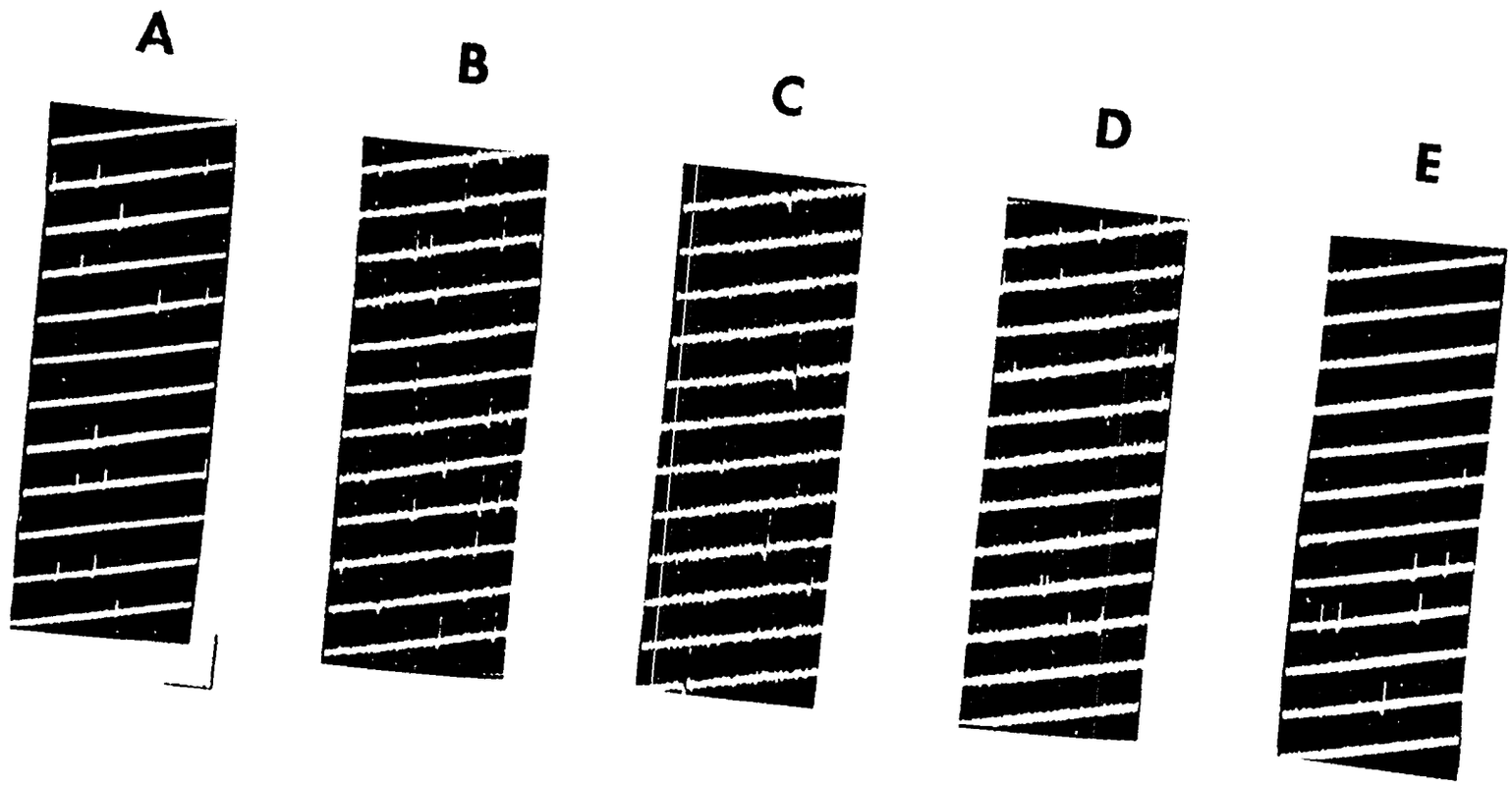


Figure 12. Film records indicating MEPP's recorded at five different end-plates. Each record represents one of the five intervals shown in Figure 11. Calibrations: vertical 1.0 mV, horizontal 50 msec, the same for all records. A. initial 1.25 mM Ca control, 5.6 MEPP's/sec; B. 10 mM Ca, 11 MEPP's/sec; C. intermediate 1.25 mM Ca control, 2.5 MEPP's/sec; D. 10 mM Ca + 1.25 mM Ba, 6.2 MEPP's/sec; E. final 1.25 mM Ca control, 3.7 MEPP's/sec.

diaphragm *in vitro* for several hours results in oscillations of MEPP frequency and a gradual decline in membrane potential. Control MEPP frequency in 1.25 mM Ca gradually declines before and after irrigation with 10 mM Ca (Figure 11). This decline in control frequency also follows irrigation with 10 mM Ca + 1.25 mM Ba. It was possible, however, that irrigation with variable solutions between control samplings could have been responsible for the apparent reduction in MEPP frequency.

In order to study the effect of time as the only variable on MEPP frequency, a control experiment was devised. End-plates from the extreme ventral and extreme dorsal regions of a hemidiaphragm preparation were monitored during seven hours of irrigation with 1.25 mM Ca control solution. Every hour MEPP frequency was recorded from 10 cells from each region. It was found that during seven hours of irrigation with 1.25 mM Ca the mean MEPP frequency did not change in either region. However, MEPP frequency tended to oscillate from hour to hour from 2.00 to 3.60 MEPP's/sec. Since the seven hour mean frequency from one region was 2.96 MEPP's/sec, it was possible that hour to hour oscillations would account for a 32% change in control frequency.

It was obvious, then, that some compensation for hour to hour drift in frequency had to be made. An interpolation method was devised. Assuming that the hour to hour drift in MEPP frequency was linear, the two controls of an experiment were averaged (see dotted lines in Fig. 11). The average of the two controls was considered the more accurate control mean. Thus, using the interpolation technique, a better estimate of the effect of Ca or Ca plus Ba on MEPP frequency could be obtained.

As explained in Methods, MEPP frequencies were calculated three

ways, geometric and arithmetic means and the means of square roots. While the interpretation of the results was the same, each method is based upon a different set of assumptions.

Geometric means of MEPP frequencies. Previous investigators have used geometric means of MEPP frequencies because means of logs fit a normal distribution somewhat better than arithmetic means (Gage and Quastel, 1966; Hubbard et al., 1968a). The means of the square roots of MEPP frequencies follow a normal distribution, whereas geometric means tend to give a skewed distribution (see Methods). However, for purposes of direct comparison of this work with previous investigations, the geometric means will be presented first. Figure 13 illustrates the results from 10 diaphragms comparing MEPP frequency in four Ca concentrations with and without Ba. In order to have a common basis for comparison between diaphragms, only the absolute increases in frequency were considered. Each absolute difference from control in an individual experiment was then added to the mean of all controls in all experiments (3.69 MEPP's/sec - horizontal bar, Fig. 13). For example, in the experiment described by Figure 11, the interpolated control MEPP frequency (4.78) was subtracted from the MEPP frequency observed in 10 mM Ca (7.92 MEPP's/sec). This absolute difference in frequency (3.14 MEPP's/sec) was added to the mean of all control means (3.69/sec), and the new corrected frequency (6.83 MEPP's/sec) was plotted as in Figure 13. A similar correction was made for the MEPP frequency in 10 mM Ca + 1.25 mM Ba. In this way the data from all 10 diaphragms are collected and plotted on a single more meaningful graph.

The data shown in Figure 13 confirm previous observations that

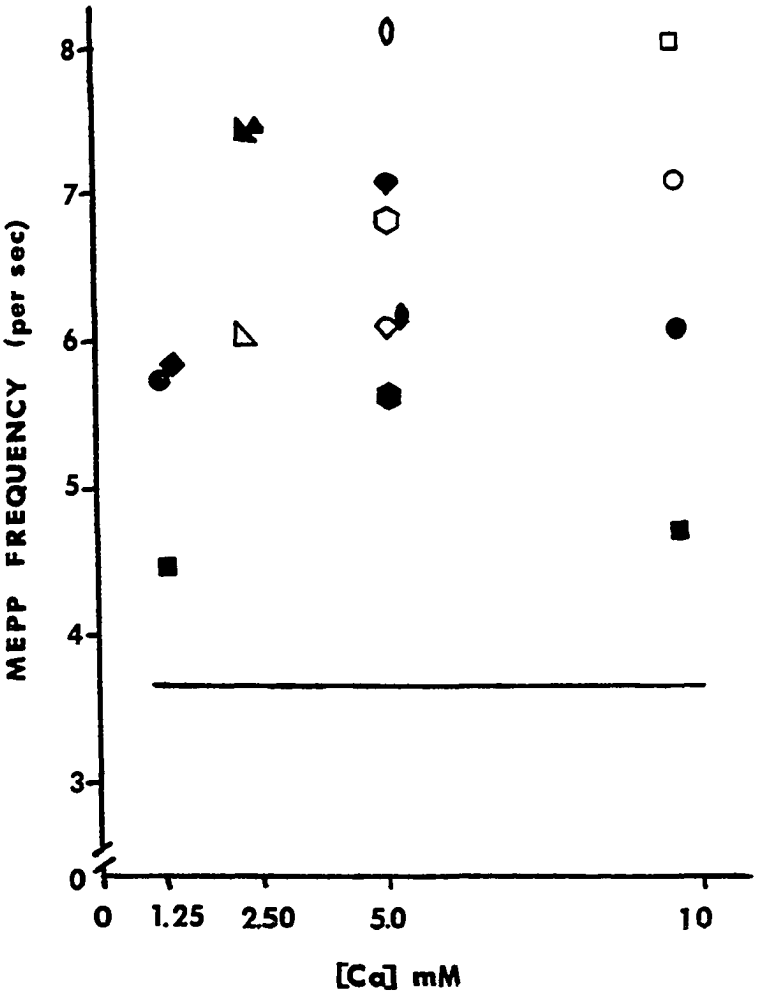


Figure 13. Geometric means of MEPP frequencies in four concentrations of Ca with and without Ba. Ordinate: MEPP frequency (per sec). Abscissa: Ca concentration (mM). Each plotted point represents a geometric mean frequency in 17-31 cells. Open symbols for Ca alone; filled symbols for Ca plus Ba. Each symbol type represents a separate experiment similar to Figure 11. Horizontal bar indicates mean control MEPP frequency of all 10 diaphragms, used as common comparison for all changes (see text).

MEPP frequency is directly related to Ca concentration. Moreover, MEPP frequency in 1.25 mM Ca was increased by the addition of 1.25 mM Ba in all three experiments. Two experiments at 2.50 mM Ca showed an increase in MEPP frequency when 1.25 mM Ba was added. When diaphragms were irrigated with 5.0 mM Ca, the effect of Ba was less clear. In one diaphragm MEPP frequency increased with the addition of 1.25 mM Ba, whereas in two other experiments in 5.0 mM Ca MEPP frequency decreased when Ba was added. This suggestion of a reversal in the effect of Ba was confirmed in 10 mM Ca, where in both diaphragms MEPP frequency decreased when 1.25 mM Ba was added. Therefore, at low concentrations of Ca, 1.25 mM Ba increases MEPP frequency, considering the distribution of geometric means. At higher Ca concentrations, however, a reversal occurs and MEPP frequency decreases when Ba is added.

Arithmetic means of MEPP frequencies. It may be shown by elementary statistical theory (see Methods) that the arithmetic mean is an unbiased estimate of the population mean of a Poisson distribution. Note that the geometric mean used in the above discussion does not have this desirable property. Therefore, by using arithmetic means of MEPP frequencies one can pool the results from the two to three diaphragms at each concentration to achieve a mean of means. Figure 14A shows the pooled arithmetic means of all experiments. These pooled means have been fitted to a common graph by the method described for Figure 13. In addition, in any single experiment the controls preceding and following the variable Ca solution were interpolated, as for Figure 13. The relationship between MEPP frequency and Ca with and without Ba was the same whether geometric of arithmetic means of frequencies were considered.

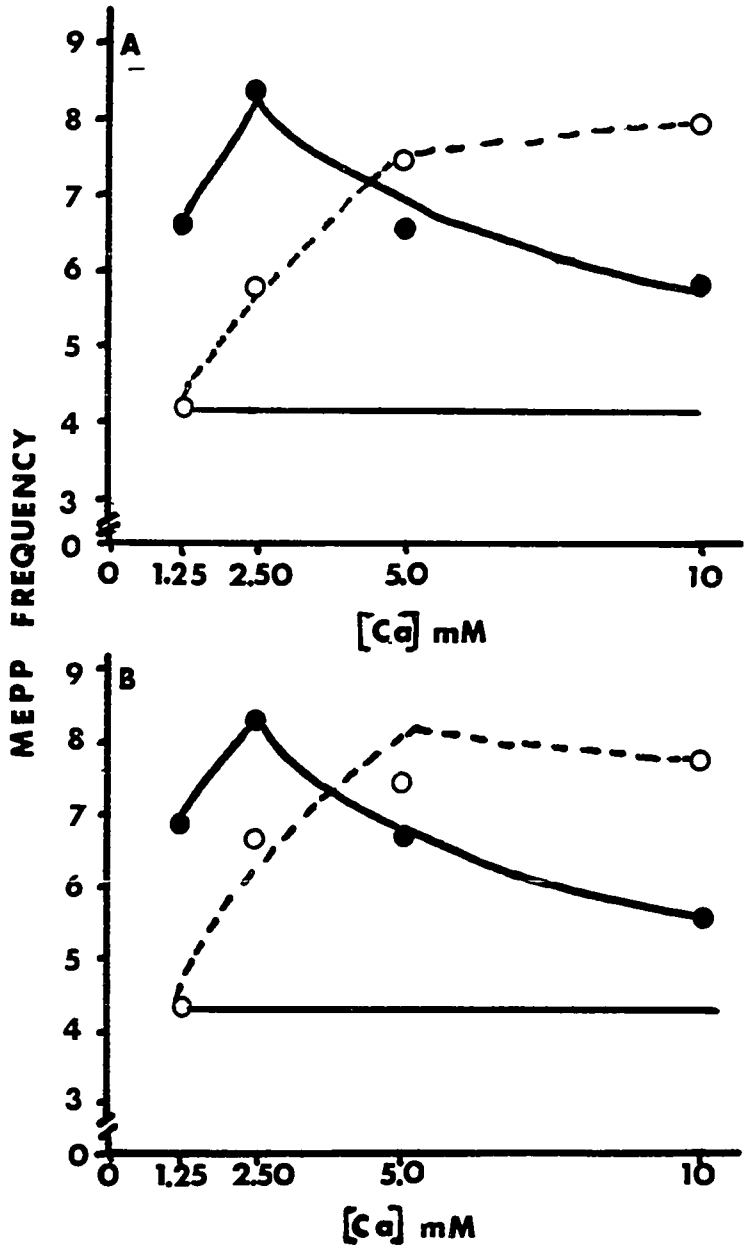


Figure 14. Arithmetic means of MEPP frequencies in four concentrations of Ca with and without Ba. Ordinates: MEPP frequency (per sec). Abscissa: Ca concentration (mM). Each point represents the mean of 2-3 diaphragms. Horizontal bar indicates mean frequency in 1.25 mM Ca controls. Lines are transposed from straight lines fitted by eye on log-log coordinates. A. Changes in MEPP frequencies from a control point derived by interpolation. B. Changes in MEPP frequencies from the control rate measured before irrigation with the variable solution. Open circles for Ca alone, closed circles Ca + Ba.

At low concentrations of Ca, the addition of Ba increased MEPP frequency. At higher concentrations of Ca, however, the addition of Ba acted to decrease MEPP frequency. The crossover point of the two curves occurs between 2.50 and 5.0 mM Ca.

Validity of the interpolation method. In order to get an estimate of MEPP frequency in the Ca concentrations between those studied, the data were replotted on log-log coordinates (Figure 15). The open circles of Figure 14A containing only Ca are replotted in Figure 15A. It is obvious that the log of MEPP frequency forms a linear relationship with the log of Ca concentration, and that the slope of this line changes between 5.0 and 10 mM Ca. A similar change in slope in this region of Ca concentration was observed when end-plate potential amplitude was compared with Ca on log coordinates (Dodge and Rahamimoff, 1967). The concentrations between those measured were estimated from the straight line of Figure 15A and plotted on the linear coordinates of Figure 14A, forming the hyperbolic curve (dashed line).

A similar line was drawn for the means of experiments in Figure 14A which contained Ba in addition to Ca (filled circles). Figure 14A shows that in the presence of Ba, the estimated line does not fit the experimental points as well as in Ca alone. The data in Figure 14A were based on interpolated controls. That is, the control before and after the variable were averaged, and this MEPP frequency was considered the better estimate of the control rate. This method of interpolation does not seem to be as reliable for Ca plus Ba as for Ca alone, judging from Figure 14A. It is possible that Ba has some irreversible effects on MEPP frequency which would invalidate the use of the interpolated method.

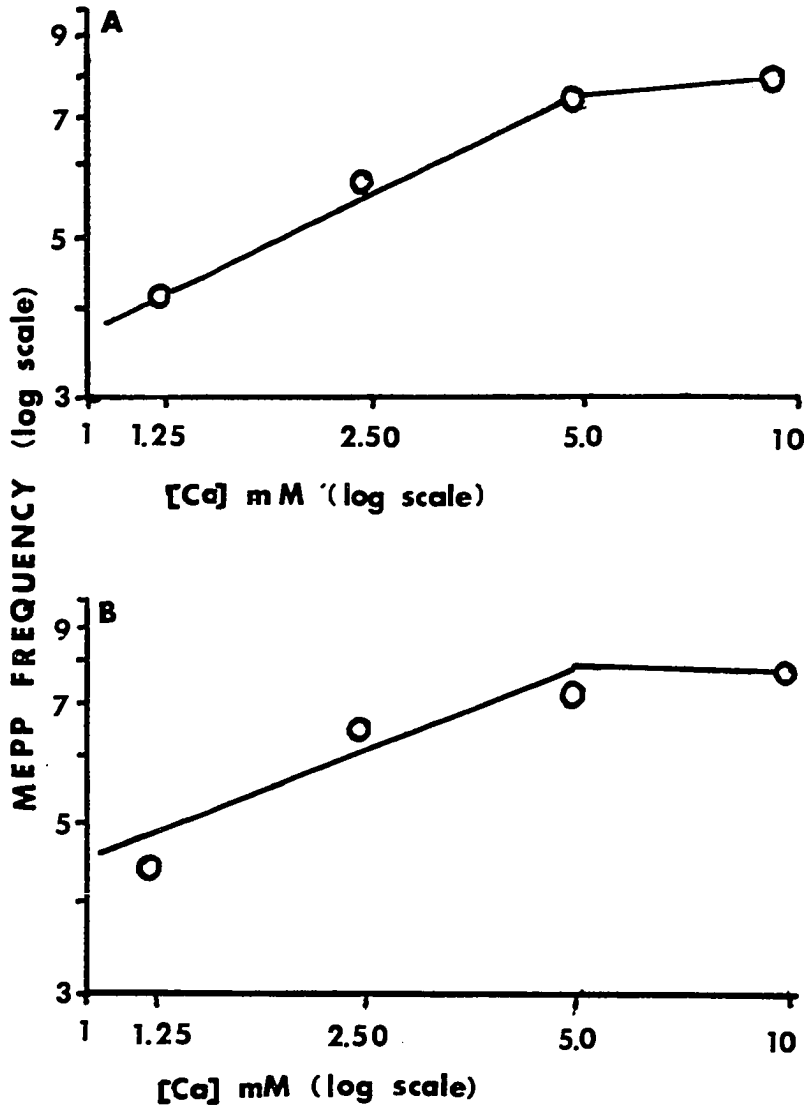


Figure 15. MEPP frequency as a function of Ca concentration on log-log coordinates. Ordinates: MEPP frequency (log scale). Abscissa: Ca concentration (log scale). A. Data from Figure 14A with Ca only (open circles, O) in which the control was obtained by interpolation. B. Data from Figure 14B with Ca only (open circles, O) based upon the preceding control only. Straight lines were fitted by eye.

This apparent irreversibility was confirmed by experiments to be described later. Therefore, the data from the Ca + Ba experiments were replotted in Figure 14B. In this case only the control which preceded the variable solution was used as the experimental control. The return to control after Ba was not considered. At worst this would leave rate changes with time uncompensated. It was found that MEPP frequencies in solutions with Ca and Ba fitted the estimated line much better when the final control was not included. When the means for Ca alone were considered in a similar manner (Figure 14B, open circles), the points did not fit the estimated line very well. Therefore, the best way of describing the effects of Ca concentration on MEPP frequency is to consider the change in frequency from an interpolated control. When Ba is added, however, the best estimate is based upon the change in frequency from the preceding control, discounting the return to control.

MEPP frequency and the normal curve. While the arithmetic mean of a Poisson distribution is the maximum likelihood estimator (best estimate of true population mean), the arithmetic mean must be transformed into its square root in order to fit the normal curve (see Methods, p. 38). Square roots of MEPP frequencies were taken from all cells in the ten experiments and the means are shown in Figure 16. Comparison of two normal curves (Ca with and without Ba) assumes that the data were obtained by the same method. If MEPP frequencies are based on two different types of controls, then the difference in frequencies is confounded by the difference in methods. No valid conclusion can be drawn from such estimates. Therefore, for the purpose of symmetry in controls both curves in Figure 16 are based on preceding controls only. This constitutes a

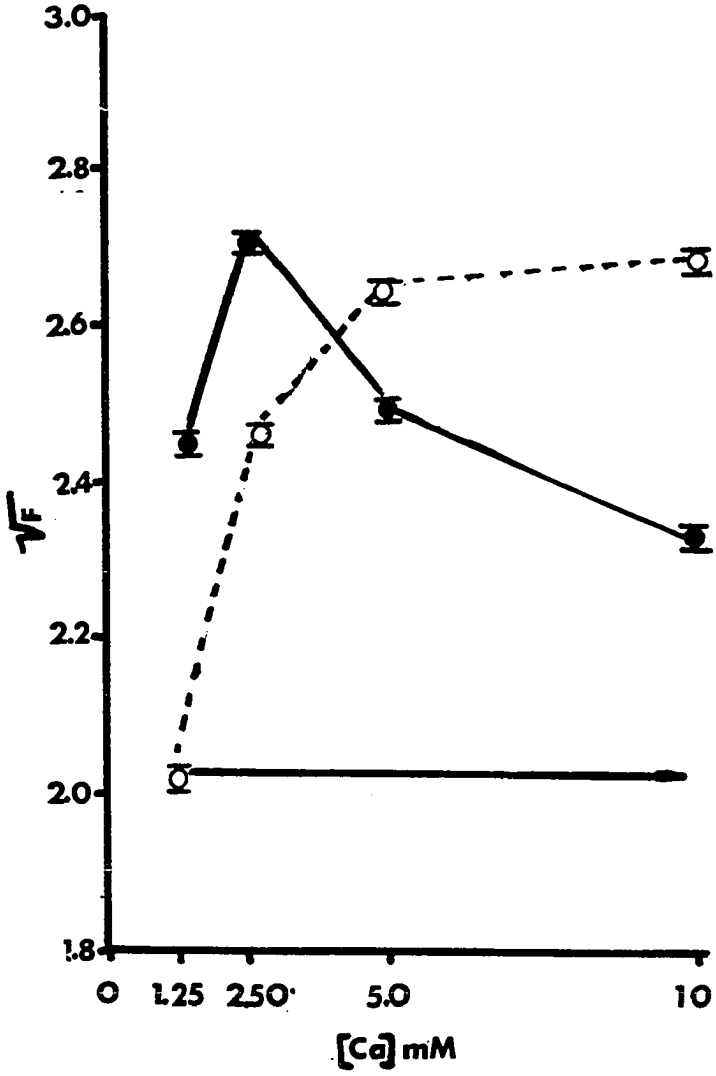


Figure 16. Means of square roots of MEPP frequency in four concentrations of Ca with and without Ba. Ordinate: square root of MEPP frequency. Abscissa: Ca concentration. Open circles (O) indicate Ca alone. Filled circles (●) indicate Ca plus 1.25 mM Ba. Bars above and below each point represent ±1 S.E. of the mean. All MEPP frequencies based upon increases from preceding control.

biased estimate of true means, but a good estimate of the normal curve.

Because of this biased estimate of the true mean, as well as the relatively small number of diaphragms (two to three) at each Ca concentration, the analysis of variance could not show the curves to be different. However, in 9 of the 10 experiments Ba shifted the curve in the directions shown in Figure 16. Moreover, on the basis of individual experiments, at least one experiment in each group showed a significant change in MEPP frequency when Ba was added. In accordance with the intersection of the two curves between 2.50 and 5.0 mM Ca, significance at the extremes of the concentration range was greater ($p < .01$) than toward the middle of the range ($p < .05$).

On the basis of geometric, arithmetic and square root means of MEPP frequencies, it is concluded that 1) increasing Ca concentrations increase MEPP frequency, and 2) Ba has a bilinear effect on MEPP frequency over the range of Ca concentration studied. At low concentrations of Ca the addition of 1.25 mM Ba increases MEPP frequency. At higher concentrations of Ca, Ba decreases MEPP frequency.

Effect of Introducing Barium with Calcium before Calcium Alone

Throughout this report the "irreversibility" of Ba has been alluded to frequently. A moderate irreversibility by Ba on MEPP frequency was first described in the 0.5 mM Ca vs 0.5 mM Ba studies in Figure 3. Irreversible effects of Ba were also observed in experiments described in Figures 13 through 16. Ba somehow modified control MEPP frequency so that use of the control measured after Ba was misleading. In solutions containing only Ca, mean MEPP frequency is quite constant for several

hours with only hour-to-hour oscillations. Thus, if any two successive control periods in Ca solutions are compared, 50% will show an increase in MEPP frequency and 50% a decrease in frequency. This theory would account for zero net change in MEPP frequency while allowing for inevitable oscillations. Experimental observations confirmed this. Of six such comparisons of pairs of controls, four showed an increase and two showed a decrease in MEPP frequency. This is close to the expected .50 : .50 probability. When such comparisons of controls were made before and after Ba was added, seven of eight comparisons showed a decrease in MEPP frequency. Therefore, Ba acts at some site to prevent MEPP frequency from returning to control rates.

Figure 17 shows the results of three experiments similar in all respects to those in Figure 11 except that Ca plus Ba was introduced before Ca alone. The preceding control was used in estimating MEPP frequency changes in Ca plus Ba. The following control was used for solutions with Ca alone. MEPP frequency in 2.50 mM Ca with Ba was greater than without Ba. However, MEPP frequency in 2.50 mM Ca was lower than the 1.25 mM Ca control rate. This is contrary to the well established relation between Ca and MEPP frequency. In some way Ba must irreversibly affect the Ca-acceleratory site. At 5.0 and 10 mM Ca MEPP frequency decreased in Ba-free solutions, the opposite of what was expected. Again, some irreversible effect of Ba on the Ca acceleratory site is implicated.

Inhibition of Calcium Action by Barium

These results have confirmed previous reports that MEPP frequency is directly related to Ca concentration. The relationship is hyperbolic on linear coordinates, and therefore the tools of enzyme kinetics have

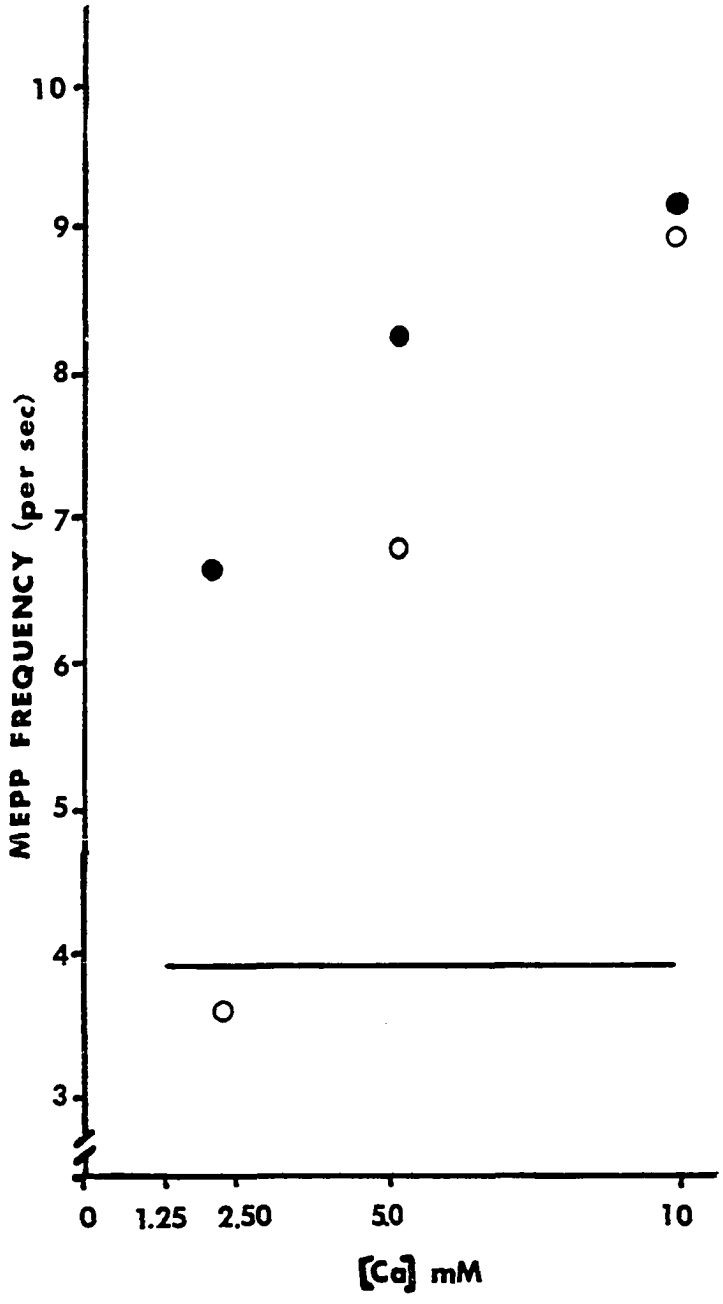


Figure 17. Effect of introducing Ca plus Ba before Ca alone. Ordinates: MEPP frequency (per sec). Abscissa: Ca concentration (mM). Open circles (○) represent Ca alone. Filled circles (●), Ca plus Ba. Data are arithmetic means of MEPP frequencies. Ca plus Ba is compared to the preceding control, while Ca alone is based on the following control. Horizontal bar has same meaning as in Fig. 13.

been employed to study the effect of Ca and other cations on MEPP frequency (Mambrini and Benoit, 1964; Gage and Quastel, 1966; Hubbard et al., 1968). Double reciprocal plots of cation concentration and MEPP frequency show that Na and Mg reduce MEPP frequency as competitive inhibitors to Ca (Gage and Quastel, 1966; Hubbard et al., 1968). The present results have shown that when Ba is present alone or together with high Ca concentrations, Ba also reduces MEPP frequency. In order to determine the type and site of the inhibition of Ba on Ca, a double reciprocal plot of Ca concentration vs. MEPP frequency was drawn (Figure 18). Contrary to the expected behavior of an inhibitor, at low Ca concentrations Ba increases MEPP frequency (right side of Figure 18). In solutions with Ca alone (open circles), the best fitting straight line obtained from Figure 15A indicates a gradual increase in frequency with increasing Ca concentration.

The curve describing the relationship between Ca and geometric means of MEPP frequency (Figure 13) was compared with data of Hubbard et al. (1968a) and Hubbard (1970). The shapes of the curves were essentially the same except that MEPP frequencies in Figure 13 were approximately twice as fast as those described previously. Fast MEPP frequencies observed in the present experiments were probably due to the absence of added Mg, lower Na and different strains of animals (see Methods, p. 31).

The slight change in slope at higher Ca concentrations shown in Figure 15A and again in Figure 18 was also observed by Gage and Quastel (1966) and Hubbard (1970) and is presumably due to saturation of receptor sites (Gage and Quastel, 1966). In order to circumvent the problem of saturation of the receptor site with Ca, the linear portion of the Ca

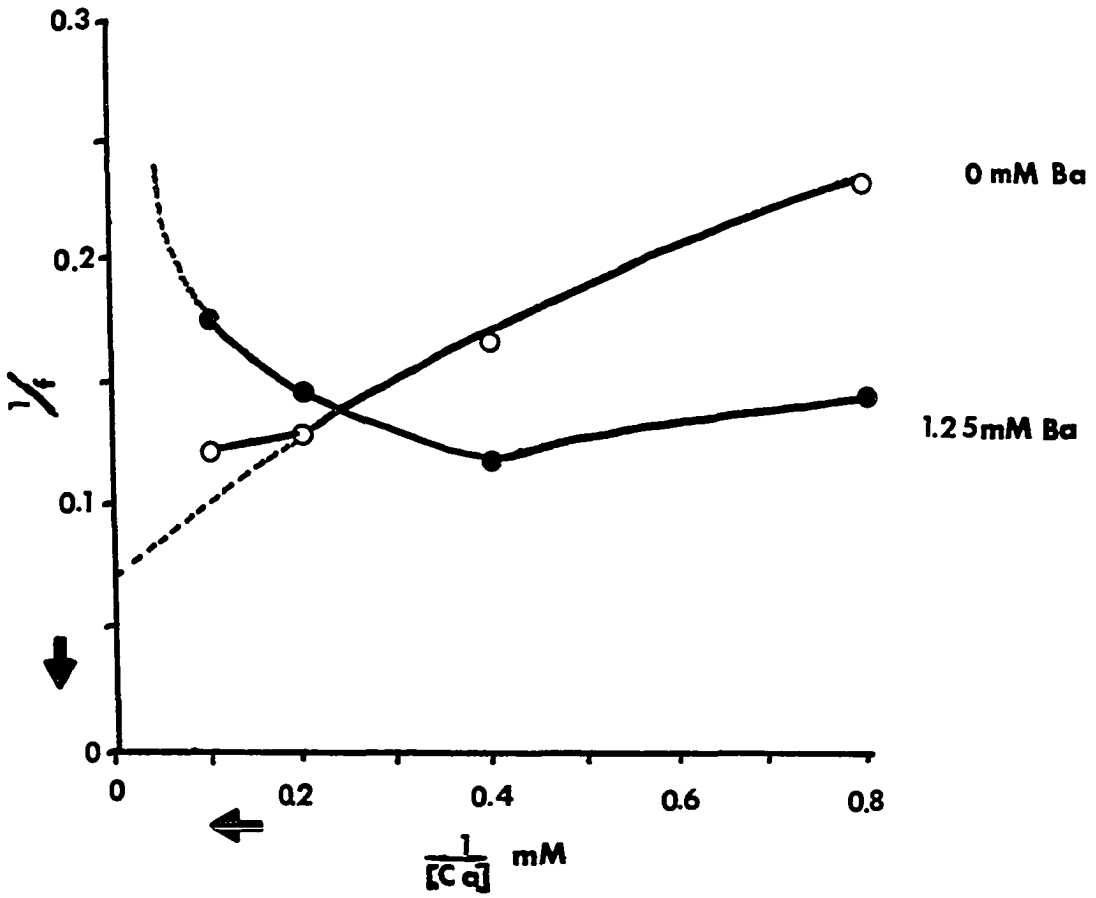


Figure 18. Double reciprocal plot of MEPP frequency vs Ca concentration. Ordinate: reciprocal of arithmetic means of MEPP frequencies. Abscissa: reciprocal of Ca concentration. Preceding controls were used for Ca plus Ba, while interpolated controls were used for Ca alone. Open circles (○), Ca alone; and filled circles (●), Ca plus 1.25 mM Ba. Arrows indicate direction of increasing Ca concentration and MEPP frequency.

curve (open circles) was extrapolated to the y-axis.

At high Ca concentrations, MEPP frequency decreases in the presence of Ba. If the Ca plus Ba curve in Figure 18 (closed circles) is extrapolated, it approaches the y-axis asymptotically, giving the appearance of saturation of the receptor site. A subsequent analysis will show the extrapolation to be invalid. The data indicate that at high Ca concentrations Ba is acting as an inhibitor to Ca. However, until an explanation is obtained for the interaction of the two curves between 2.50 and 5.0 mM Ca, no meaningful conclusions can be drawn from a discussion of inhibition kinetics.

Intersection of the Calcium - Barium Curves

It has been shown that Ba depolarizes the muscle membrane (Figure 8), and presumably this also occurs at the nerve terminal membrane. Two questions immediately arise. First, does Ba depolarize the membrane in the presence of Ca? Second, if Ba does depolarize the membrane, then how can this be dissociated from the Ca-Ba competition curves of Figure 14?

Membrane stability in the presence of calcium. To answer the first question, membrane potentials were recorded in seven of the ten experiments shown in Figure 14. Table 6 illustrates that the addition of 1.25 mM Ba depolarized the membrane at all concentrations of Ca. As Ca concentration increased, membrane potential also increased whether Ba was present or not. Table 6 (right hand column) shows that Ba was consistently less effective in depolarizing the membrane as Ca concentration was increased. Thus Ca is acting to stabilize the membrane potential in competition with the depolarizing action of Ba. Therefore, it is likely that

TABLE 6

RELATIONSHIP BETWEEN MEMBRANE POTENTIAL AND Ca CONCENTRATION WITH AND WITHOUT 1.25 mM Ba

$[Ca^{2}]$ mM	Membrane Potential (mV)		Percent of Control (based on 1.25 mM Ca)
	Ca alone	Ca + Ba	
1.25	67	48	72
2.50	69	50	75
5.0	70	52	78
10.0	74	54	81

All data based on means of cells from two diaphragms, except 1.25 mM Ca + Ba which is based on one diaphragm.

the stabilizing effect of Ca on membrane potential may be responsible for reduced MEPP frequency at higher Ca concentrations in the presence of Ba (Figure 14).

An approach to achieving a stable membrane. The only way to eliminate the possibly confounding effects of membrane depolarization on the Ca-Ba competition data is to maintain the membrane at a constant potential. One approach was to use a wide range of Ba concentrations to determine at what concentration Ba produces its maximum depolarizing effect. Solutions with four concentrations of Ba (0.25, 0.50, 1.25, and 2.50 mM) and with zero Ba were tested on seven diaphragm preparations. All solutions contained 1.25 mM Ca. Ba concentrations above 2.50 mM reduced MEPP amplitude so greatly that measuring frequency was impossible. In the Ca-Ba competition experiments previously described, 1.25 mM Ca was the control solution for all experiments. In this series, 1.25 mM Ca + 0.25 mM Ba was used as control. Note that this series was designed to overlap the series illustrated in Figure 14 at two points: a) 0 Ba + 1.25 mM Ca, and b) 1.25 Ba + 1.25 Ca. Figure 19B shows that as the concentration of Ba increases, MEPP frequency also increases. This increase in MEPP frequency is especially great in 2.50 mM Ba. Figure 19A indicates that all concentrations of Ba are effective in depolarizing the membrane. However, the maximum effect of Ba on membrane depolarization is achieved in 0.50 mM Ba with little change at higher concentrations. Therefore, at higher concentrations of Ba, membrane potential has achieved relative stability. Figure 19B shows that higher Ba concentrations produce faster MEPP frequencies, although membrane potential is relatively stable. Thus, by stabilizing the membrane potential with higher Ba

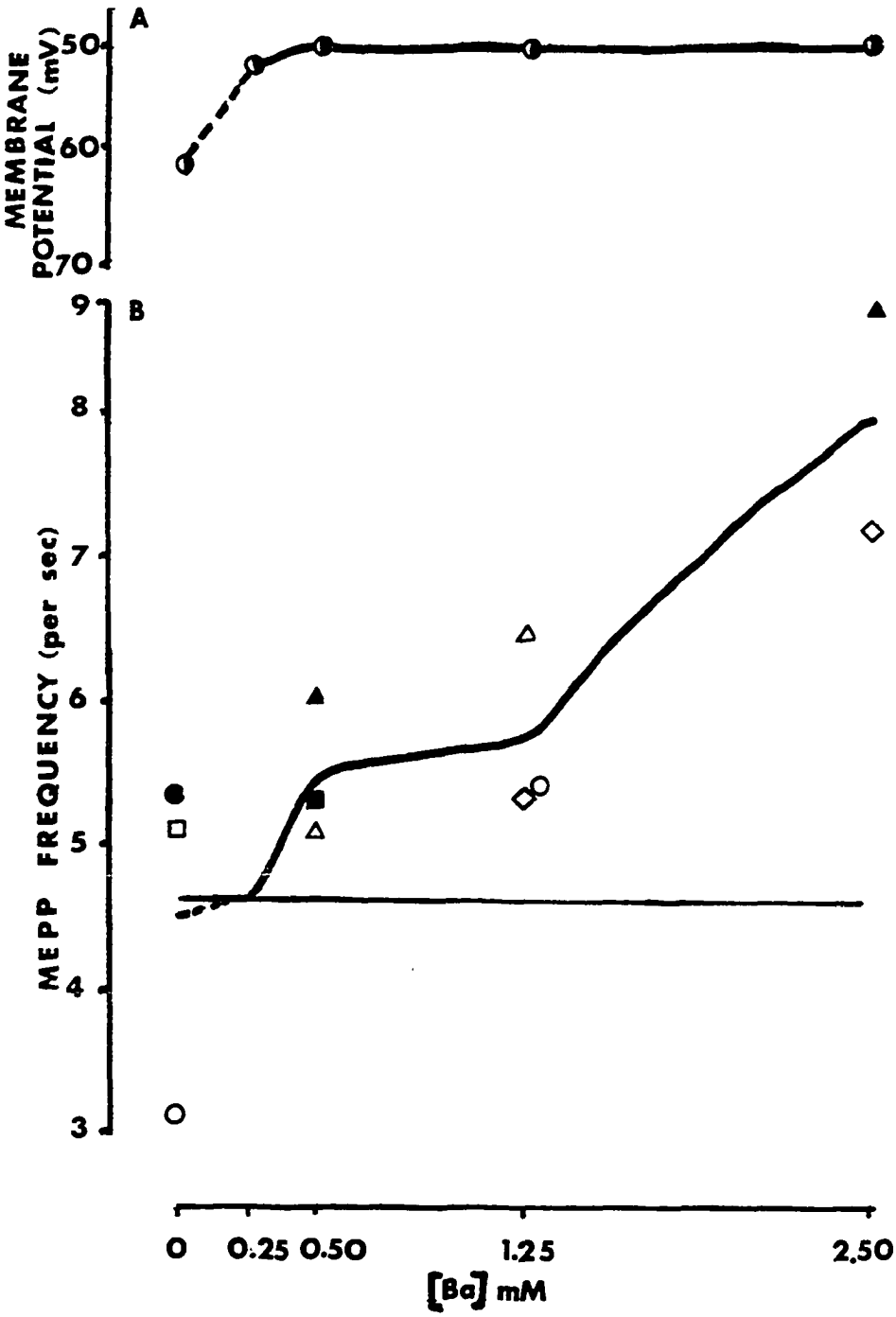


Figure 19. Effect of various concentrations of Ba with constant Ca on MEPP frequency and membrane potential. Ordinates: A. muscle membrane potential (mV); B. MEPP frequency (per sec). Abscissa: Ba concentration (mM). All data based on arithmetic means. Each symbol type represents 1 diaphragm. Dashed lines indicate that 0 mM Ba was not included on the log-log coordinates used to compute the best fitting line by eye. Horizontal bar represents means of all controls with 0.25 mM Ba and 1.25 mM Ca. All data based on interpolated controls.

concentrations it was possible to dissociate the two effects of Ba on membrane potential and MEPP frequency.

The Sites of Interaction Between Calcium and Barium

It has been shown that depolarization of membranes by Ba is caused by interference with the K conductance mechanism (Josse et al., 1965; Sperelakis et al., 1967; Pappano and Sperelakis, 1969). Therefore, Ba-induced depolarization is analogous to depolarization produced by raising external K concentration. Increasing external K results in depolarization of the membrane and increased MEPP frequency (Liley, 1956c; Takeuchi and Takeuchi, 1961). Hubbard et al. (1967) found that MEPP frequency increased by a factor of ten when the membrane was depolarized 26.7 mV by K. This relationship was replotted to fit the coordinates of Figure 20 (dashed line). Figure 20 is a summary of the interrelationship of MEPP frequency and membrane potential in the presence of various concentrations of Ca and Ba. Data from Figure 14A containing Ca + Ba were replotted in Figure 20 (filled symbols). In order to compare these data with those of Figure 19, the two overlapping points discussed above were used to determine a correction factor (1.157; see Appendix II). The two curves were adjusted so that MEPP frequencies were equal in 1.25 mM Ca + 1.25 mM Ba in each series.

Figure 20 shows that equal Ca:Ba ratios result in approximately equal membrane depolarizations. A ratio equal to 1 resulted in an 18 to 19 mV depolarization. When R = 2, the membrane is depolarized by 17 to 18 mV. When R = 4 or 5, the membrane potential is reduced by 17 mV. This indicates that membrane potential is approximately the same provided

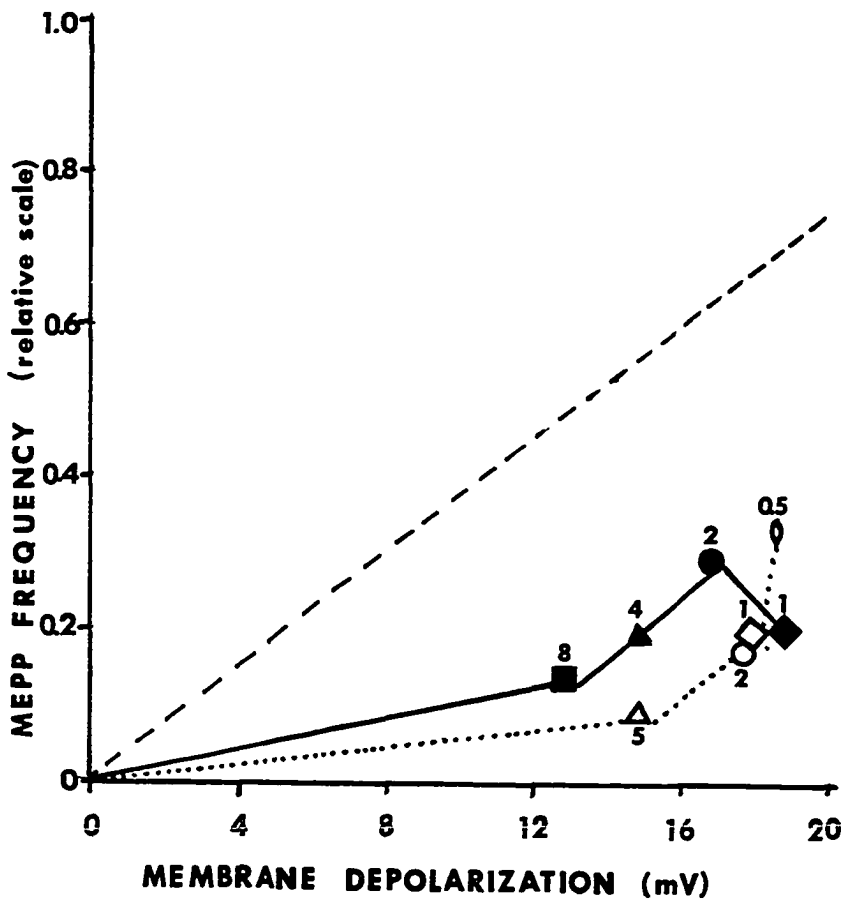


Figure 20. Dissociation of barium's effect on membrane potential from its effect on MEPP frequency. Ordinate: MEPP frequency (logarithmic, per sec). Abscissa: depolarization of membrane (mV). Dashed line indicates the effect of K-induced membrane depolarization on MEPP frequency. Solid line (closed symbols) represents four Ca concentrations in presence of 1.25 mM Ba (from Figure 14A). Dotted line (open symbols) represents four concentrations of Ba with 1.25 mM Ca (from Figure 14B). Each point represents 2-3 diaphragms. Comparable symbols represent similar Ca:Ba ratios. Note value of ratio marked adjacent to each symbol. Insert shown below indicates each symbol used in the figure with Ca and Ba concentrations below and value of the ratio above.

Ratio	8	4	2	1	5	2	1	0.5
	■	▲	●	◆	△	○	◇	◊
$\frac{[Ca^{2+}]}{[Ba^{2+}]}$	$\frac{10}{1.25}$	$\frac{5.0}{1.25}$	$\frac{2.50}{1.25}$	$\frac{1.25}{1.25}$	$\frac{1.25}{0.25}$	$\frac{1.25}{0.50}$	$\frac{1.25}{1.25}$	$\frac{1.25}{2.50}$

the ratio Ca:Ba is constant. This occurs in spite of the fact that absolute Ca and Ba concentrations may be considerably different.

At higher absolute concentrations of ions (Figure 20, filled symbols), increasing R from 2 through 8 reduces MEPP frequency. This reduction in frequency forms a line approximately parallel with the theoretical (dashed) line expected for K-induced depolarization. This theoretical line describes the effect of membrane depolarization on MEPP frequency. Therefore, the reduction of MEPP frequency at higher Ca and Ba concentrations is due primarily to stabilization of the membrane. This reduction in MEPP frequency occurred in spite of increasing Ca concentrations (R = 2 is 2.50 mM; R = 8 is 10 mM Ca). Figure 14 (filled circles) showed that as Ca was increased from 2.50 to 10 mM in the presence of Ba, MEPP frequency declined. Therefore, an explanation for the reduction of MEPP frequency in Figure 14B has been achieved. This reduction in MEPP frequency is due to a gradual repolarization of the nerve terminal membrane.

Figure 20 (filled symbols) also shows that when R was increased from 1 to 2 (Ca from 1.25 to 2.50 mM) MEPP frequency increased. Therefore, it is only when Ca:Ba is greater than 2:1 that Ca acts to stabilize membrane potential. This suggests that Ba binds to the nerve terminal membrane with a force at least twice as great as Ca. The site of this interaction is at the K conductance "channel" of the nerve terminal membrane.

The data from Figure 19 are indicated in Figure 20 as open symbols. These preparations also contained Ca and Ba but at decreased absolute concentrations, Ca of 1.25 mM and Ba of 0.25 up to 2.50 mM. There-

fore, in these experiments Ca:Ba ratios ranged from 5.0 to 0.5 (see legend, Figure 20). Increasing Ca:Ba ratios from 2 to 5 reduced MEPP frequency. Because Ca concentration was constant (1.25 mM), the reduction in MEPP frequency was due to a decrease in Ba from 0.50 to 0.25 mM. The reduction of MEPP frequency associated with this decrease of Ba concentration forms a line approximately parallel to both the theoretical line relating MEPP frequency to K-induced depolarization, and the Ca-membrane potential stabilization line (filled symbols, solid line). Therefore, MEPP frequency reduction caused by increasing R from 2 to 5 (decreasing Ba from 0.50 to 0.25 mM) is due to a repolarization of the nerve terminal membrane. As the membrane is repolarized, MEPP frequency is reduced.

The three approximately parallel lines in Figure 20 indicate that the effect of Ba on MEPP frequency and membrane potential is similar to what would be expected from an increase in extracellular K. However, MEPP frequencies of both experimental curves are lower than the theoretical curve, although parallel to it. Therefore, Ba is not as effective as Ca in providing the link between K-induced depolarization and the resultant increase in MEPP frequency. In this study Ca binds more strongly than Ba at the binding site in step I of the linkage between membrane depolarization and accelerated MEPP frequency. At site I therefore, Ca binds more strongly than Ba (see Discussion, p. 110).

When membrane depolarization is stabilized at approximately 18 mV, the effect of Ba itself on MEPP frequency can be observed. In the experiments represented by open symbols, when R is decreased from 1 to 0.5 (Ba increased from 1.25 to 2.50 mM), the increase in Ba concentration results in an acceleration of MEPP frequency. This increase in MEPP

frequency by Ba occurred in the presence of constant Ca concentration (1.25 mM). Early experiments (Figure 3) showed that when membrane potential had stabilized, Ba without Ca caused a decreased MEPP frequency. Thepresent experiments show that Ba with Ca increases frequency.

This paradox can be resolved if two sites of action for Ba and Ca are considered. Firstly, Ca binds at a site which results in an acceleration of MEPP frequency. For the present discussion this site will be denoted "S" for substrate site. In the absence of Ca, Ba binds very poorly with the S site. Thus, in the early experiments Ba could not bind as well as Ca to the S site, and MEPP frequency declined. Secondly, Ba binds to another site which does not accelerate frequency per se but makes more S sites available for Ca. This is called "site A" for activator site. Ba binds much more readily to the activator site than Ca does. When Ba is added in the presence of Ca, more S sites are made available and the effect of Ca on MEPP frequency is potentiated. However, when Ba is added in the absence of Ca, although more S sites are made available, MEPP frequency declines since little Ca is there to bind.

The following analysis shows that Ca is more effective at the S site than Ba. If R = 1 and R = 2 of both curves in Figure 20 are compared, an inequality is derived:

$$\frac{1.25\ Ca}{0.50\ Ba} \simeq \frac{1.25\ Ca}{1.25\ Ba} \ll \frac{2.50\ Ca}{1.25\ Ba}$$

where each ratio represents the MEPP frequency observed at these concentrations. Doubling Ba concentration produces far less increase in MEPP frequency than doubling Ca concentration. Thus Ca is more effective than Ba in its capacity to accelerate MEPP frequency, although Ba does not

inhibit the acceleration. Therefore Ca binds more firmly than Ba at the S site, although Ca binds less firmly than Ba at the A site.

Summary of Major Observations

Substitution of Ba for Ca in rat diaphragm muscles changed MEPP frequency in three phases. During the first 10 min after exposing muscles to Ba, MEPP frequency was accelerated. Frequency then declined and stabilized below control rates after 40 min. Finally, after 80 min MEPP frequency often rose above control rates. This third phase did not occur when Ca was present with Ba.

The initial increase of MEPP frequency by Ba is explained by its reduction of K conductance and depolarization of the nerve terminal membrane. The predominant effect of Ba substitution is depression of MEPP frequency. Ba substitutes poorly for Ca at a binding site (I) that links depolarization to MEPP frequency acceleration. Consequently MEPP frequency declines as Ca diffuses away from this binding site (I). Ba further reduces frequency by substituting poorly at the MEPP frequency acceleratory site (S). When Ba is added in addition to normal concentrations of Ca, Ba increases MEPP frequency, perhaps by association with an activator site (A). However, with elevated concentrations of Ca, addition of Ba reduces MEPP frequency. Such elevated concentrations of Ca may stabilize the Ba-depolarized nerve terminal membrane, and thereby depress MEPP frequency.

Therefore, Ca and Ba interact at four sites in the series of reactions linking membrane depolarization with transmitter release. The sites of interaction and the relative binding strengths of Ca and Ba are as follows:

1. K conductance (gK) "channel" in the nerve terminal membrane: Ca < Ba

2 and 3. Linkage between depolarization of the nerve terminal membrane and Ca-induced MEPP frequency increase: Step I Ca > Ba

Step II (S site) Ca > Ba

4. Activator site (A) associated with the MEPP frequency acceleratory site (S): Ca < Ba

CHAPTER IV

DISCUSSION

Present Observations Compared with Previous Reports of Barium Action

Effects of Barium on Spontaneous Release

Initial reports. The first report of the effects of Ba on spontaneous release of transmitter was by Feng (1937a) with toad sciatic nerve-sartorius muscle preparations. Muscles exposed to 2 to 10 mM Ba and 1 to 2 mM Ca twitched spontaneously because of a Ba-induced "spontaneous release of acetylcholine...from nerve endings." Observations reported here confirm the spontaneous twitching of rat diaphragms in Ba. All experiments described by Feng contained Ca in addition to Ba. Figure 19 in Results shows that when a diaphragm is exposed to Ba in addition to Ca, spontaneous release is always increased. Consequently, results presented here confirm this early report. Yet when the effect of Ba in the absence of Ca is considered, mammalian transmitter release may be different from release in amphibians.

Boyd and Martin (1956a) reported a personal communication from del Castillo stating that Sr and Ba both accelerate MEPP's above rates in equimolar Ca in the frog neuromuscular junction, presumably the toe muscle. Ba was especially effective in accelerating MEPP frequencies.

Ca dependent spontaneous release may be less in the frog than in the mammal (Fatt and Katz, 1952). In view of this possible difference, comparison of Ba action between the two phyla may be equally hazardous. It is also possible that the actions of Ba are the same in both animals, but methods of measurement were quite different. Figure 2 described two diaphragms irrigated with 1.0 mM Ba at 3 ml/min. Both experiments showed an increase in MEPP frequency which was sustained for 30 to 50 min. Doubling the flow rate decreased the latency of the Ba induced MEPP frequency reduction. It is possible that del Castillo observed only the first phase of MEPP frequency, the increase caused by membrane depolarization, because of different irrigation procedures.

Calcium substitution experiments. A more detailed account of the effect of Ba on MEPP frequency was reported by Elmqvist and Feldman (1965). They described a multipuncture experiment with a rat diaphragm incubated for 3 hours in 1 mM EDTA in order to chelate most of the tissue Ca. Because of the low free $[Ca^{2+}]$ MEPP frequency was well below control rates. Irrigation with 2 mM Ba restored MEPP frequency to double the rate with 2 mM Ca. The high MEPP frequency in Ba was reported to be maintained for at least 3 to 4 hours. It was also reported that two fibers with 130 and 65 MEPP's/sec were not included in computing MEPP frequency.

Multiple puncture experiments described in the present report sampled MEPP frequencies every minute for up to 140 min after the introduction of Ba (see Methods). Elmqvist and Feldman (1965) did not state whether samples were taken every minute. However, other experiments described in their report sampled several fibers at 15-40 min intervals rather than continuously. It is possible that not all of the phases of

changing MEPP frequency were observed. If MEPP frequencies had been recorded during the first 20 to 30 min in Ba, most likely an accelerated MEPP frequency would have been observed. Even rates of 130 and 65 MEPP's/sec should have been included in computing MEPP frequencies during this time (see Figure 3). MEPP frequency slows after 20 to 40 min and subsequently increases after 80 min in Ba alone (Figure 10). If 30 to 40 minutes elapsed before frequency samples were taken, the second (reduced) frequency phase could have been missed entirely. The statement by Elmqvist and Feldman (1965) that the Ba-induced increase in MEPP frequency was maintained for at least 3 hours could have resulted from infrequent sampling during the third MEPP frequency phase.

Elmqvist and Feldman (1965) also reported experiments in which diaphragms were incubated for 4 to 6 hours in solutions containing 20 mM K, 1 mM EDTA and 2 mM Ba but no Ca. Addition of 20 mM K ordinarily results in dramatic acceleration of MEPP frequency if Ca is present. When Ca is chelated by EDTA, the increase in MEPP frequency by high K is not seen. Their report indicated that Ba was capable of substituting for Ca in producing the K induced rise in MEPP frequency. Observations in the present report indicate that the initial rise in MEPP frequency (phase I) is observed only because Ca has not yet been leached from the nerve terminal receptor sites I and S. As Ca is gradually removed, Ba cannot substitute for Ca and the increased MEPP frequency is not sustained, declining below control rates. Elmqvist and Feldman (1965) found that Ba was at least as effective as Ca in substituting for the accelerated release of transmitter by 20 mM K. Our observations indicate that in 5 mM K, Ba cannot substitute for Ca. The difference in experimental procedure

is immediately obvious. The MEPP frequency increase resulting from membrane depolarization described by Elmqvist and Feldman was observed in the presence of 20 mM K, while the gradual decline in frequencies described in this report was observed in the presence of normal (5 mM) K. The K^+ itself accelerates MEPP frequency in addition to those MEPP's resulting from its membrane depolarization effect (Gage and Quastel, 1965). Also, after long periods of exposure K can act at the Ca-complex sites (Hubbard et al., 1967). Since it was shown in Results (p. 96) that relative to Ca, Ba binds poorly at sites S and I but strongly at sites A and gK (K conductance channel), it is possible that the synergistic action of Ba plus high K could have produced a net increase in MEPP frequency. This explanation is supported by the similar latency by either Ba (350 msec) or Ca (250 msec) for restoration of MEPP frequency when effused by ionophoresis from a micropipette. This may indicate relatively equal accessibility to all sites, although this is quite speculative.

Based upon observations presented in this report, it is concluded that Ba substitutes poorly for Ca at sites I and S. After an initial acceleration of MEPP frequency due to membrane depolarization, Ca is leached away from site I and frequencies return toward control. Leaching of Ca from site S reduces MEPP frequency below control rates. Differences reported by others are explained on the basis of interspecies variation or different experimental techniques and conditions.

Effects of Barium on Release by Nerve Impulses

Feng (1937a) observed that stimulation of the frog sciatic nerve resulted in a more forceful twitch of the sartorius muscle if Ba were

added to Ringer's solution containing 2 mM Ca. Blioch et al. (1968) and Miledi (1966) observed that Ba could partly substitute for Ca in frog sartorius and cutaneous pectoris nerve-muscle preparations. Ba was inconsistent in its replacement of Ca when applied ionophoretically to the neuromuscular junction region.

Hubbard et al. (1968b) have shown that transmitter release by a nerve impulse is best explained by a great increase in the activation of a membrane carrier site "X" associated with three Ca ions, Ca_3X. Thus, while spontaneous and evoked release are analogous, resulting from a common series of reactions, evoked release activates a specific complex (Ca_3X), while spontaneous release is produced by any complex (X, CaX, Ca_2X, Ca_3X). The site of interaction between Ca and Ba in activation of the Ca_3X (stimulus activated) complex may be different from the site of interaction for spontaneous release. Results from the present investigation substantiate this. It was shown (p. 95) that Ca and Ba interact at two sites in the linkage between membrane depolarization and MEPP frequency increase. Ba was a very poor substitute for Ca at the MEPP frequence acceleratory site (site S or II). Ba was also less effective than Ca at site I, described as the first link between membrane depolarization and MEPP frequency rise. No further quantitative analysis of site I was possible. These observations are consistent with reports of Ba substituting for Ca in evoked transmitter release, Ba being neither as effective nor as consistent as Ca in evoked release.

Explanations for Three Phases of Barium Action

Irrigation with Barium at Rapid Flow Rates

Figure 2 showed that the depression of MEPP frequency by Ba was

observed earlier when the rate of flow was doubled from 3 to 6 ml/min or faster. Increasing flow rate provides a greater diffusion gradient with the synaptic compartment, and consequently, the nerve terminal membrane. It was not surprising therefore that the depressant phase of Ba on MEPP frequency occurred earlier at faster flow rates. However, conclusions drawn from Figure 2 should be tempered with the understanding that these were early experiments and many differences other than flow rate existed between the three experiments.

Multiple puncture experiments indicate that in a population of muscle fibers changes in MEPP frequency by Ca, Na or K are relatively complete within 15 to 40 min when irrigating at 3-6 ml/min (Hubbard et al., 1968a; Gage and Quastel, 1965 and 1966). At fast flow rates the Ba-depressed MEPP frequency approached stability during the 21-40 min period shown in Figure 3. Therefore, the sites of action by Ca, Na, K and Ba on MEPP frequency are approximately equally accessible.

First Phase of Barium Action

Action of barium on potassium conductance. Depolarization of the muscle membrane by Ba was expected. Similar effects of Ba have been observed on membranes of earthworm somatic muscle, barnacle striated muscle, lobster stretcher muscle, grasshopper extensor muscle, frog sartorius muscle, chick cardiac muscle, cockroach giant axon, frog peripheral nerve, frog spinai ganglion and Limulus photoreceptor cells (Ito et al., 1970; Hagiwara and Naka, 1964; Werman et al., 1961; Werman and Grundfest, 1961; Jenden and Reger, 1963; Josse et al., 1965; Sperelakis et al., 1967; Volle, 1970; Sperelakis and Lehmkuhl, 1966; Pappano and Sperelakis, 1969; Narahashi, 1961, 1966; Lorente de No and Feng, 1946; Nishi and Soeda,

1964; Gorn, 1969).

Barium reduced K conductance in resting membranes, and this is associated with a rapid and reversible increase in membrane resistance (Werman et al., 1961; Nishi and Soeda, 1964; Josse et al., 1965; Sperelakis et al., 1967; Volle, 1970). Resting membrane potential depends upon K conductance (Hodgkin, 1958). Therefore, a decrease in K conductance results in a decrease in resting membrane potential. Ba blocks or binds "gK (K conductance) channels," reducing efflux and thereby resulting in a decrease in membrane potential. When extracellular K is raised, K efflux diminishes due to a reduced chemical diffusion gradient, and this reduced efflux results in a lower membrane potential. Both Ba and K therefore act through a common mechanism (diminished resting membrane potential). On the basis of common mechanisms, Figure 20 is justified.

Membrane depolarization and MEPP frequency. Depolarization of the nerve terminal membrane results in an acceleration of MEPP frequency. Because Ba and K act through the same mechanism, equal membrane depolarization should yield equal MEPP frequency, whether K or Ba were used to induce the depolarization. Figure 20 shows that this was not observed. For a given depolarization of the membrane, MEPP frequency is not as great in Ba as in K -- the three lines are parallel but not equal. There are two possible explanations for this discrepancy. Depolarization of the membrane may be just one step in a series of reactions between membrane depolarization and accelerated MEPP frequency. Equal membrane depolarization by any source should yield equal acceleration of MEPP frequency. It is possible that another site of Ba action between membrane depolarization and transmitter release is involved. A theoretical Ca_3X

complex (described on p. 11) has been proposed (Hubbard et al., 1968b). This complex is greatly activated by a reduction of membrane potential. Ba acts at some site between membrane depolarization and MEPP frequency increase, but whether it acts at the Ca_3X complex is speculative. Therefore, to be less specific Ba acts at site I in the series of reactions between membrane depolarization and MEPP acceleration. As concluded in Results (p. 95), Ca > Ba at site I, shown by barium's diminished effect at this site. The discrepancy between expected and observed MEPP frequencies may be explained by the theoretical curve describing the effect of K-induced depolarization on MEPP frequency which is itself measuring two actions of K. It is possible that K acts to depolarize the membrane, as well as accelerating site I. This would exaggerate the theoretical curve in Figure 20. However, this is highly unlikely because K-induced changes in membrane potential fitted potentials predicted by the Nernst equation, and MEPP frequencies were the same (Liley, 1956b; Katz, 1962; Hubbard et al., 1967).

The data shown in Figure 9 are redrawn in Figure 21 to show that the observed change in MEPP frequency is actually an algebraic sum of two curves. Line a indicates that the observed MEPP frequency has reached a maximum within 10 min in Ba. Membrane potential has stabilized by this time (Figure 9). If the observed MEPP frequency is the maximum effect of depolarization, then after 10 min this MEPP frequency would be maintained at about 15/sec (line b). However, MEPP frequency begins to decline within 10 min of exposure to Ba. Figure 21 raises three questions concerning sites of action of Ba. 1) Is the observed initial increase in MEPP frequency representative of the maximum increase resulting from

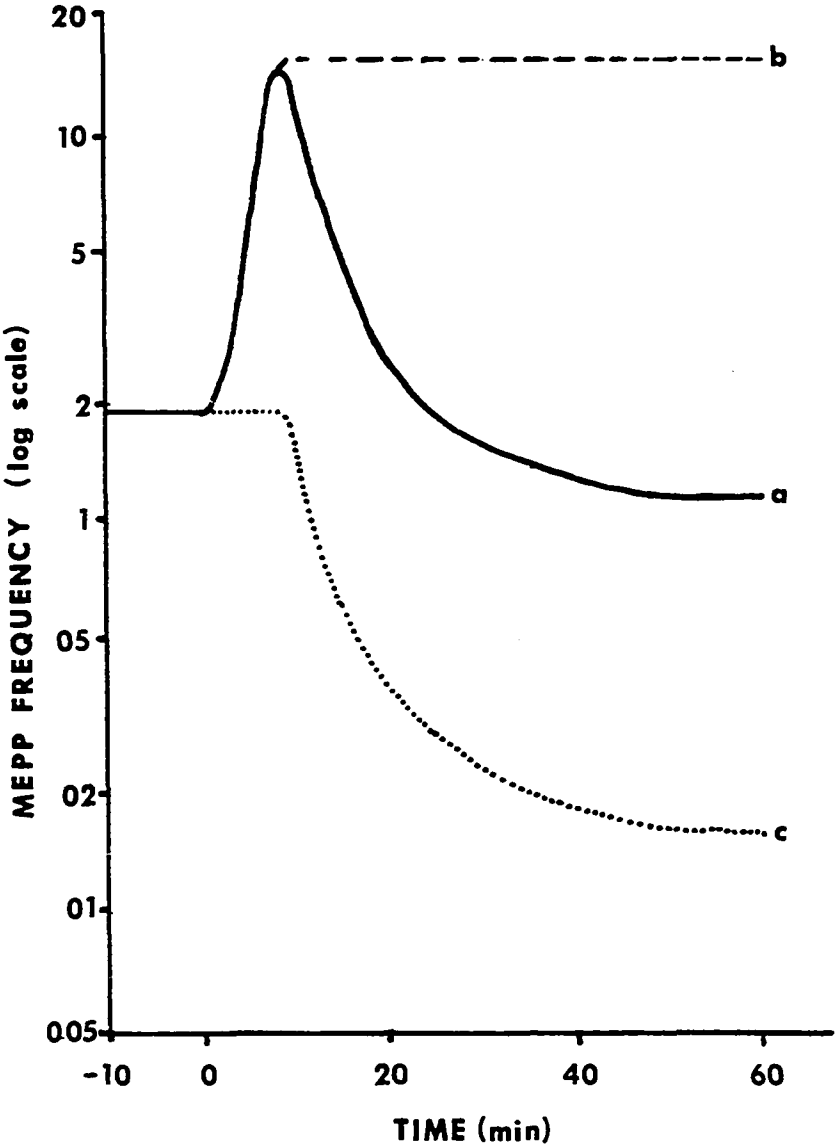


Figure 21. Separation of Ba-induced MEPP frequency curve into its components. Ordinate: MEPP frequency, log scale. Abscissa: time in minutes. Zero time indicates introduction of 0.5 mM Ba. Solid line (a) represents observed MEPP frequency, data same as Figure 9. Dashed line (b) represents plateau of increased MEPP frequency due to membrane depolarization. Dotted line (c) represents the total decline in MEPP frequency from the fast rates expected from line (b).

membrane depolarization? 2) What is the basis for the latency prior to MEPP frequency reduction (curve c)? 3) What are the implications of the 92% reduction from the expected MEPP frequency predicted by the theoretical curve?

Maximum initial increase. The observed initial increase in MEPP frequency achieves a maximum (14.5/sec) consistent with the predicted frequency change (12/sec) associated with a 17 mV depolarization (Hubbard et al., 1967).[1] Results presented here (Figure 20) indicate that the maximum acceleration was not achieved in the presence of Ba. The experiment that showed the highest initial increase in MEPP frequency was used for Figure 21. In most experiments the initial increase was much less or even absent (see Figure 3). Therefore, if all the experiments are considered, Ba does reduce the acceleration of MEPP's caused by membrane depolarization. Since the initial increase shown in Figure 21 is the highest recorded, this represents the maximum rate due to membrane depolarization, and MEPP frequency would be expected to plateau after this has been reached (Gage and Quastel, 1965; Hubbard et al., 1967).

Second Phase of Barium Action

Latency of phase II. It has been shown by others that K depolarizes the membrane before it acts at the MEPP frequency acceleratory site (Gage and Quastel, 1965; Hubbard et al., 1967). This action is complete within 10 min, the same time as required for Ba to produce its

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[1] Hubbard et al. (1967) showed that when the membrane is depolarized by 27 mV, MEPP frequency increases tenfold. Figure 9 shows that in this experiment Ba reduced the membrane potential from 67 to 50 mV, a 17 mV depolarization. Thus $17 \div 27 \times 10 = 6.3$ fold increase in MEPP frequency is predicted. If control MEPP frequency is 1.90/sec, then predicted MEPP frequency is $1.90 \times 6.3 = 12$/sec.

effect on membrane potential. All tested cations require 15-40 min to achieve their maximum action on the MEPP frequency acceleratory site. The action of Ba at this site was also complete within 40 min. Therefore, the latency between the actions of Ba on the membrane and at the acceleratory site is within the range observed for other cations.

Two components of MEPP frequency reduction. Line c in Figure 21 indicates the total frequency reduction necessary to account for the observed MEPP frequencies (line b), its minimum at 8% of control rates. There are two components of this frequency reduction. The first part of line c is due to a poor substitution of Ba for Ca at site I, the second part is due to a similarly poor affinity of Ba for site S (the acceleratory site). It is difficult to determine the relative contribution of each action of Ba to line c. There are three possibilities. Firstly, Ba may act at site I to reduce the depolarization-induced frequency to a rate above control. In this case in order to account for the MEPP frequency observed after 40 min, Ba must reduce release from the S site by an amount greater than the difference from its control rate. In line b the observed MEPP frequency after 40 min is 61% of control, while this reduction in all experiments ranged from 36-67% of control (Figure 3). Hubbard et al. (1968a) have shown that in Ca-free solutions MEPP frequency declines to 41-67% of control in rat diaphragm, and Miledi and Thies (1970) found MEPP frequencies 30-45% of control in Ca-free solution. Thus the observed reduction of frequency from control is within the range of frequencies expected in Ca-free solutions. MEPP frequency in the absence of added Ca has been attributed to a Ca-independent fraction of release (Hubbard et al., 1968a), but more likely is due to Ca remaining chelated

to the membrane (Miledi and Thies, 1970). That the nerve terminal binds Ca strongly was confirmed by ionophoretic application of Ca, restoring transmitter release even in the presence of 1 mM EGTA (Miledi and Thies, 1970). In order to reduce MEPP frequency below rates observed in Ca-free solution, Ba would have to replace this strongly bound Ca. Since Ba has a much lower affinity for site S, it is highly unlikely that Ba could replace the residual bound Ca. Therefore, Ba probably acts at site S only as a poor substitute for Ca, and could not account for an additional component of MEPP frequency reduction.

Secondly, it is possible that Ba acting at site I causes a reduction of MEPP frequency to rates slightly below control. It is not known what role site I may have in spontaneous transmitter release. However, if it can be assumed that site I is the Ca_3X complex, Hubbard et al. (1968a, b) have shown that all four carrier complexes (X, CaX, Ca_2X, and Ca_3X) are capable of spontaneous release. Inactivation of Ca_3X (site I?) could reduce slightly spontaneous release from the S site, implying that Ba reduces MEPP frequency even below Ca-free rates. Again, since rates in Ba alone were approximately those expected in Ca-free solutions, it is unlikely that Ba acts at site I to reduce MEPP frequency below control rates.

Thirdly, the most likely explanation for line c is that as Ca is leached away from site I in the presence of Ba, the link between membrane depolarization and release is broken, and MEPP frequency returns to control rates. Because Ba is also a poor substitute for Ca at site S, as Ca diffuses away from this site, MEPP frequency declines to rates expected in a Ca-free solution.

In summary, Ba acts at the K conductance (gK) "channel" to depolarize the nerve terminal membrane. Because Ca is still present at site I, depolarization leads to an increase in MEPP frequency within the first 10 min in Ba solutions. Frequency does not become stable (line b), but rather as Ca diffuses away from site I, MEPP frequency declines. Because Ba binds poorly to site S, as well as site I, MEPP frequency continues to decline below control rates, achieving stability after 40 min.

Third Phase of Barium Action

The second rise in MEPP frequency produced by prolonged exposure to Ba (phase III) is not temperature dependent (Figure 10). The cause of this second rise in frequency remains obscure. Two characteristics of this second rise in frequency were observed. Firstly, in only one experiment was the second rise in rate observed within the first 80 min exposure to Ba. Usually phase III was observed after 80 min, indicating either that the site of action is very remote to Ba or that the reaction rate affected is very slow. In any event the extremely long latency indicates that it probably is not acting at any previously described cation binding sites capable of influencing MEPP frequency. Secondly, in solutions with added Ca phase III was not observed over a range of Ba concentrations (0.25 and 1.25 mM Ba). Therefore, while the cause of phase III remains an enigma, it did not obscure the other observed actions of Ba.

Temperature Dependent Effects of Barium

Observations described in Results indicated that lowering temperature 10°C resulted in a less pronounced increase in MEPP frequency during phase I (Figure 6) and a faster steady state frequency (phase II;

Table 5). In addition, Table 5 shows that Ba-induced depolarization of the membrane is less at lower temperatures, though not significantly so. There are two explanations for this. There are at least two temperature dependent reactions involved in spontaneous transmitter release (Hubbard et al., 1967; Hubbard, 1970), and these display both positive and negative temperature dependence. Reducing temperature by 10°C could account for both positive and negative temperature dependence in the presence of Ba. A second temperature effect on MEPP frequency is ionization of solutes. Decreasing temperature from 30°C to 20°C results in a 6.6% reduction in Ba solubility, from 38.2 to 35.7 g/100 ml H_2O (Weast and Silby, 1966). However, for the same temperature reduction Ca is 27% less soluble (from 102 to 74.5 g/100 ml H_2O). This comparison of solubilities shows that reducing temperature by 10° causes Ca to be four times less soluble than Ba. While the temperature effect on solubility is an appealing answer because it would explain all three temperature dependent effects of Ba described above, it should be accepted with reservation. Solubility properties measured at such great salt concentrations (35 to 102 g/100 ml), need not be true in solutions with much lower concentrations. Therefore, the temperature dependence observed in the present series of experiments is largely due to temperature effects on reaction rates, although relative ionizations of Ba and Ca may have a slight influence.

In addition, temperature dependence of Ba action may be influenced by more Ca than Ba being dissolved in the same volume of water at any given temperature. At 30°C, $BaCl_2 \cdot 2H_2O$ has a maximum solubility of 38.2 g/100 ml H_2O, while $CaCl_2 \cdot 2H_2O$ has a maximum solubility of 97.7 to

326 g/100 ml H_2O (Weast and Silby, 1966). Based upon different maximum solubilities at the same temperature, it is possible that actions distinguishing Ca from Ba could reflect solubility differences. Yet, there are two reasons against accepting this hypothesis. Firstly, the weight of Ba used in these experiments was 0.006 to 0.061 g/100 ml of solution (Table 1, row IV). Maximum solubility is measured in solutions with approximately 1200 to 2900 times more Ba or Ca. Just as with the effects of changing temperature by 10°C, drawing conclusions from solutions with such high salt concentrations and applying these to solutions with much lower Ca or Ba concentrations may be invalid. Secondly, since ionic strength usually was held constant, relative activities of Ca to Ba did not change in different solutions at the same temperature. Therefore, differences in MEPP frequency between solutions reflect different effects of Ca and Ba more than differences in their ionization.

Model for Calcium - Barium Interaction

Surface Action of Calcium

Ca binds to the external surface of the nerve terminal membrane (Katz and Miledi, 1965; Miledi and Slater, 1966), and possibly is moved through the membrane by a carrier molecule "X" (del Castillo and Katz, 1954a; Jenkinson, 1957; Dodge and Rahamimoff, 1967; Katz and Miledi, 1967b; Hubbard *et al.*, 1968a, b). In the scheme of Hubbard *et al.* (1968a, b), carrier X can complex with 1, 2 or more Ca ions and release transmitter spontaneously, while release evoked by nerve impulses occurs when the Ca_3X complex of this series is activated. Depolarization of membranes leads to faster influx of Ca into nerve and skeletal muscle cells

(Fluckiger and Keynes, 1955, Keynes and Lewis, 1956; Hodgkin and Keynes, 1957; Bianchi and Shanes, 1959). Birefringence changes during the action potential in squid axon indicate a conformational change in membrane proteins (Cohen et al., 1968; Carnay and Barry, 1969), and conformational changes may represent a flux of charge carriers through the membrane (Wei, 1969; Hubbard, 1970). Since spontaneous and evoked release occur through a common mechanism (Hubbard et al., 1968b; Hubbard, 1970), Ca may act on the membrane carrier X to change its conformation, thus permitting movement of the CaX complex from external to internal membrane surface (Hodgkin and Keynes, 1957; Birks and MacIntosh, 1957; Hubbard et al., 1968a; Hubbard, 1970). Therefore, binding of Ca to the external surface of the nerve terminal membrane may lead to conformational changes and subsequently, transmitter release.

Allosteric Transitions and the Calcium Carrier

Allosteric effects of proteins are indirect interactions between distinct specific binding sites, resulting in a conformational change in protein structure - an allosteric transition (Monod et al., 1963). The primary function of allosteric proteins in biological systems is to amplify and translate low energy signals (Monod et al., 1965). Many proteins consist of two or more identical subunits or protomers. An association of identical protomers leads to the formation of a dimer (two protomers) or a tetramer (four protomers) with the tertiary bond region serving as an axis of symmetry to the associated protein. The non-covalent bonding between protomers results in changes in their quaternary structure, and this is modified by "allosteric ligands." The term

"ligand" is used here in a different context than in the discussion of EGTA and other chelating agents (see Introduction, p. 15, and Appendix I). In the present consideration an allosteric ligand is a molecule which binds to a specific site on a protomer, and thereby influences interprotomeric bonding and consequently protein conformation. A ligand may be a substrate, an inhibitor or an activator, and either facilitates or inhibits the primary function of the protein.

While allosteric transitions have been investigated primarily in proteins which are enzymes, the theory applies to any protein composed of identical subunits and capable of being modified by suitable ligands. If the Ca carrier were also an allosteric protein, then its activity would be modified by ligands according to predictions based on activities of enzymes. In the present context the Ca carrier X may contain at least three protomers (Hubbard et al., 1968a, b) and possibly four (Dodge and Rahamimoff, 1967). The ligand, Ca, serves as a "substrate" for the carrier protein and forms a CaX complex with it. When Ca binds to the protomer, the relatively small decrease in free energy permits a much closer apposition of identical protomers, and many more symmetrical bonds are formed. The result is a greater decrease in free energy, and a change in conformation of the protein. Therefore, the low energy signal involved in the binding of Ca to the protomer results in the amplification of the energy of ligand binding to a change in quaternary protein structure.

The presence of a substrate molecule bound to one protomer cooperates in binding further molecules of substrate to the other protomers. This cooperative action of substrate is typical of allosteric proteins (Monod et al., 1965), and is characterized by a sigmoidal relationship

between substrate concentration and reaction velocity, rather than the hyperbolic relationship typical of Michaelis-Menten kinetics. Dodge and Rahamimoff (1967) and Hubbard et al. (1968b) have shown that a sigmoidal relationship exists between Ca and quantal content of the end-plate potential. Thus, the presence of one Ca ion (substrate) cooperates in further Ca binding. Equations based upon cooperative Ca-carrier binding indicate that a common mechanism is involved in both spontaneous and nerve impulse evoked transmitter release (Hubbard et al., 1968a, b).

Barium as An Activator Ligand

If a ligand other than substrate acts at a second site on the same protomer to facilitate the allosteric transition, this ligand is called an activator. The presence of an activator reduces substrate cooperativity and shifts the sigmoidal substrate-rate relationship toward a hyperbolic curve as a limit. Since the monomeric, dimeric, and tri- or tetrameric forms of the protein all exist at equilibrium, the binding of an activator ligand to a protomer causes a shift in the equilibrium toward a conformational state more favorable to the association of the complete protein. It is possible that the proposed Ca carrier also contains an activator site which would reduce the cooperativity of substrate (Ca) binding. The results (p. 96) indicate that Ba may act at this activator site, assuming that Ca binds cooperatively.

A sigmoidal relationship between Ca concentration and end-plate potential quantal content has been observed by Dodge and Rahamimoff (1967) and Hubbard et al. (1968b). A sigmoidal curve was observed also for the effects of Ca on MEPP frequency (Hubbard et al., 1968a; Mambrini and Benoit, 1964). Hubbard et al. (1968a) have shown that Ca acts on a common

mechanism (carrier) in accelerating MEPP frequency and increasing quantal content. The results described here indicate that if Ca acts cooperatively on a membrane carrier, then Ba exhibits properties typical of an activator to such a system. It was shown in Results (p. 93) that solutions with Ba plus Ca accelerate MEPP frequency above rates in Ca alone up to 2.50 mM Ca. At 5 and 10 mM Ca MEPP frequency is affected by membrane repolarization so that the direct effect of Ba on MEPP frequency is obscured. Figure 22 shows the data of Figure 18 replotted to include only the lower Ca concentrations in the presence of Ba. In order to make a more valid comparison, all four concentrations of Ca without Ba were fitted by eye to the best straight line on log-log coordinates (not shown) and replotted in Figure 22. This differs from Figure 18, wherein the highest concentration of Ca was not included in the extrapolation to the ordinate. The lower curve with Ba in Figure 22 assumes that measured MEPP frequencies would lie on the dotted line if it were possible to measure MEPP frequency in Ba devoid of its effects on membrane potential. Figure 22 shows that curves with and without Ba might intercept at a common point. Note that the addition of Ba to the substrate Ca increases MEPP frequency at all concentrations of Ca. This action of Ba is typical of an allosteric activator (Monod et al., 1965). Ba acts at an activator site (A) making more carrier available for Ca and consequently MEPP frequency increases.

Carrier Model -- an Isologous Tetramer

Homologous allosteric effects involve cooperation between identical ligands to change the conformation of a protein. Heterologous allosteric effects result from interaction between different ligands. The

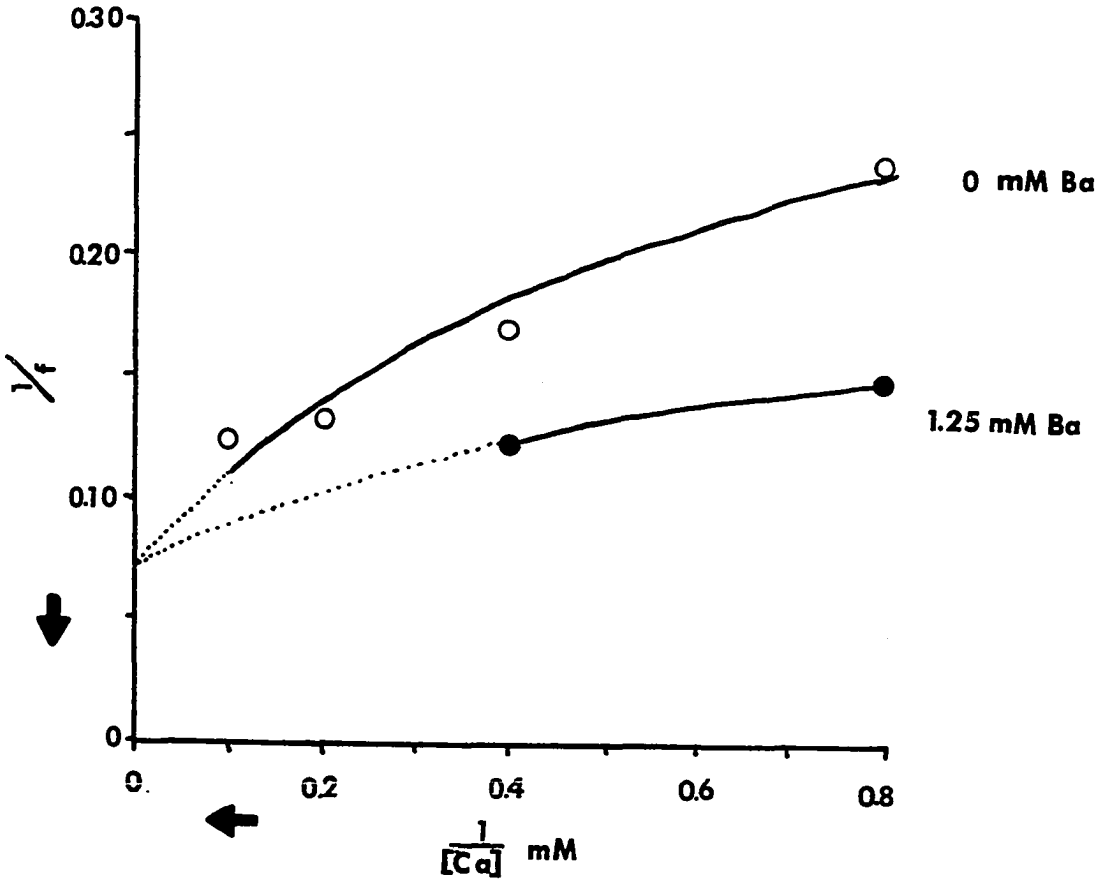

Figure 22. Double reciprocal plot of MEPP frequency vs Ca concentration, including 10 mM Ca. Data same as Figure 18 except higher concentrations of Ca + Ba are not included here. Ordinate: reciprocal of arithmetic means of MEPP frequencies (per sec). Abscissa: reciprocal of Ca concentration (mM). Preceding controls were used for Ca + Ba; interpolated controls used for Ca alone. Dotted lines indicate extrapolations of the solid lines to the ordinate. Note the solid line for Ca alone was drawn to include all four concentrations. Arrows indicate directions of increasing frequencies and concentrations.

cooperative action of Ca shows homologous cooperativity at its substrate binding site. Until the present report, heterologous cooperative interaction on this protein had never been observed.

The protein site of interaction has been shown to consist of four Ca binding sites. Since by definition each protomer contains only one substrate binding site, the allosteric protein must consist of at least four protomers--a tetramer. Figure 23 describes the interaction between Ca, Ba and Mg on this tetramer. When a substrate has a different binding affinity for each of two extremes of protein conformation, the substrate-protein interaction represents the K system. In the V system substrate binds equally with both extremes of protein conformation (Monod et al., 1965). Only in the K system will an activator reduce cooperativity - Ba has this effect. The model shown in Figure 23 (upper scheme) contains two basic assumptions. Firstly, the kinetics of Ca binding to the allosteric protein represent the K system. Secondly, the association of protomers probably forms an isologous tetramer. Most allosteric proteins studied are isologous, so that the region of bonding produces a two-fold axis of rotational symmetry (Figure 23, both schemes). Isologous associations between protomers can exist only in multiples of two, and since four sites of Ca action have been described, Figure 23 shows a model of an isologous tetramer.

According to Hubbard et al. (1968a, b), the carrier protein X acts to release transmitter even in the absence of Ca (or Ba). However, Miledi and Thies (1970) have shown that some Ca probably remains bound to the carrier site even in the presence of a strong chelating agent. Therefore, release from carrier X with no bound Ca is questionable. The

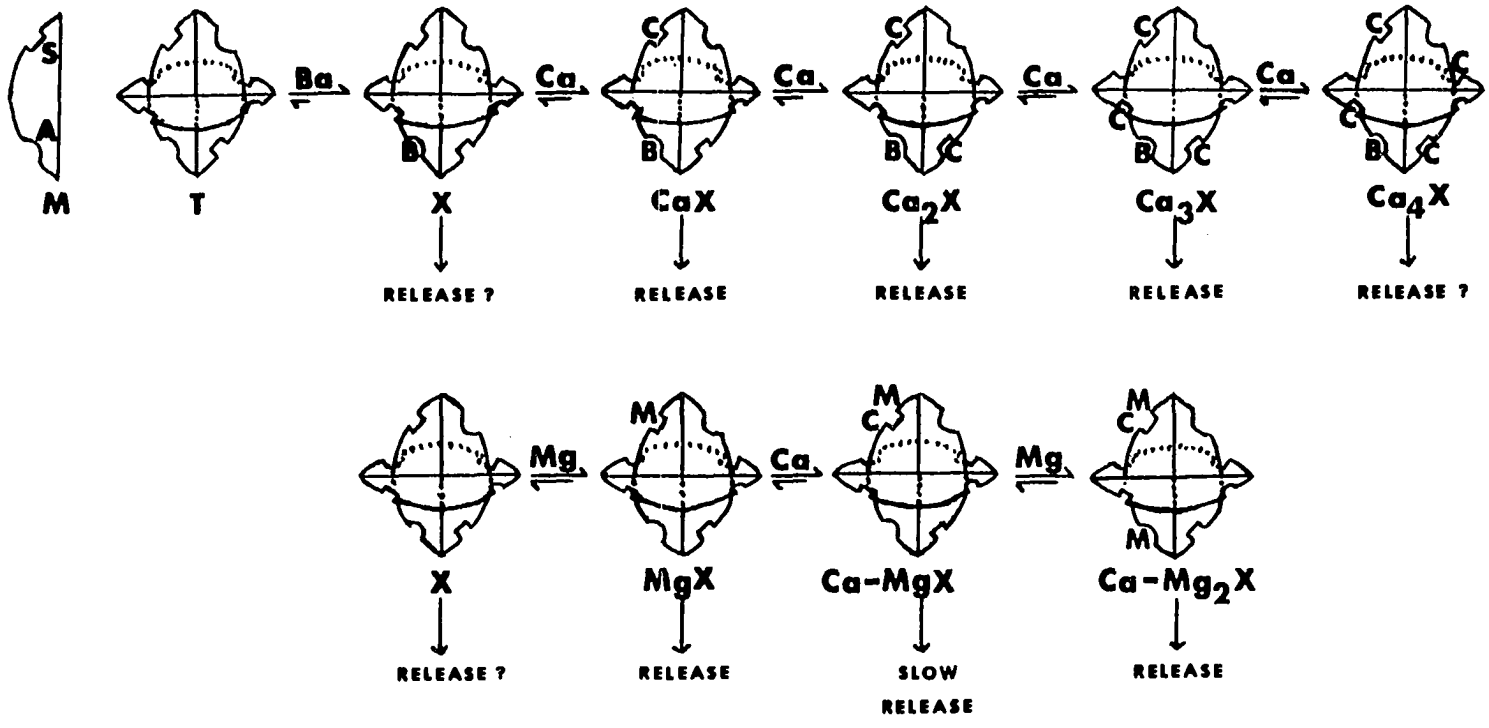

Figure 23. Model representing interactions of Ca, Ba and Mg on binding sites of an isologous tetramer. Upper scheme from left to right: a representative monomer (M) containing one substrate (S) site and one activator (A) site; T represents a tightly bound form of the tetramer incapable of producing transmitter release unless activated by Ba to the "X" form; association of 1 or more Ca^{2+} to the X form of the tetramer produces gradual cooperative interaction of Ca binding toward a hypothetical Ca_4X complex as a limit. Lower scheme: the same tetramer model illustrating interactions between Ca and Mg for site S and binding of high concentrations of Mg at site A.

binding of one Ca ion to a protomer produces a conformational change in the tetramer leading to a greater symmetry. Accompanying the conformational change is a decrease in free energy so that the reaction proceeds spontaneously to the right. Thus by mass action more unbound protein is made available for Ca binding. By this process Ca ions cooperate in changing the conformation of the protein. This cooperativity produces several Ca-carrier complexes all in equilibrium (CaX, Ca_2X, Ca_3X and Ca_4X). The existence of a Ca_4X complex is based upon the observation that four Ca binding sites interact in the frog neuromuscular junction (Dodge and Rahamimoff, 1967). Others have observed three sites of interaction, and each Ca complex is capable of release (Hubbard et al., 1968b; Jenkinson, 1957). More evidence is needed to account for the step between Ca-carrier binding and transmitter release. One theory suggests that the greater symmetry in protein conformation may make protein more hydrophobic and thus more soluble in the membrane lipid. Diffusion of the mobile carrier from the external to internal membrane surface would be facilitated by more Ca and therefore greater symmetry. At the internal membrane surface the presence of the carrier may serve as the link to transmitter release, but it is not due to the presence of Ca itself (Miledi and Slater, 1966). Normally vesicles appear to be in contact with specific areas of the presynaptic membrane (Hubbard and Kwanbunbumpen, 1968). At these areas the positive charge on the vesicle may bind electrostatically with the negative membrane charge (Hubbard, 1970; Landau and Kwanbunbumpen, 1969). It is possible that the carrier present on the internal surface of the membrane catalyzes the fusion of vesicular and nerve terminal membranes, subsequently releasing the contents of the

vesicle into the synaptic space.

Figure 23 (lower scheme) shows the way Mg in the presence of Ca may act as an inhibitor to MEPP frequency. In this system Mg is a substrate analog in that it can bind to the S site but not as well as Ca. In the absence of Ca, Mg can bind to the carrier X and facilitate spontaneous release though at a rate less than Ca (Hubbard et al., 1968 a b). Mg acts at two sites in the presence of Ca. Low Mg reduces MEPP frequency, while high Mg accelerates frequency when Ca is added. Figure 23 (lower scheme) shows that in the presence of Ca, low Mg competes with Ca at site S and reduces MEPP frequency. However, at higher concentrations Mg may combine with the activator site. If Mg were to combine with the A site, by mass action it would facilitate the binding of Ca to the S site. Therefore, it is possible that the second site of Mg action observed by Hubbard et al. (1968a) is the A site. Here Ba is a more potent activator than Mg.

Figure 3 in Results showed that Ba in the absence of added Ca permits transmitter release, although at a very low rate. Ba is thus a poor substitute for Mg and an even less effective substitute for Ca at the S site. When Ca is added to Ba containing solutions, MEPP frequency is greater than in Ca alone. It is possible that an activator, Ba, binds to an inactive form of the protein carrier T (Figure 23, upper scheme) producing a more active protein form X. The X form is equivalent to the usual R form in allosteric transitions (Monod et al., 1965; Mahler and Cordes, 1966). Ca readily binds to the active X form, and since more X form is available, MEPP frequency is higher. Ba thus facilitates Ca binding while it reduces the cooperative effect of Ca. The result is a

$[Ca^{2+}]$ - rate relation approaching a hyperbolic curve as a limit in solutions containing Ca plus Ba. This is implied by the results described in Figure 14A. If Ca, Ba and Mg were compared with respect to their relative potencies at sites A and S, two selectivity patterns emerge. At site S, Ca > Mg >> Ba; at site A, Ba > Mg > Ca.

Group Specific vs Stereospecific Ligands

Monod et al. (1965) state that group specific ligands (such as ions, SH reagents and detergents) may not behave as allosteric effectors, because they may bind functionally similar groups rather than geometrically similar sites. With respect to the present discussion, the argument seems one of semantics. Classically, the substrate is defined as that unique site on a protomer which when bound by an appropriate ligand yields the lowest free energy state. In the present context the unique substrate site is defined by the selectivity patterns Ca > Mg >> Ba. It is possible that a different site on the protomer could yield a greater conformational change and thus lower free energy, but this remains to be shown. Nevertheless, it is possible that in a single protomer two anionic groups may have identical selectivity patterns for Ca, Mg and Ba. If this were true, then the cooperative interactions described above might represent interactions on a single monomer. Whether the interaction is truly cooperative or not, the results indicate that the A and S sites are distinct because of the variability in selectivity patterns.

Isosteric vs Heterosteric Effects

Because Ba has a larger nonhydrated (ionic) radius than Ca, the question of stereospecificity of the binding site arises. Do the selec-

tivity patterns observed for the S and A sites represent actual differences in affinities (isosteric effects) or differences in steric hindrance to a common binding site (heterosteric effects)? Because Ba and Ca showed approximately equal accessibility to both A and S sites (p. 102), it is unlikely that the selectivity patterns reflect primarily differences in steric hindrance. However, the isosteric basis for selectivity patterns could be confirmed by a series of experiments using various concentrations of Ba but no added Ca. It was shown in Results (p. 97) that Ba binds weakly to the S site but strongly to A. In solutions without Ca, Ba acts as both activator and substrate. If increasing Ba concentrations in the absence of Ca produced faster MEPP frequencies, and if the relationship approached a hyperbola, then Ba at site A would be facilitating the binding of Ba (substrate analog) at site S. The implications of these experiments would be that if Ba could serve as its own activator, then the S site is not a steric hindrance to Ba. On the other hand, if these experiments did not show a hyperbolic relationship between Ba concentration and MEPP frequency, steric hindrance of Ba to site S may be the cause.

Irreversible Effects of Barium

The effects of Ba on MEPP frequency had an irreversible component (Results, p. 83). Because MEPP frequency failed to achieve expected rates in Ca (Figure 17), an irreversible binding of Ba to the S site may occur. Since reversibility of substrate to its binding site is presumed in allosteric kinetics, the potentially confounding effects of a partial irreversibility by Ba are apparent. Therefore, all conclusions drawn from observed cooperative actions of Ba on MEPP frequency should take into

account the fact that the action of Ba has an irreversible component. Nevertheless, Figures 3 and 17 indicated that Ba action was only slightly irreversible. While the slope of the Ba curve in Figure 22 may be an overestimation due to an irreversible Ba component, its essential relationship to the Ba-free curve is not changed. Therefore, the major action of Ba on the S site is compatible with the basic assumptions of allosteric kinetics.

Cation Selectivity Patterns

Selectivity Pattern at the S Site

Divalent cations. Hubbard (1961) and Hubbard et al. (1968a) observed that in the absence of Ca, Mg could accelerate MEPP frequency although rates were slightly lower in Mg than in Ca. Results presented here indicate that Ba was much less effective than Ca in accelerating MEPP frequency. Ba acts at site S primarily as a poor substitute for Ca and probably does not reduce MEPP frequency below the residual rate observed in Ca-free solutions (Discussion, p. 110). Dodge et al. (1969) observed that Sr at 1-5 mM had no effect on MEPP frequency in the frog and only occasional effects at 7-10 mM Sr. This indicates that Sr is also a poor substitute for Ca at site S. Since Ba has many secondary effects on rat diaphragm muscle, concentrations equimolar to Sr were not studied. Therefore, the relative affinities of Sr and Ba are not known.

The divalent cation selectivity sequences (from p. 18) are listed again below

I $Ba > Sr > Ca > Mg$

II $Ba > Ca > Sr > Mg$

III Ca > Ba > Sr > Mg

IV Ca > Ba > Mg > Sr

V Ca > Mg > Ba > Sr

VI Ca > Mg > Sr > Ba

VII Mg > Ca > Sr > Ba

The initial choice of Ba in studying MEPP frequency was based upon the selectivity isotherms for divalent cations calculated by Diamond and Wright (1969). Since Ba has the largest ionic radius and Ca is the most biologically prevalent of the four alkaline earth divalent cations, ratios of Ba:Ca potencies can predict all seven selectivity patterns for the four divalent cations. However, since the membrane strongly binds Ca, an irreducible MEPP frequency is always observed (50% of rates in 1.25 mM Ca). Therefore, this system will not permit observations of Ba:Ca ratios less than .50:1.00. In order to determine which selectivity pattern represents binding site S, relative action potencies must be determined qualitatively. It was shown (p. 125) that Ca is slightly more potent than Mg and much more than Ba or Sr at site S. On the basis of relative binding affinity, either sequence V or VI is the selectivity pattern representative of this site. While it is not possible to determine which of the two sequences actually represents the S site, either pattern indicates that site S is a relatively strong binding site with a large negative field strength (Diamond and Wright, 1969).

The high anionic field strength of site S, the MEPP frequency acceleratory site, may be due to closely spaced charges of very high field strength.[2] Sites displaying high field strength cannot be defined

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[2]In this context the conceptual term "site S" may actually

in terms of charge spacing based on divalent cation selectivities alone. Therefore, since site S displays a high field strength, both mono- and divalent cation sequences must be examined.

Monovalent cations. The relative affinities of monovalent and divalent cations to an anionic point charge will depend upon intercharge spacing. This phenomenon was described by Eisenman (1962), based upon studies of halogen-oxide crystals as representative of point negative charges of varying field strength. Monovalent cations generally have greater affinity for widely spaced fixed anions than divalent cations have. Conversely, closely spaced charges attract divalent cations more readily (Eisenman, 1962). It should be possible theoretically to resolve the question of intercharge spacing by comparing the relative effectiveness of mono- with divalent cations. If a monovalent selectivity sequence indicates high field strength, then the charges may be widely spaced. On the other hand, if a low field strength point charge is indicated by monovalent cation affinities, closely spaced fixed negative anions could be inferred.

In addition to point charge spacing it is also possible to estimate the pKa of the binding site by measuring both order and magnitude of monovalent cation selectivities over a wide range of pH. At lower pH protons would screen the fixed negative charges, and the field strength of the binding site would be reduced. At higher pH, fewer protons would

---

represent one strong negative charge or several closely associated weaker charges constituting a strong point charge. In strict ion exchange terminology a "site" is a fixed negative charge in the membrane. To prevent confusion, use of the word "site" in this discussion will be restricted to the general locus on a protein which binds a cation. Therefore, an allosteric site theoretically could consist of one or more fixed negative charges.

screen the fixed anion, and its field strength would be higher. Knowledge of monovalent cation selectivity with respect to pH would permit estimation of the pKa of the binding site from the pKa isotherms calculated by Eisenman (1961, cited in Diamond and Wright, 1969).

At the frog neuromuscular junction Na and Li decrease MEPP frequency, possibly by competing with Ca for the MEPP frequency acceleratory site S (Birks et al., 1968; Kelly, 1968). Lithium was not observed to have this effect in the rat diaphragm (Gage and Quastel, 1966). K can accelerate MEPP frequency distinct from its membrane depolarizing function (Gage and Quastel, 1965). With respect to cation selectivities, nothing can be concluded on the basis of the monovalent studies conducted thus far. Because five monovalent cations (Li, Na, K, Rb and Cs) display similar properties as alkali earth metals, at least four must be shown to compete with Ca before selectivity sequences can be applied validly. Studies of mono- and divalent cation interactions necessarily involve a heterovalent-concentration phenomenon which may tend to confound the data (Reichenberg, 1966). Therefore, resolution of intercharge spacing and pKa of binding site must await more complex studies of monovalent cation selectivities.

In summary, on the basis of divalent cation selectivities the MEPP frequency acceleratory site (site S) has been shown to be a relatively high field strength binding site consisting of either one very strong fixed negative charge or several moderately strong anions, closely spaced. Studies of monovalent cations will further describe spacing of the fixed negative charges, as well as the pKa of the binding site.

Other Sites of Calcium-Barium Interaction

In addition to Ca-Ba interaction at site S, the results presented here (p. 97) showed three other sites: site gK (the K conductance channel), site I in the depolarization-MEPP frequency linkage, and site A or the activator site.

Interaction at the gK (K conductance) site. Figure 8 showed that Ba was more effective than Ca in depolarizing the membrane. This was probably due to reduced K conductance in Ba. Mg had little effect on membrane potential. Therefore, the cation selectivity sequence for this gK blocking site is Ba > Ca, Mg. Table 7 lists a literature survey of the effects of the alkaline earth metals on depolarizing membrane resting potentials. Since the resting membrane potential is primarily determined by K conductance, these observations are relevant to the present discussion. Table 7 shows that all membranes studied display the same selectivity pattern for the gK site, with one exception in the order of Mg-Ca binding in frog sartorius muscle (Jenden and Reger, 1963). Therefore, in almost all membranes the gK "channel" binds Ba $\geq$ Sr $\geq$ Ca $\geq$ Mg, implying differences only in magnitudes of binding rather than order. According to the series of selectivity sequences (p. 125), this negative site has a very low field strength. Magnitude differences between membranes may arise from variable numbers of anions forming a coordination complex with the cation as a point negative charge.

Interaction at the I (coupling) site. It was observed (p. 96) that Ca was bound more firmly than Ba at a site which linked Ba- (or K-) induced membrane depolarization with an acceleration of MEPP frequency -- site I. With the amount of data available it is difficult to determine

TABLE 7

REDUCTION OF RESTING MEMBRANE POTENTIAL FROM LEVELS IN CALCIUM

Relative Depolarizing Ability	Membrane Type	Reference
Ba > Ca > Mg	Squid Giant Axon	Blaustein and Golsman (1968)
Ba > Ca	Cockroach Giant Axon	Narahashi (1961)
Ba > Ca	Frog Sartorius Muscle	Volle (1970)
Ba > Sr, Ca	Frog Sartorius Muscle	Sperelakis *et al.* (1967)
Ba > Sr > Mg > Ca	Frog Sartorius Muscle	Jenden and Reger (1963)
Ba, Sr > Ca	Crustacean Muscle	Fatt and Ginsborg (1958)
Ba, Sr > Ca	Lobster Muscle	Werman and Grundfest (1961)
Ba, Sr > Ca	Grasshopper Muscle	Werman *et al.* (1961)
Ba > Sr > Ca	Chick Heart Monolayers	Pappano and Sperelakis (1969)

whether site I is identical to the Ca_3X complex (Hubbard et al., 1968b), the Ca-Sr-Ba interaction site in induced transmitter release (Miledi, 1966; Dodge et al., 1969) and the site for "phasic" release (Miledi and Thies, 1970). At best the data presented here indicate no inconsistency with such an identity. The affinity sequence at the site activated by neural stimulation is Ca > Sr > Ba > Mg (Miledi, 1966; Dodge et al., 1969; Hubbard et al., 1968b). In the present investigation the observed sequence for site I was Ca > Ba. The physiological mechanisms which link membrane depolarization with transmitter release must be better understood before cation selectivities for site I can be interpreted.

Interaction at the A (activator) site. The activator site on the "carrier" protein displays the following cation selectivity: Ba > Mg > Ca (p. 123). This sequence does not fit any of the affinity patterns predicted by Sherry (cited in Diamond and Wright, 1969). There are four possible explanations for this. Firstly, it may be that Mg does not act at the activator site. The very high concentrations of Mg (9-12 mM) used by Hubbard et al. (1968a) to produce the frequency potentiating effect of Mg indicate that new Ca may be made available to site S by high Mg substituting for Ca stores. Secondly, since such high concentrations of Mg are used before its effects at site A are observed, it is possible that Ca itself is a better activator but its effect is obscured by a greater affinity for site S (cooperative interaction). Thirdly, site A may represent one of the few exceptional ion exchangers, deviating from the usual cation affinities observed in both biological and non-biological systems. Fourthly, site A may be indistinct from site I. The hyperbolic relationship between Ca concentration and MEPP frequency in the presence

of Ba (Figure 14) may reflect the Ba-depolarizing effect which was lost when Ca was leached away from site I in Ca-free solutions. However, in support of the existence of a distinct site A, double reciprocal plots of Ca vs rate (Figure 22), as well as characteristics displayed by Ba typical of an activator, favor acceptance of a site A as different from site I.

Biochemical Implications of Cation Selectivity Patterns

In addition to describing field strength and spacing of point charges in the receptor site, selectivity patterns provide a mechanism for comparing functional membrane binding sites with ion exchange sites in artificial membranes and organic polymers whose structure is known. Such comparisons are meaningful because of the tendency in both biological and non-biological systems to maintain fixed charges in only a few of the many possible geometric configurations.

The MEPP frequency accelerating site (site S) was shown (p. 126) to display affinity properties similar to those predicted by sequence V and VI. Bungenberg de Jong (1949) compiled a list of selectivity patterns derived from potencies of cations in reversing the negative charge on amphoteric colloids. Although the magnitudes, as well as sequences, of affinity patterns would define more precisely the S site, there are several important implications. Selectivity sequences V and VI were observed with egg lecithin, a phosphate colloid of egg lecithin plus 50% cholesterol, gelatin, casein, sphingomyelin and soya bean phosphatide. In addition Lehninger (1968) suggested that strong Ca-binding sites on nerve membranes may be sialic acid residues of membrane gangliosides which

are negative at pH 7. A ganglioside is a membrane bound glycoprotein containing many oligosaccharide branch chains terminating in sialic acid residues. In support of Lehninger (1968) Derry and Wolfe (1967) observed that the highest concentration of neuronal gangliosides occur in the membrane fraction of nerve endings. Extracted pellets of gangliosides from brain indicate a binding affinity sequence Ca > Mg > Na > K (Quorles and Folch-Pi, 1965; Spence and Wolfe, 1967). While the nerve terminal membrane may contain many charged sialic acid residues exposed to extracellular space, only one charge or small group of charges may be situated at a critical position. Interaction of Ca with this specific residue could induce a conformational change in the adjacent protein carrier (modification of Lehninger, 1968). At present it is not possible to assign this model to any of the four Ca-Ba sites of interaction previously described. Further investigations of selectivity sequences in both intact tissues and isolated membrane fractions are needed before structural and functional properties of nerve terminal membranes coincide.

Cation selectivity sequences are not to be used as a substitute for structural and functional analysis of transmitter release. Affinity patterns merely provide insight into an exceedingly complex series of mechanisms whereby the depolarization of the nerve terminal membrane results in the release of acetylcholine.

CHAPTER V

SUMMARY

In the rat phrenic nerve-hemidiaphragm preparation substitution of Ba for Ca produced a triphasic change in MEPP frequency. Ba produces an initial increase in MEPP frequency followed after 10 min by a gradual decline in frequency that stabilizes below control rates after 40 min. After 80 min MEPP frequency often rises to rates above control. The third phase is abolished when Ca is added to the solution with Ba.

Ba initially reduces K conductance, thereby depolarizing the nerve terminal membrane and increasing MEPP frequency. Within 10 min of exposure to Ba, Ca has diffused away from a binding site (I) which links depolarization to MEPP frequency acceleration. Ba substitutes poorly for Ca at this binding site (I) causing a fall in MEPP frequency. Ba further reduces frequency by substituting poorly for Ca at the MEPP frequency acceleratory site (S). In solutions with normal concentrations of Ca, Ba increases MEPP frequency perhaps by association with an activator site A. However, with elevated concentrations of Ca, addition of Ba reduces MEPP frequency. At those elevated concentrations Ca may stabilize the Ba-depolarized nerve terminal membrane, and consequently MEPP frequency declines.

A model is proposed accounting for Ca, Ba and Mg interaction on

a membrane carrier protein. Ca binds cooperatively with 1 to 4 binding sites on a proposed tetramer. Mg competes for these sites with Ca. Ba increases MEPP frequency by binding at an activator site A and reducing the sigmoidicity of the Ca-rate relation.

Cation selectivity sequences indicate that the MEPP frequency acceleratory site (S) consists of a point negative charge with a relatively high field strength. Estimation of intercharge spacing and acid dissociation constant (pKa) awaits analysis of monovalent cation binding affinities.

APPENDIX I

Calculation of Free Lanthanum, Calcium and Magnesium in the Presence of EGTA

Initially it was hoped that La could be used to study the Ca Mg binding site on the nerve terminal membrane. However, as the following calculations will show, in the presence of EGTA the free $[La^{3+}]$ was too low to be an effective Ca-substitute and the study of La was not pursued.

EGTA is an organic ligand containing four potential anionic sites dependent upon the pH of the solution. The top of Table 8 shows the association constants for the binding of H^+ to each of the four anionic sites. In addition, the possible reactions of Ca, Mg and La are also described and association constants are listed in the bottom of Table 8. These are the "true" association constants (K) which assume that all the form HL^{3-} of L^{4-} is free to combine with free metal ions. However, in real solutions some HL^{3-} becomes unavailable by dissociating H^+ or combining with additional H^+ depending upon the pH, while L^{4-} may combine with H^+ to form HL^{3-}. Therefore, an "apparent association constant" (K') must be derived for each of the metal ligand reactions listed in the bottom of Table 8, and using these values of K' the free concentrations of cations can be calculated.

Calculation of Apparent Association Constants

True (ideal) association constants. Complexes of the form MeHL

TABLE 8

ASSOCIATION CONSTANTS (K) AND REACTIONS USED FOR CALCULATION OF FREE CATION CONCENTRATIONS IN EGTA SOLUTIONS

Association constant	Association reaction	log K
K_1	$H^+ + L^{4-} \rightleftharpoons HL^{3-}$	9.46[a]
K_2	$H^+ + HL^{3-} \rightleftharpoons H_2L^{2-}$	8.85
K_3	$H^+ + H_2L^{2-} \rightleftharpoons H_3L^{1-}$	2.68
K_4	$H^+ + H_3L^{1-} \rightleftharpoons H_4L$	2.00 (about)
K_{MeL}[b]	$Ca^{2+} + L^{4-} \rightleftharpoons CaL^{2-}$	11.0[c]
	$Mg^{2+} + L^{4-} \rightleftharpoons MgL^{2-}$	5.21
	$La^{3+} + L^{4-} \rightleftharpoons LaL^{1-}$	15.55
K_{MeHL}	$Ca^{2+} + HL^{3-} \rightleftharpoons CaHL^{1-}$	5.33
	$Mg^{2+} + HL^{3-} \rightleftharpoons MgHL^{1-}$	3.37

[a]Values for association constants from Portzehl, Caldwell and Rüegg (1964).

[b]MeL indicates metal (Ca, Mg or La) bound to Ligand (EDTA).

[c]Values for association constants from Sillen and Martell (1964).

with EDTA containing a proton and a rare earth simultaneously do not occur in the equilibrium mixture (Wheelwright, Spedding and Schwarzenbach, (1953). If it were assumed that the same phenomenon holds for EGTA, then LaHL would not exist at equilibrium and need not be included in the present calculations. Therefore, the divalent metals, Mg and Ca, may combine with three species of ligand, L^{4-}, HL^{3-} and H_2L^{2-}. Equations describing the true association constants are

$$K_{MeHL^-} = \frac{[MeHL^-]}{[Me^{2+}]\ [HL^{3-}]} \quad (1)$$

$$K_{MeL^{2}} = \frac{[MeL^{2-}]}{[Me^{2+}]\ [L^{4-}]} \quad (2)$$

$$K_{MeH_2L} = \frac{[MeH_2L]}{[Me^{2+}]\ [H_2L^{2-}]} \quad (3)$$

Apparent (real) association constants. Equations for the apparent association constants are

$$K'_{MeHL^-} = \frac{[MeHL^-]}{[Me^{2+}]\ [\text{Total L added - total } Me^{2+} \text{ added}]} \quad (4)$$

$$K'_{MeL^{2}} = \frac{[MeL^{2-}]}{[Me^{2+}]\ [\text{Total L added - total } Me^{2+} \text{ added}]} \quad (5)$$

$$K'_{MeH_2L} = \frac{[MeH_2L]}{[Me^{2+}]\ [\text{Total L added - total } Me^{2+} \text{ added}]} \quad (6)$$

Equation 6 can be neglected because of the low probability of the formation of H_2L^{2-}, a necessary reactant to equation (6). The direct reaction $Me^{2+} + H_3L^-$, of course, is impossible.

Compensation for unavailable HL^{3-}. The difference between K_{MeHL^-} and K'_{MeHL^-} is the amount of the species HL^{3-} available for com-

bination with Me^{2+} (Portzehl et al., 1964). In K_{MeHL^-} it was assumed that $[HL^{3-}]$ was equal to $[L_{tot}$ added$]$, whereas in K'_{MeHL^-} a correction is made for this error. Therefore, $\frac{[L_{Total}]}{[HL^{3-}]} > 1$

and dividing equation (1) by equation (4),

$$\frac{K_{MeHL^-}}{K'_{MeHL^-}} = \frac{[L_{Total}]}{[HL^{3-}]} > 1 \qquad (7)$$

There are four possible side reactions making HL^{3-} unavailable to Me^{2+} (Top of Table 8, Portzehl et al., 1964). Firstly, some HL^{3-} dissociates to the L^{4-} form, thus,

$$\text{(association)} \quad K = \frac{[HL^{3-}]}{[H^+][L^{4-}]}$$

$$\text{(dissociation)} \quad \frac{1}{K_1} = \frac{[H^+][L^{4-}]}{[HL^{3-}]}$$

and

$$\frac{[L^{4-}]}{[HL^{3-}]} = \frac{1}{[H^+]\,K_1} \qquad (8)$$

which is the fraction of HL^{3-} going to L^{4-}. Secondly, some HL^{3-} associates with H^+ to form H_2L^{2-}, thus

$$K_2 = \frac{[H_2L^{2-}]}{[H^+][HL^{3-}]}$$

and

$$\frac{[H_2L^{2-}]}{[HL^{3-}]} = K_2\,[H^+] \qquad (9)$$

which is the fraction of HL^{3-} associating to form H_2L^{2-}. Thirdly, some HL^{3-} associates with $2H^+$ to form H_3L^-, thus,

$$K_3 = \frac{[H_3L^-]}{[H^+]\;[H_2L^{2-}]}$$

and substituting equation (9) for $[H_2L^{2-}]$,

$$K_3 = \frac{[H_3L^-]}{[H^+][H^+][HL^{3-}]K_2}$$

and $$\frac{[H_3L^-]}{[HL^{3-}]} = [H^+]^2 \quad K_2K_3 \qquad (10)$$

which is the fraction of HL^{3-} associating to form H_3L^-. Fourthly, some HL^{3-} associates with 3 H^+ to form H_4L, thus,

$$K_4 = \frac{[H_4L]}{H \quad H_3L^-}$$

Substituting equation (10) for $[H_3L^-]$ gives

$$K_4 = \frac{[H_4L]}{[H^+][H^+]^2\ [HL^{3-}]\ \ K_2K_3}$$

and $$\frac{[H_4L]}{[HL^{3-}]} = [H^+]^3 \quad K_2K_3K_4 \qquad (11)$$

which is the fraction of HL^{3-} associating to form H_4L. The total amount of ligand (L) added to the solution is

$$[L_{Tot}] = [L^{4-}] + [HL^{3-}] + [H_2L^{2-}] + [H_3L^-] + [H_4L] \qquad (12)$$

In order to place equation (12) into the form of equation (7), dividing through by $[HL^{3-}]$ gives

$$\frac{[L_{Tot}]}{[HL^{3-}]} = 1 + \frac{[L^{4-}]}{[HL^{3-}]} \quad \frac{[H_2L^{2-}]}{[HL^{3-}]} \quad \frac{[H_3L^-]}{[HL^{3-}]} \quad \frac{[H_4L]}{[HL^{3-}]} \qquad (13)$$

Substituting into equation (12) the values obtained in equations (8), (9), (10), and (11),

$$\frac{[L_{Tot}]}{[HL^{3-}]} = 1 + \frac{1}{[H^+]K_1} + K_2[H^+][H^+]^2K_2K_3 + [H^+]^3K_2K_3K_4 \qquad (14)$$

It was stated in the Introduction that pH 6.0 was to be maintained in all solutions. Therefore, substituting into equation (14) the association

constants for K_1, K_2, K_3 and K_4 (top of Table 8) and $[H^+] = 10^{-6}$ M, it is shown that

$$\frac{L_{Tot}}{HL^{3-}} = 708.98 \qquad (15)$$

Equation (7) showed that

$$\frac{K_{MeHL^-}}{K'_{MeHL^-}} = \frac{[L_{Tot}]}{[HL^{3-}]} > 1$$

consequently,

$$\frac{K_{MeHL^-}}{K'_{MeHL^-}} = 708.98 \qquad (16)$$

Using the value for the ratio derived in equation (16) and the association constants from Table 8, K'_{MeHL} was calculated for each cation (Ca, Mg and La) and is shown in the top row of Table 9. A derivation similar to that shown by equations (7) through (16) resulted in a ratio for K'_{MeL}, such that

$$\frac{K_{MeL}}{K'_{MeL}} = \frac{[L_{Tot}]}{[L^{4-}]} = 10^{6.31} \qquad (17)$$

Again, K'_{MeL} was calculated for each cation and is shown in second row of Table 9. Because K_{MeH_2L} is insignificant in comparison to $K_{MeL_{Tot}}$, K'_{MeH_2L} was not included in computing $K'_{MeL_{Tot}}$. The apparent association constants (K') for each cation were summed to $K'_{MeL_{Tot}}$. The total K' was used to estimate free $[La^{3+}]$, $[Ca^{2+}]$ and $[Mg^{2+}]$.

Calculation of free cation concentrations. Estimations of free La, Ca and Mg concentrations were based on the method of Portzehl et al. (1964) except that in the present report three cations had to be considered rather than two. The basic form of the equation was

TABLE 9

CALCULATED APPARENT ASSOCIATION CONSTANTS (K') AND FREE [Me]

Apparent Association Constant	La	Ca	Mg	Ligand
K'_{MeHL}	(a)	2.965×10^{2}	3.312	
K'_{MeL}	1.739×10^{9}	4.895×10^{4}	0.031	
K'_{MeH_2L}	(b)	(c)	(c)	
$K'_{MeL_{Tot}}$	1.739×10^{9}	4.924×10^{4}	3.343	
Me Partition				
$[Me_{added}]$	5×10^{-3} M	3×10^{-5} M	10^{-5} M	10^{-2}
$[Me_{bound}]$	4.994×10^{-3}	2.987×10^{-5}	1.30×10^{-7}	5.21×10^{-3}
$[Me_{free}]$ 1st Approx.	6.44×10^{-6}	1.257×10^{-7}	9.87×10^{-6}	4.96×10^{-3}
$[Me_{free}]$ 2nd Approx.	5.78×10^{-10}	1.22×10^{-7}	9.845×10^{-6}	

[a]Complexes of the form MeHL for La probably do not occur in equilibrium mixtures.

[b]Theoretically impossible combination.

[c]Calculations sufficiently exact without calculating these constants.

$$K'_{MeL} = \frac{[MeL]}{[Me\ free]\ [L\ free]}$$

which can be converted to

$$K'_{MeL} \doteq \frac{[Me_{added} - Me_{free}]}{[Me_{free}][L_{added} - Me_1L - Me_2L - Me_3L]}$$

where Me_1, Me_2 and Me_3 are La, Ca and Mg. Maximum "buffering" capacity of a ligand occurs when [Ca added] / [Ligand total] = 0.5. In the present context the "Ca added" was expanded to include the sum of all metal ions added. Therefore, the selection of amounts of ligand and metals to be added (listed in the bottom of Table 9) was made with this ratio in mind. The simplest initial experiments would have been to observe the effects of La alone in solution. For this case no Ca or Mg would be added to the solution, while the maximum concentration of La utilized in these experiments would have been 5.0 mM. Therefore, 10 mM EGTA was used in the calculations.

For the first approximation, $[Me_2L]$ and $[Me_3L]$ were assumed to be equal to their added concentration (residual amounts in Ca-free and Mg-free solutions). Thus a first approximation for free $[La^{3+}]$ calculated and shown in Table 9 was repeated for each cation. These concentrations were used in estimating the $[L_{free}]$ remaining in solution, and this $[L^{4-}_{free}]$ was used to calculate the second approximations to free cation concentrations. Table 9 indicates that even when 5.0 mM La is added to a solution with EGTA, only 5.78×10^{-10} La remains unbound. This concentration was far below a theoretically acceptable range (1.0 to 0.1 mM). Consequently, the effects of La on MEPP frequency in the presence of EGTA were not pursued. While it may have been possible to stabilize free

cation concentrations using another chelating agent, this was not attempted since the study of the effects of Ba on MEPP frequency promised to provide much more information about receptor sites.

APPENDIX II

Method of Fitting Data of Figures 14A and 19 to a Common Graph

Comparison of MEPP Frequencies in Identical Solutions

Experiments shown in Figure 19 were done with 0.25 mM Ba and 1.25 mM Ca in control solutions. An interpolation method was used to compute controls for Figure 19. Interpolated controls were also used for the data shown in Figure 14A, although these tended to give less exact agreement with an estimated curve. Both curves shown in Figure 20 were based upon interpolated controls. This made the inclusion of both curves in a common graph more valid.

The design of the experiments illustrated in Figure 19 included two concentrations of Ba and Ca in common with experiments shown in Figure 14. Consequently, two points of reference from both series of data could be illustrated on a single graph. These two points were for 1.25 mM Ca, and 1.25 mM Ca plus 1.25 mM Ba. The arithmetic mean MEPP frequency of all controls (1.25 mM Ca) in Figure 14A was 4.23/sec. Mean MEPP frequency in 1.25 mM Ca shown in Figure 19 was 4.51/sec. Therefore MEPP frequency in 1.25 mM Ca was 6.6% higher in the data of Figure 19 than Figure 14A. In computing data for Figure 20, the curves were assumed to intersect the origin at almost a common point (6.6% difference).

The second point of overlap was at 1.25 mM Ca plus 1.25 mM Ba.

In the constant Ba experiments (Figure 14A) mean MEPP frequency was 6.64/sec, while in constant Ca experiments (Figure 19) MEPP frequency was 5.74/sec in solutions containing 1.25 mM Ca plus 1.25 mM Ba. This represented a MEPP frequency reduction of 13.5% in the data of Figure 19 compared with Figure 14A. If data from the two series of experiments were plotted on Figure 20 with no compensation for this 13.5% difference in MEPP frequency at these concentrations, the absolute differences between the points of the two curves would be an underestimate of their true differences.

Calculation of a Correction Factor

In order to have equal MEPP frequencies at 1.25 mM Ca plus 1.25 mM Ba for both series, a correction factor was calculated. Let f_1 = MEPP frequency in this solution in the series of experiments in which Ba was constant (Figure 14A). Let f_2 = MEPP frequency in this solution in the series of experiments in which Ca was held constant (Figure 19). Then,

$$\left(\frac{f_1 - f_2}{f_2} + 1\right) = \text{CF (Correction Factor)}$$

Specifically, if f_1 = 6.64 MEPP's/sec, and f_2 = 5.74 MEPP's/sec, then

$$\left(\frac{6.64 - 5.74}{5.74} + 1\right) = 1.157$$

This correction factor (1.157) was used for computing the corrected V/C ratios shown in Table 10. Log V/C of MEPP frequencies (Table 10, far right) were plotted in Figure 20. Data from the upper half of Table 10 are plotted as closed symbols in Figure 20, and from the lower half of Table 10 as open symbols.

TABLE 10

CORRECTION OF ARITHMETIC MEAN MEPP FREQUENCIES AND CONVERSION TO LOGARITHMS (LOG V/C) USED TO COMPARE TWO SERIES OF DATA IN FIGURE 20

$[Ca^{2+}]$ mM	$[Ba^{2+}]$ mM	Means of MEPP frequencies (V/C)[a]	Correction Factor	Corrected V/C	Log V/C
1.25	1.25	$\frac{6.64}{4.23}$		1.57	0.196
2.50	1.25	$\frac{8.40}{4.23}$		1.98	0.297
5.0	1.25	$\frac{6.64}{4.23}$		1.57	0.196
10.0	1.25	$\frac{5.86}{4.23}$		1.38	0.140
1.25	0.25	$\frac{4.63}{4.23}$	1.157	1.27	0.104
1.25	0.50	$\frac{5.48}{4.23}$	1.157	1.50	0.176
1.25	1.25	$\frac{5.74}{4.23}$	1.157	1.57	0.196
1.25	2.50	$\frac{7.97}{4.23}$	1.157	2.18	0.338

[a]V/C = Variable/Control MEPP frequencies.

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