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Safety factor at the neuromuscular junction

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Abstract

Reliable transmission of activity from nerve to muscle is necessary for the normal function of the body. The term 'safety factor' refers to the ability of neuromuscular transmission to remain effective under various physiological conditions and stresses. This is a result of the amount of transmitter released per nerve impulse being greater than that required to trigger an action potential in the muscle fibre. The safety factor is a measure of this excess of released transmitter. In this review we discuss the practical difficulties involved in estimating the safety factor in vitro. We then consider the factors that influence the safety factor in vivo. While presynaptic transmitter release may be modulated on a moment to moment basis, the postsynaptic features that determine the effect of released transmitter are not so readily altered to meet changing demands. Different strategies are used by different species to ensure reliable neuromuscular transmission. Some, like frogs, rely on releasing a large amount of transmitter while others, like man, rely on elaborate postsynaptic specialisations to enhance the response to transmitter. In normal adult mammals, the safety factor is generally 3–5. Both pre- and postsynaptic components change during development and may show plasticity in response to injury or disease. Thus, both acquired autoimmune and inherited congenital diseases of the neuromuscular junction (NMJ) can significantly reduce, or even transiently increase, safety factor.

Contents

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; AChRs, acetylcholine receptors; ARIA, acetylcholine receptor inducing activity; μCTX, μ-conotoxin; EDL, extensor digitorum longus; EMG, electromyogram; EPC, endplate current; EPP, endplate potential; FIM, familial infantile myasthenia; K_{Ca}, Ca^{++}-activated K^+-channels; LEMs, Lambert-Eaton myasthenic syndrome; MFS, Miller–Fisher syndrome; MG, myasthenia gravis; minEPC, miniature endplate current; minEPP, miniature endplate potential; NMJ, neuromuscular junction; R_{in}, input resistance; SCS, slow-channel syndrome; SkM1/2, skeletal muscle-specific isoforms of voltage gated Na^+-channels; TTX, tetrodotoxin; TTX-R, TTX-resistant; TTX-S, TTX-sensitive; VGCCs, voltage-gated Ca^{++} channels; VGKCs, voltage-gated K^+-channels; VGSCs, voltage gated Na^+-channels..

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1. Neuromuscular transmission is a highly reliable process

The transmission of signals from nerve to muscle is an extremely reliable process. In normal humans, no indication of a failure of neuromuscular transmission is seen even during the most extreme voluntary exertion. This is mainly because more transmitter is released from the motor nerve terminal by each nerve impulse than is required to excite the muscle fibre. This ensures that during prolonged, high-frequency activation of muscles, when the amount of transmitter released per nerve impulse declines substantially, transmission does not fail. The existence of this reserve capacity for transmitter release from the nerve has given rise to the notion of a 'safety factor' for neuromuscular transmission.

An understanding of features of the neuromuscular junction (NMJ) that underlie this safety factor is of interest at several levels. At a basic level, there is interest in how the NMJ carries out its function and how the essential features of the NMJ arise during development. There is the broader issue of how the efficacy of chemical synapses throughout the nervous system is controlled, both during development and in the mature animal. Finally, an understanding of the safety factor of neuromuscular transmission is of clinical importance, providing the basis for understanding the nature of pathological conditions in which neuromuscular transmission is impaired and for devising effective therapies for those conditions.

In recent years, a number of new insights into the functional organisation of the vertebrate NMJ have been gained. These have led to a more complete understanding of the process of neuromuscular transmission and how its efficacy is ensured. It is therefore timely to examine the basis of the safety factor. In this review, we will first outline the functional organisation of the NMJ and then consider the concept of the safety factor and how it may be defined and measured. This will be followed by a detailed look at the structural and biological features of the NMJ that influence the safety factor. Finally, we will consider the clinical relevance of the safety factor.

Although there is much interesting new information about the regulation of synaptic efficacy at invertebrate NMJs (Davis and Goodman, 1998), we have restricted this review to vertebrates.

2. Functional organisation of the NMJ

Many features of the structural and functional organisation of the NMJ influence the safety factor. Excellent detailed descriptions of the anatomy of the neuromuscular junction can be found elsewhere (Couteaux, 1973; Salpeter, 1987; Engel, 1994b). Here, we describe briefly the main structural features of the NMJ.

2.1. Presynaptic structure and transmitter release

In mammals and most higher vertebrates, each skeletal muscle fibre is innervated at a single site by a single myelinated motor axon. At the NMJ, the motor axon loses its myelin sheath and forms a number of branches which make synaptic contact with the muscle fibre (Fig. 1). At the ultrastructural level (Fig. 2), the nerve can be seen to contain a large number of small clear synaptic vesicles which contain the transmitter acetylcholine (ACh), numerous mitochondria and a core of filamentous proteins. In favourable views, some of the synaptic vesicles can be seen to form clusters around small dense spots on the presynaptic membrane, the so-called 'active zones' (Fig. 3). The active zones consist of an ordered array of intramembranous particles which can be seen in freeze-fracture preparations. It is at these sites, located opposite to the openings of the postsynaptic folds (Section 2.2), that vesicle exocytosis and the release of ACh occur during neuromuscular transmission (Ceccarelli and Hurlbut, 1980; Heuser and Reese, 1981).

The release of transmitter is triggered by an increase in the concentration of free Ca^{++} within the nerve terminal. This results from the opening of voltage-gated Ca^{++} channels (VGCCs) by the depolarisation of the nerve impulse. There is strong evidence that these channels correspond to at least some of the intramembranous particles that make up the active zones (Robitaille et al., 1990, 1993a). The Ca^{++} flowing through these channels acts rapidly on the small fraction of vesicles 'docked' within less than 100 nm of the active zones (Augustine et al., 1992; Stanley, 1997). There is also increasing evidence that depolarisation itself may play some role in determining the time course of quantal release (Parnas et al., 2000). In addition to Ca^{++} channels, several forms of potassium channel are present in the nerve terminal, including voltage-gated and Ca^{++}-activated potassium channels (VGKC, K_{Ca}). There is some evidence that K_{Ca} channels are components of the active zones (Robitaille et al., 1993a,b). The potassium channels are likely to limit the duration of nerve terminal depolarisation and hence the extent of Ca^{++} entry and transmitter release. In recent years, though many of the molecules involved in exocytosis have been identified (Calakos and Scheller, 1996; Fernandez-Chacon and Sudhof, 1999), many details of their functional interactions remain to be elucidated.

Each vesicle appears to contain 5000–10 000 molecules of ACh (Martin, 1965; Kuffler and Yoshikami, 1975). The ACh contained in a single vesicle is often referred to as a 'quantum' of transmitter,

though its amount and effect on the postsynaptic membrane shows considerable statistical variation. Spontaneous exocytosis of the contents of individual vesicles occurs at most motor nerve terminals at a rate of several per minute to several per second. These give rise to small depolarisations ($\sim$1 mV) of the muscle fibre membrane, the miniature endplate potentials (minEPPs Fig. 4). A nerve impulse causes the release of about 20–200 quanta, depending on the species (see Section 6.1), within a fraction of a millisecond. The quanta act together to give rise to a larger depolarisation of the muscle cell surface, the end-plate potential (EPP; Fig. 4). The number of quanta released by a nerve impulse at a given NMJ is known as the 'quantal content' of the EPP. The quantal content can be determined electrophysiologically and is a commonly used measure of transmitter release (see Section 4.2.2).

2.2. Postsynaptic structure and transmitter action

At many NMJs the most striking structural features of the postsynaptic region are the deep infolding of the sarcolemma (Fig. 5). These are particularly prominent in human NMJs (see Section 6.3.2). Different ion channels are present in different regions of the postsynaptic membrane infoldings. The crests of the folds contain a very high density of acetylcholine receptors (AChRs) (Fig. 2) (Salpeter, 1987) which may explain the characteristic curvature of the membrane in this region. The openings of the folds are aligned with the active zones in the nerve terminal (Fig. 3) (Engel, 1994b), an arrangement that allows the transmitter to act on a sub-millisecond time scale. In recent years, it has become clear that in the depths of the folds, while there are few AChRs, voltage-gated sodium channels (VGSCs) are present at high density (Flucher and Daniels, 1989; Wood and Slater, 1998b) (Fig. 6).

The arrangement of ion channels in the postsynaptic folds is well suited for the generation of action potentials. Released ACh diffuses to the postsynaptic membrane where it binds to the AChRs, causing the opening of cation-selective ion channels and allowing a net flux of positive charge into the cell. The effect of this current is a depolarisation of the membrane which is greatest within the folds where the high density of VGSCs is highest (Flucher and Daniels, 1989). When the depolarisation is adequate to open these channels, the threshold for action potential generation is reached (Fig. 4). The value of the threshold is influenced by the structure of the postsynaptic folds themselves and the distribution of ion channels within it (see Section 5.2). It has recently been suggested that the structure and molecular organisation of the postsynaptic apparatus makes highly efficient use of the transmitter released from the nerve, helping to ensure that every nerve impulse normally gives rise to a muscle action potential (Martin, 1994; Wood and Slater, 1995, 1997).

2.3. Synaptic cleft and termination of transmitter action

Between the nerve and muscle cells, there is a synaptic cleft some 50–100 nm wide. Within this cleft, and surrounding each nerve and muscle fibre, is a single

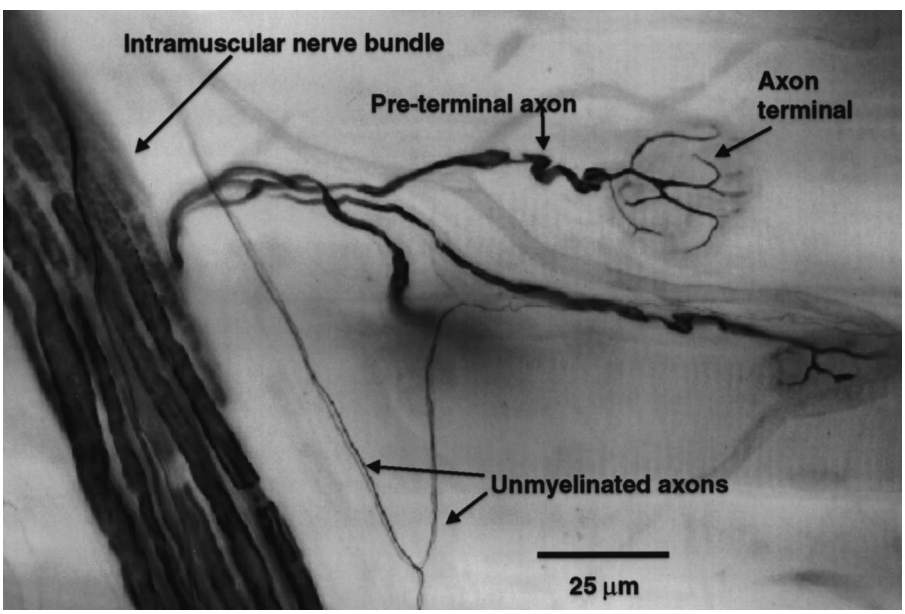

Fig. 1. Motor innervation of mouse muscle fibres. Each muscle fibre is innervated by a single myelinated preterminal motor axon which extends from the intramuscular nerve bundle. In the region of contact with the muscle, the myelin sheath is lost and the axon forms numerous terminal branches. Unmyelinated axons, presumably innervating blood vessels, are also present. Silver impregnation.

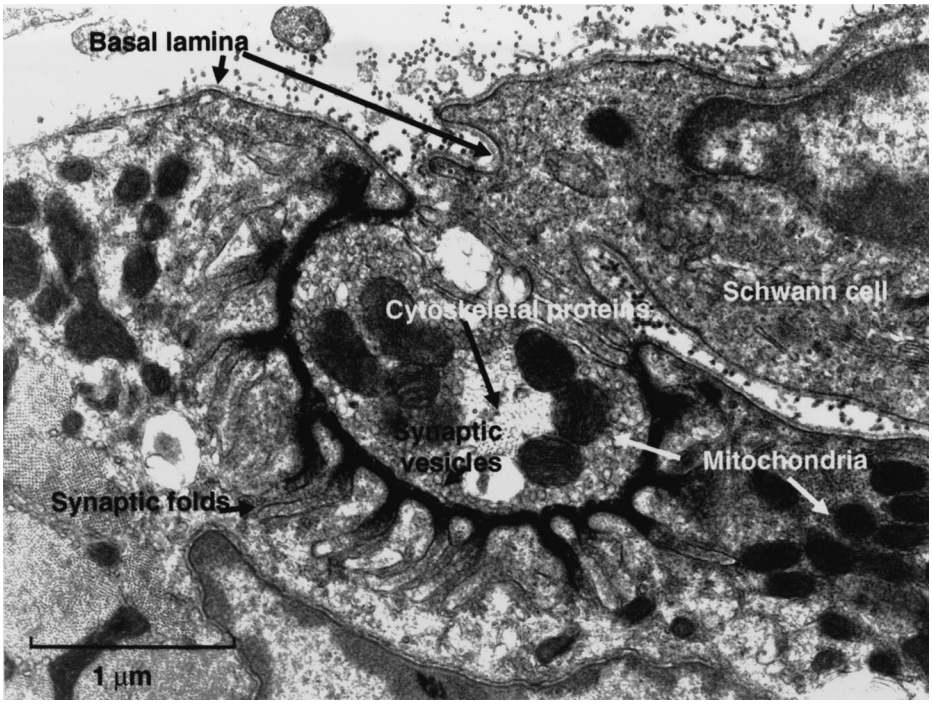

Fig. 2. Electron micrograph of a mouse neuromuscular junction. The nerve terminal, which occupies a depression ion the muscle fibre surface, contains synaptic vesicles, mitochondria and a core of cytoskeletal proteins. (It is the latter that are revealed by the silver impregnation in Fig. 1). The postsynaptic surface of the muscle fibre contains numerous infoldings about 0.6 μm deep. Acetylcholine receptors, labelled here with an electron-dense reaction product indicating HRP-α-bungarotoxin, are concentrated in the plasma membrane at the crests of the folds. The nerve terminal is capped by processes of a terminal Schwann cell (from Lyons and Slater, 1991, with permission).

layer of condensed extracellular matrix, the basal lamina (Figs. 3 and 5). Associated with the synaptic portion of the basal lamina are a number of proteins and glycoproteins that are not present in non-synaptic regions, including acetylcholinesterase (AChE), laminin β2, agrin and ARIA (Sanes and Lichtman, 1999). Most relevant of these to a consideration of the safety factor is AChE, the enzyme that rapidly cleaves ACh, thus limiting both the temporal and spatial extent of transmitter action (see Section 5.2.1).

3. The Concept of safety factor

3.1. Origin of the concept of a safety factor in excitable cells

The reliability of neuromuscular transmission normally results from the release of more quanta of ACh than are required to initiate an action potential. The term safety factor (or safety margin-the terms are used interchangeably) for neuromuscular transmission is used to describe this excess. However, the concept of safety factor for cell excitation was initially developed in the context of the initiation and propagation of action potentials in myelinated axons. Early experimental studies suggested that the longitudinal current flowing within a myelinated axon from an active to an inactive region was 5–10 times greater than that required to initiate an action potential at the node of Ranvier (Hodgkin and Rushton, 1946). Other studies pointed out that the critical factor for the initiation of an action potential is not the amplitude of the current applied at a point but the total positive charge (current $\times$ time) entering the cell (Rushton, 1937). Of particular importance is that the charge transferred, and the membrane depolarisation it causes, should be distributed along the axon in such a way that enough VGSCs are opened to allow a net influx of positive charge (Jack et al., 1975), thus setting in train the regenerative events leading to the action potential. In the context of neuromuscular transmission, this means that the critical factor for triggering an action potential is that the positive charge entering the muscle fibre, as a result of transmitter action, should result in the opening of enough VGSCs to initiate a similarly regenerative response in the surrounding muscle fibre membrane.

Experimental studies of muscle excitation during voluntary contractions in normal human subjects suggest that failure of neuromuscular transmission rarely occurs even during extreme exertion (Bigland-Ritchie et al., 1978, 1982). Many subsequent studies of isolated nerve-muscle preparations have established that substantially more transmitter is released from the nerve, and more positive charge enters the muscle at the NMJ,

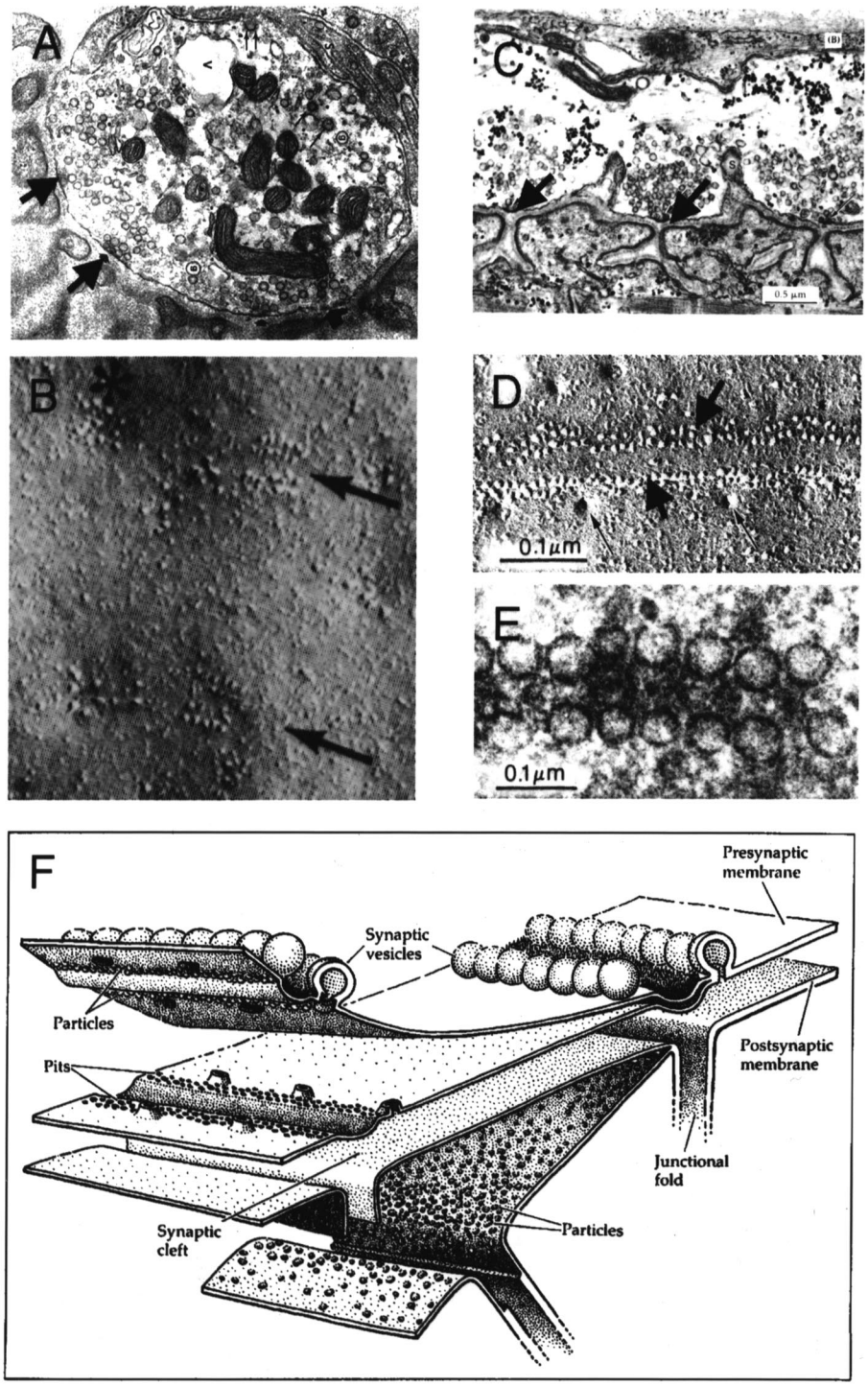

Fig. 3. Active zones in the presynaptic membrane at mammalian (left column) and frog (right column and lower diagram) NMJs. In the upper electron micrographs (A, C), transverse sections through active zones (indicated by arrows) are shown; these are often much more distinct in frogs than in mammals. In both groups, the active zones lie immediately opposite the openings of the synaptic folds In freeze-fracture preparations, the active zones both mammals (B) and frogs (D) are seen to be made up of two parallel rows of intramembranous particles (arrows). In frogs, these rows are up to 1 μm long and run perpendicular to the long axis of the motor axon terminal. In mammals the rows are much shorter, each one containing about 20 particles, and apparently randomly oriented in the plane of the membrane. In views on the cytoplasmic side of the active zones, synaptic vesicles can be seen aligned with the much smaller intramembranous particles (E). The diagram in (F) depicts the 3-dimensional arrangement of intramembranous particles. (A, B from Engel, 1994b, with permission of The McGraw-Hill Companies; C, F reprinted with permission of U.J. McMahon; D from Heuser et al., 1974 with permission; E from Couteaux and Pécot-Dechavassine, 1973 with permission.)

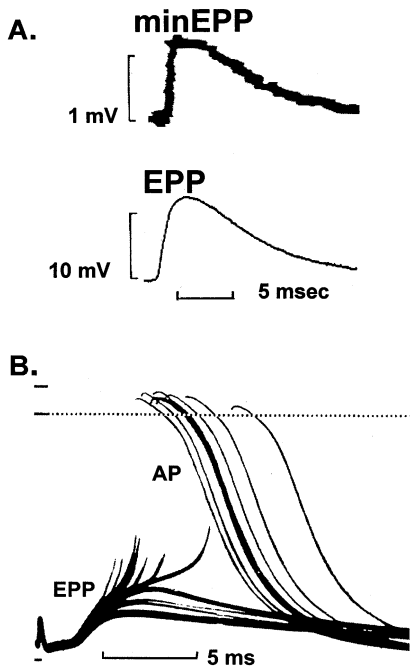

Fig. 4. Synaptic events recorded at the human neuromuscular junction. The action of individual quanta of ACh molecules generate minEPPS, usually 0.5–1 mV in amplitude. In response to a nerve impulse, a number of minEPPs sum to generate an EPP. When the peak of the EPP is greater than the critical threshold, an action potential (AP) is generated in the muscle fibre. In normal muscle, this happens every time and the EPP is not visible. The recordings illustrated are from a patient with myasthenia gravis, in whom the effect of ACh, and hence the amplitudes of minEPPs and EPPs are greatly reduced so many EPPs are sub-threshold. (B from Elmqvist et al., 1964).

than is required to depolarise the muscle fibre to the threshold for action potential generation (Wood and Slater, 1997). The safety factor of neuromuscular transmission is thus an expression of how much greater an effect the nerve has on the muscle fibre than is required to generate an action potential.

3.2. Definitions of the safety factor for neuromuscular transmission

A number of different, detailed working definitions of the safety factor of neuromuscular transmission have been proposed, generally reflecting the type of experiment carried out. In some early studies, the safety factor was defined as the fraction of AChRs that could be pharmacologically blocked before action potential generation was prevented (Paton and Waud, 1962; Chang et al., 1975). In most studies, however, electrophysiological methods have been used to define the safety factor as the ratio of the postsynaptic effect of the transmitter released by a single nerve impulse to the effect required to trigger a muscle fibre action potential. Frequently, safety factor has been expressed in terms of the depolarisation of the postsynaptic membrane, most often as the ratio of the estimated peak amplitude of the endplate potential to the threshold depolarisation required to generate an action potential (Harris and Ribchester, 1979; Kelly and Robbins, 1983; Engel, 1994a). Another approach has been to estimate the magnitude of the postsynaptic current flowing in response to a nerve impulse (Magleby, 1994). In such studies the safety factor has been defined as the excess current generated in response to a nerve impulse over that required to reach the action potential threshold. Since the quantal content of the EPP is the feature of neuromuscular transmission that varies most during normal neuromuscular activity (see Section 5.1.2), it has seemed logical to us to ask in any given circumstance: how many more quanta of transmitter are released than are required to excite the muscle fibre? We have therefore defined the safety factor in terms of the number of ACh quanta actually released compared to the number which must act to generate an action potential (Wood and Slater, 1997).

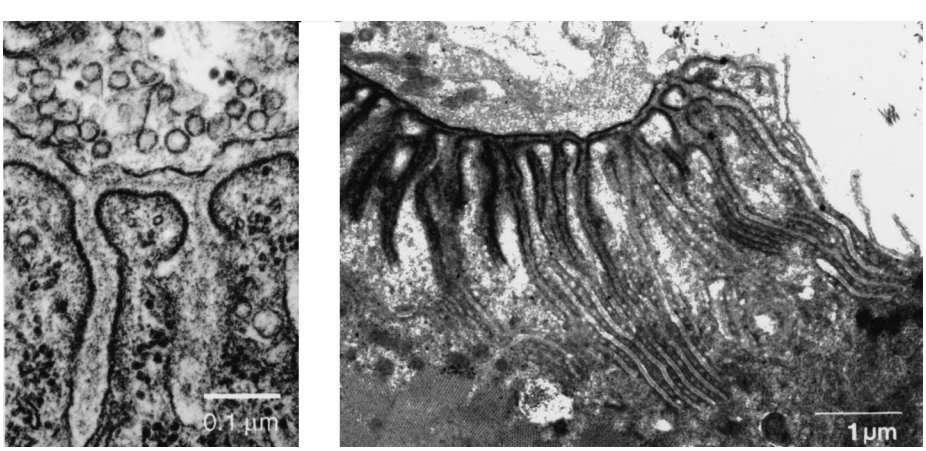

Fig. 5. Postsynaptic folds at NMJs in rat (left panel) and man (right panel). The membrane at the crests of the folds is thickened, probably reflecting the high density of AChRs there. In humans the folding is often particularly extensive. In the example shown, periodic increases in density are seen in the interfold cytoplasm, suggesting attachment between the membranes of adjacent folds. A compact extracellular basal lamina extends into the folds.

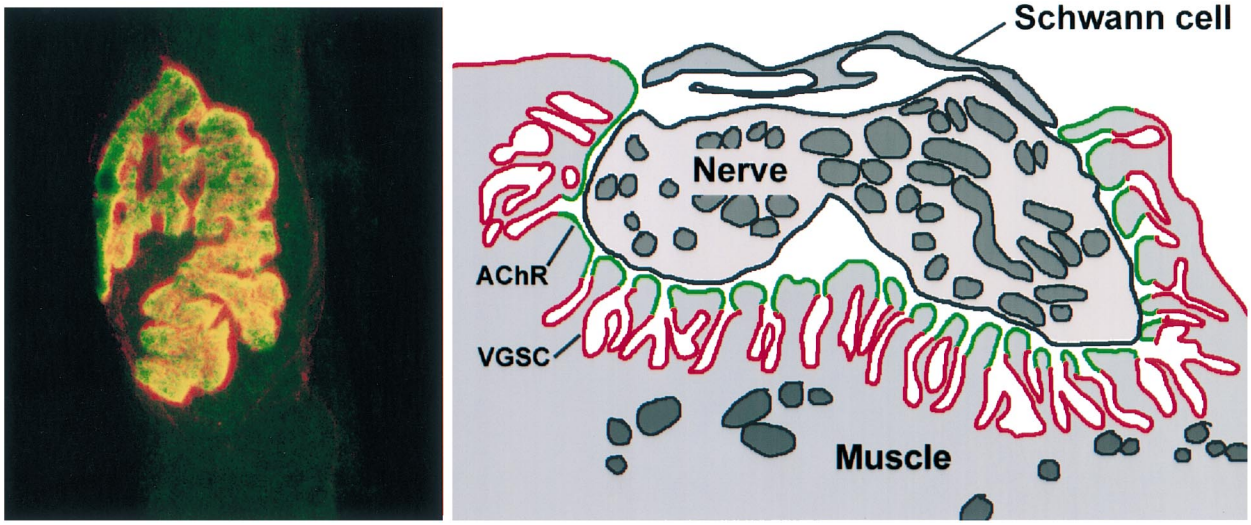

Fig. 6. Molecular organisation of the postsynaptic folds. The membrane of the folds contains two distinct domains. At the crests, there is a high concentration of AChRs (shown in green), the AChR-clustering protein rapsyn and utrophin. In the depths of the folds, there is a high concentration of VGSCs (shown in red) together with ankyrinG. In addition, a number of other proteins present in the non-junctional membrane are present here, including β-spectrin, dystrophin, and proteins associated with dystrophin. In en face views of suitably labelled whole NMJs, proteins in the depths of the folds (VGSCs, labelled red) appear to extend laterally beyond those at the crests of the folds (AChRs, labelled green). (A) from (Wood and Slater, 1998b). (Left panel reproduced from The Journal of Cell Biology, 1998, Vol. 140, pp. 675–684 by copyright permission of the Rockefeller University Press.)

Whichever definition of safety factor has been used, there are substantial practical difficulties involved in estimating its magnitude. A detailed comparison of the values of safety factor obtained by these methods is presented below (Section 4.4).

4. Estimating safety factor at the NMJ

For most definitions of safety factor, estimating its value requires assessing both the amount of transmitter released per nerve impulse and the amount of transmitter required to initiate an action potential. The many technical problems associated with direct biochemical measurement of ACh released from motor nerve terminals have led most workers to estimate transmitter release from electrophysiological recordings of postsynaptic effects. From such recordings, it is possible to obtain an estimate of the number of quanta of transmitter released or of the total effect of those quanta on the postsynaptic membrane. However, to do this, it is necessary to block the generation of muscle fibre action potentials, both to allow the effect of the transmitter to be measured in the absence of the action potential and to prevent the mechanical disturbance resulting from contraction. This leads to a fundamental difficulty in determining the safety factor; it is inherently impossible to determine both the amount of transmitter normally released by a nerve impulse and the amount required to trigger an action potential in the same experimental conditions. Many different approaches have been taken to resolving this problem, with more or less success. In this section, we present and comment on a number of them. Further consideration can be found elsewhere (Prior et al., 1993; Isaacson and Walmsley, 1995).

4.1. Blocking action potential generation

When isolated nerve muscle preparations are immersed in a suitable physiological bathing fluid, nerve stimulation usually elicits muscle fibre action potentials and subsequent contraction. In order to make an accurate estimate of the full-sized effect of the nerve impulse on the muscle fibre, muscle fibre action potentials must be abolished. Whatever method is used, problems arise since the procedures used to abolish muscle contraction or reduce the EPP to sub-threshold levels often interfere with the transmitter release process or with action potential generation.

4.1.1. Presynaptic block

Reducing the ratio of Ca^{++} to Mg^{++} concentrations in the bathing fluid can be used to reduce transmitter release to levels that are too low to generate an action potential (del Castillo and Katz, 1954). This method has the essential limitation that it does not allow the full effect of nerve activity to be measured. Nonetheless, its simplicity makes it potentially useful for assessing the relative values of quantal content in different situations (Alshuaib and Fahim, 1990; Lyons and Slater,

1991). However, it must be borne in mind that even this use depends on the assumption that the sensitivity to Ca^{++} is constant in the situations being compared, and this may not be so.

4.1.2. Postsynaptic block

In early studies, the response of the muscle to ACh was partially blocked with D-tubocurarine so that the amplitude of the EPP was reduced to a sub-threshold level (Fatt and Katz, 1951). Using this approach in mammals, 80% or more of the AChRs must be blocked to ensure that contraction is fully abolished and intracellular recordings can be made (Paton and Waud, 1962; Chang et al., 1975). There are two major problems with interpreting data from D-tubocurarine-blocked preparations. Firstly, in the presence of D-tubocurarine, EPPs at many NMJs are very small and minEPPs are too small to measure. Therefore, when a measure of the quantal content is required, indirect estimates based on the analysis of variation of EPP amplitudes must be used and these are inherently inaccurate in many situations (see Section 4.2.2) (Martin, 1955; Slater et al., 1992). Secondly, D-tubocurarine itself may reduce the amount of transmitter released by interacting with presynaptic nicotinic autoreceptors (see Section 5.1.3.1) (Glavinovic, 1979b; Magleby et al., 1981; Bowman et al., 1988; Ferry and Kelly, 1988).

An alternative approach, used successfully in a number of studies, is to damage the muscle fibres, usually by cutting them some distance from the NMJ (Barstad and Lilleheil, 1968; Glavinovic, 1979c; Maselli et al., 1991; Slater et al., 1992). This causes depolarisation of the membrane, usually to a resting potential of about -40 mV. This, in turn, leads to inactivation of the voltage-gated sodium channels, thereby blocking action potential generation in the muscle. There are several disadvantages to this approach. The low resting membrane potential and reduced electrical 'input resistance' of the fibres mean that both EPPs and minEPPs are small and difficult to record. To some extent, this effect can be counteracted by injecting a steady current into the muscle fibre through a second intracellular electrode to restore the resting potential locally to a more negative value. A further complication is that extensive damage to the muscle results in the leakage of K^+ into the extracellular space. This may depolarise the intramuscular nerve branches, leading to a failure of propagation of the nerve impulse into the presynaptic terminal. In spite of these limitations, this approach has provided much useful information.

Recently, a natural toxin has become available which blocks action potentials in the muscle but not in the nerve. In principle, this is the ideal approach to the study of evoked release. μ-conotoxin GIIIB (μCTX) is a component of the venom of the cone snail *Conus geographus*. In initial studies, it was found that μCTX blocks muscle sodium channels while having no effect on those from nerve or brain (Cruz et al., 1985; Moczydlowski et al., 1986). In subsequent studies, concentrations of μCTX were found which blocked action potentials in muscles of guinea pig (Muraki et al., 1991), mouse (Gonoi et al., 1989), rat (Plomp et al., 1992) and frog (Sosa and Zengel, 1993) muscles but not in their nerves. However, at high concentrations, μCTX has been shown to block sodium currents in mouse nerves (Braga et al., 1992). While it is generally believed that this relative specificity for muscle VGSCs is a feature of all vertebrate NMJs, this is unfortunately not the case. In both human and chicken tissues, μCTX blocks the nerve impulse at concentrations lower than that required to block muscle fibre action potentials (Plomp et al., 1995; Wood and Slater, 1998a). This limit of the specificity of μCTX for muscle VGSCs raises the possibility that in some circumstances, its use may lead to partial block of the nerve impulse and the reduction of transmitter release. However, at low concentrations, the use of μCTX to block muscle fibre action potentials is currently the best way to study full-sized EPPs in some species.

4.2. Estimating transmitter release from electrophysiology

The effect of the released transmitter on the muscle fibre can be measured and expressed in several ways. The most straight forward is to record and measure the peak amplitude of full-sized EPPs in a situation in which the muscle fibre action potential has been blocked. While the amplitude of the EPP provides one useful measure of the effect of the transmitter, it is usually an inaccurate measure of the amount of transmitter released (see below). When it is desirable to express the safety factor in terms of release, there are two ways of dealing with this inaccuracy. One is to attempt to 'correct' for it mathematically and the other is to record the flow of ACh-induced current across the membrane, rather than potential, since the current is more directly related to the amount of transmitter released. In what follows, we comment more fully on each of these approaches and their limitations.

4.2.1. Potentials or currents?

When using intracellular electrodes, it is possible to record either voltage or current transients (Fig. 7). There are advantages and disadvantages to both types of recording and the appropriate choice may be influenced by the method of blocking action potential generation (see Section 4.1). Assuming the resting membrane potential is near normal values, in a suitably blocked preparation, a single microelectrode can be used to record EPPs in response to nerve stimulation (see Sections 4.2.2 and 5.1.2). To obtain an accurate measure-

ment, it is necessary to ensure that the recording electrode is close to the NMJ (< 100 μm) since the capacitative properties of the muscle membrane result in the amplitude of the EPP declining as the recording electrode is placed increasingly far from the NMJ (Fatt and Katz, 1951; Betz et al., 1984). This effect is usually overcome by limiting recordings to those where the rise time of the EPP is faster than some preset value (e.g. 1 ms), since rise time is also a function of distance from the NMJ (Fatt and Katz, 1951; Betz et al., 1984). While the amplitude of the EPP provides a measure of transmitter effect, it is not an accurate reflection of the amount of transmitter released from the nerve. This is because of the non-linear relationship between the amount of transmitter released and the resulting depolarisation of the muscle fibre membrane (see Section 4.2.2). Each EPP results from the action of a number of quanta. When each quantum acts on the postsynaptic membrane it causes an increase in cation conductance which leads to a change in the membrane potential. Each increment of conductance represents a smaller and smaller fraction of the total conductance change as the number of quanta increases. As a result, the amplitude of the EPP increases less rapidly than the number of quanta (Martin, 1955). As a result of this non-linear summation of quantal effects, the amplitude of the EPP underestimates the number of quanta which give rise to it (McLachlan, 1978; Slater et al., 1992). Furthermore, this confounding factor becomes greater as the quantal content increases.

Various formulae for adjusting the EPP amplitude to take account of this 'non-linear summation' have been devised (Martin, 1976; Stevens, 1976; McLachlan and Martin, 1981), such as;

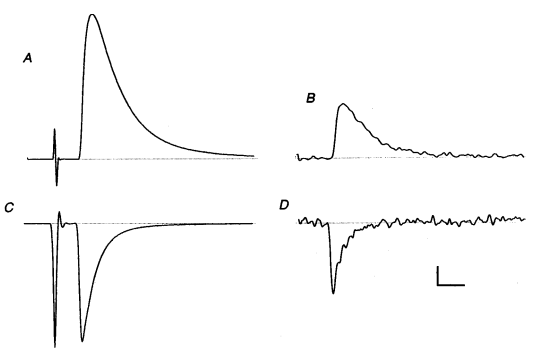

Fig. 7. Voltage and current transients recorded at rat NMJs in the presence of μCTX. Full-sized EPPs (A) and minEPPs (B) can be recorded in the presence of μCTX which blocks muscle fibre action potentials. minEPPs are not affected. By using a two-electrode voltage-clamp arrangement, the corresponding flows of current, the EPCs (C) and minEPCs (D), can be recorded. The time course of the current transients is faster than that of the potentials and reflects more closely the kinetics of the ACh-induced ion channels. Similarly the peak of the currents is the best estimate of the number of channels opened by ACh. The vertical calibration bar represents 4 mV (A), 0.25 mV (B), 30 nA (C), 1 nA (D). The horizontal bar represents 3 ms for all traces (from Wood and Slater, 1997).

$$v' = v/(1 - v/E),$$

where v' is the adjusted EPP amplitude, v is the recorded EPP amplitude and E is the difference between the resting potential and the reversal potential for transmitter action (the potential at which no net current flows across the cell membrane when ACh-gated channels are opened, usually between 0 and -15 mV).

In practice, however, there are considerable uncertainties involved in using these corrections. This is because, in addition to the effect of non-linear summation, the magnitude of the peak depolarisation of the recorded EPP is also influenced by the capacitative properties of the muscle fibre membrane. Efforts have been made to account for this, for example by introducing an additional variable (f) into some of the equations (Martin, 1976) as follows;

$$v' = v/(1 - fv/E).$$

Unfortunately, the value of f can only be determined empirically, greatly reducing the usefulness of the whole approach.

One way around the difficulties raised by non-linear summation is to make recordings of synaptic currents rather than potentials. To do this, two microelectrodes need to be inserted close together into a muscle fibre at the NMJ. In this way the muscle fibre can be voltage-clamped and nerve evoked endplate currents (EPCs) can then be recorded (Fig. 7) (Takeuchi and Takeuchi, 1959; Magleby and Stevens, 1972; Glavinovic, 1979c; Slater et al., 1992). This method provides a more direct estimate of transmitter release since the recorded currents are linearly related to the number of ion channels opened by released ACh (McLachlan, 1978). It also has the advantage of allowing the membrane potential to be maintained at any desired value. However there is a potential problem with this method, that of inadequate voltage control also referred to as poor space clamp. This arises since the effectiveness of voltage regulation decays rapidly with distance from the electrodes. This problem can be minimised if care is taken to avoid damaging the muscle fibre and to place the electrodes close to the NMJ. With these precautions, the use of two-electrode voltage clamp to record synaptic currents is the best method available to estimate the effect of released transmitter.

4.2.2. Estimating quantal content

When, as in our studies (Wood and Slater, 1995, 1997), it is desired to express the safety factor in terms of the number of quanta normally released by a nerve impulse, it is necessary to determine the quantal content. A number of ways to do this have been devised. The basic approach involves determining the ratio between the effect of the numerous quanta of ACh re-

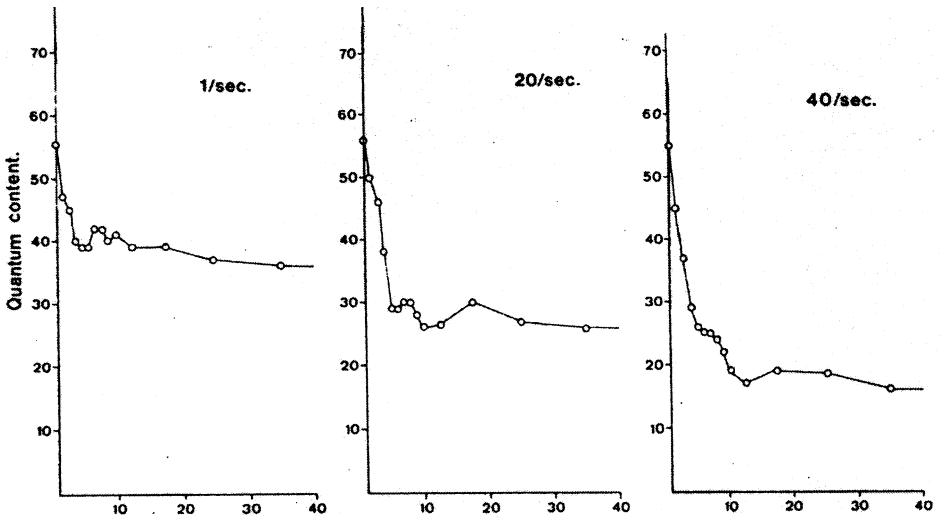

Fig. 8. The quantal content depends on the frequency of activation. Isolated human intercostal nerve-muscle preparations were stimulated at different frequencies. The muscle fibres were pretreated with glycerol to block action potentials. During stimulation, there is an initial rapid decline of quantal content. This is followed by a slower decline which is not seen in this figure (from Kamenskaya et al., 1975).

leased by a single nerve impulse (e.g. the amplitude of EPPs or EPCs) and that of individual, spontaneously released quanta (e.g. the amplitude of minEPPs or minEPCs). Since there is statistical variation in both the effect of individual quanta and of the number of quanta released, even at the same NMJ in the same experiment, mean amplitudes of a suitable number of events must always be used in these calculations. Another important feature of quantal release is that the number of quanta released per nerve impulse varies depending on the pattern of activity (Section 5.1.2) and the degree of short term modulation (Section 5.1.3). In isolated nerve-muscle preparations from frog and mammals, the response to frequencies greater than 1 Hz results in a gradual decline in quantal release which is itself frequency dependent (Fig. 8). In what follows, values for quantal content have been derived from studies in which the frequency of stimulation was not greater than 1 Hz.

Ideally, the samples of spontaneous quantal events and full-sized evoked responses should be recorded from the same NMJ. Since the amplitude of synaptic events depends on membrane potential, it is important to record both spontaneous and evoked events at the same membrane potential, or that some correction for differences in membrane potential are made. We have often used two intracellular electrodes in a current clamp configuration to maintain a constant membrane potential that is sufficiently negative to allow minEPPs to be recorded even from cut fibre preparations (Slater et al., 1992). In many species, spontaneous quantal events occur at a frequency of about 1 Hz and 50–100 events can be recorded with no difficulty. In some cases, such as normal humans or immature rodents, the frequency is much lower-of the order of a few per minute. One way around this, which is effective in human nerve-muscle preparations, is to stimulate the nerve at high frequency (50 Hz) for up to 10 seconds. This results in a great increase in the frequency of quantal events which lasts from 10–20 s (Magleby, 1994). In our experience, the mean amplitude of minEPPs recorded this way does not differ significantly from those recorded with out stimulation, though this may not be true for more exhaustive stimulation (Van der Kloot, 1991; Glavinovic, 1995).

Once the effect of spontaneous and evoked transmitter release has been measured from current or voltage recordings, then the quantal content (m) can be calculated in a number of ways. The most direct is to calculate the ratio of the amplitude of the EPC to that of the minEPC;

m = EPC amplitude/minEPC amplitude.

This 'direct' method can also be used with EPPs and minEPPs but then corrections must be made for both variations of resting membrane potential and non-linear summation (see Section 4.2.1) and these are likely to lead to inaccuracies in the final value;

m = NLScorrEPP amplitude/minEPP amplitude,

where 'NLScorrEPP amplitude' is the amplitude of the EPP corrected for non-linear summation. Values of quantal content obtained in comparable conditions using the 'direct' method, with either potential or current recording, are between 20 and 200 (see Section 6.2).

Early studies of quantal release demonstrated that there were fluctuations in the number of quanta released per nerve impulse. In situations where the extracellular Ca^{++} concentration was relatively low (see Section 4.1.1), some nerve impulses failed to elicit a

response at all, while others gave rise to EPPs that varied in amplitude in a stepwise manner. This variation was statistical in nature (del Castillo and Katz, 1954) and could be described by assuming that it obeyed a binomial distribution. In particular, the mean number of quanta released per nerve impulse could be well determined from the equation;

$$m = np,$$

where m is the average number of quanta released per nerve impulse, n is the number of 'units capable of responding to a nerve impulse' (often interpreted as the number of vesicles of ACh available for release) and p is the probability of any of those 'units' responding, i.e. being released (Martin, 1965; Glavinovic, 1979a). In practice, the physical interpretation of both n and p remains uncertain, and therefore impossible to determine independently of their combined effect on quantal release (Glavinovic, 1979a). It was further found that when the release probability p is very low, the probability of transmission failing increases. Under these conditions, for example low Ca^{++} and high Mg^{++}, the distribution of EPP amplitudes of a large number of events can be described by the Poisson distribution;

$$p_x = m^x/x!\, e^{-m},$$

where p is the probability of occurrence of a response resulting from x quanta and m is mean number of quanta per response, or quantal content. This analysis has the feature that a single parameter, e.g. the number of 'failures' (i.e. the trials where the number of quanta released was zero), is adequate to describe the whole population of responses and, hence, to predict the mean quantal content.

In early studies where D-tubocurarine was used to abolish action potential generation, such a large number of AChRs were blocked that the amplitude of the minEPP was too small to be recorded. To estimate quantal content in the absence of a measured value of quantal size, the assumption was made that the responses could be described by the Poisson distribution. On this basis, the mean quantal content was estimated from a measure of the variance of the amplitude of the EPP. While this seems an attractive method to be used in preparations blocked by D-tubocurarine, it turns out that the Poisson approximation is only justified in situations where the probability of release from the nerve terminal, and hence the quantal content, is very low. When, as in solutions of normal Ca^{++} concentration, this is not true, the 'variance' method greatly overestimates the quantal content (Fig. 9) (Martin, 1965; McLachlan, 1978; Slater et al., 1992). For a long time, the use of the 'variance' method to estimate quantal content led to the belief that the quantal content at mammalian, including human, NMJs was around 200, similar to that in frog (Ginsborg and Jenkinson, 1976). It is now clear that at a stimulus frequency of 1 Hz, the true value at rat NMJs is between 50 and 100 (Glavinovic, 1979a; Plomp et al., 1992; Wood and Slater, 1995) while in humans it is lower still, 20–50 (Fig. 17, Section 6.2) (Cull-Candy et al., 1980; Engel et al., 1990; Plomp et al., 1995; Slater et al., 1992).

In summary, the most accurate method for measuring quantal content, at least in rats and mice, is to record EPCs and minEPCs from muscle in which action potentials have been blocked with μCTX and to use the direct method to calculate quantal content. In other species, where μCTX cannot be used, it may be necessary to resort to other less direct approaches as described above.

4.3. Measuring action potential threshold

Having obtained a measure of transmitter release per nerve impulse, it is then necessary to estimate how much transmitter is 'required' to generate an action potential. The threshold for action potential generation in skeletal muscle is usually defined as the smallest abrupt depolarisation from the resting potential needed to trigger an action potential. Most commonly, this has been determined using two intracellular electrodes; passing rectangular pulses of current through one while recording the resulting changes in membrane potential with the other (Fig. 10). In many such experiments, depolarisation to an absolute membrane potential of about −55 mV, close

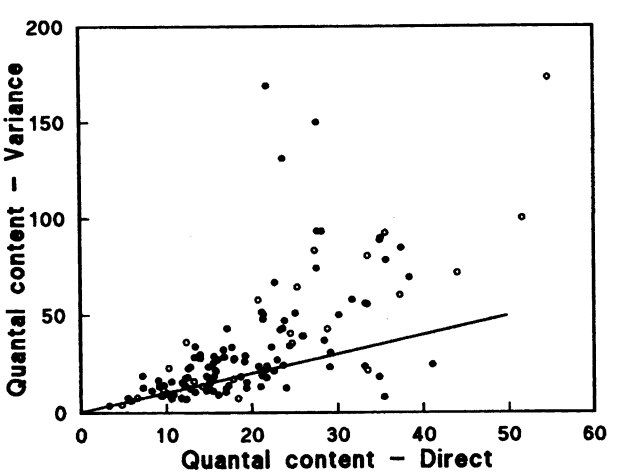

Fig. 9. Comparison of estimates of quantal content at human NMJs based on the 'variance' and 'direct' methods (see text for details). When the quantal content is high, as in normal physiological conditions, the variance method over-estimates the quantal content calculated by the direct method (from Slater et al., 1992 by permission of Oxford University Press).

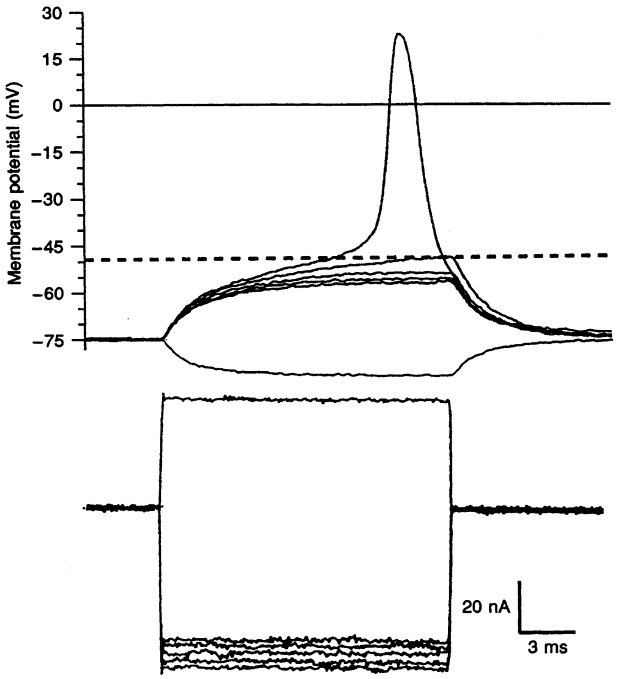

Fig. 10. Determination of the threshold for muscle fibre action potential generation. These properties can be determined by inserting two microelectrodes into the muscle fibre. Pulses of current are passed through one (lower traces) while the resulting changes in membrane potential are recorded with the other (upper traces). Note that outward current, shown increasing upwards, causes a hyperpolarisation of the muscle fibre, shown increasing downwards. For changes of membrane potential that do not reach the threshold, the ratio of the change in voltage to the change in current defines the 'input resistance' of the muscle fibre. As the depolarisation of the muscle fibre approaches threshold, about -50 mV in this example, this relationship becomes non-linear and an action potential is soon generated (from Wood and Slater, 1995).

to the activation threshold for voltage-gated sodium currents in mammalian muscle (Adrian and Marshall, 1977), was adequate to trigger an action potential.

Recent insights into the functional organisation of the postsynaptic region suggests that the threshold depolarisation at the NMJ may be smaller than in the rest of the fibre. This is a consequence of the combined effect of a high density of VGSCs in the depths of the postsynaptic folds and the effect of the folds themselves on the flow of current within the postsynaptic membrane (see Secton 5.2.3). These considerations imply that values of threshold obtained away from the NMJ are a poor approximation to the events that occur during neuromuscular transmission.

A more appropriate approach, therefore, is to use the action of ACh released from the nerve to determine the threshold. This can be done by using D-tubocurarine to block, partially, the response of the muscle to ACh so that occasional NMJs can be found where the amplitude of the EPP is close to threshold (Wood and Slater, 1995, 1997). In this case, some EPPs exceed the threshold while others do not (Fig. 11) (Wood and Slater, 1997). The threshold depolarisation determined in this way is about half that measured using rectangular current pulses (Boyd and Martin, 1956; Wood and Slater, 1995). For example, in the case of rat EDL muscles (Wood and Slater, 1995) with a resting potential of -75 mV, an EPP of 12 mV just reaches threshold while a step depolarisation of nearly 30 mV is required to trigger an action potential away from the NMJ (Wood and Slater, 1997). This reduction of threshold at the NMJ is close to that predicted on theoretical grounds (Martin, 1994).

In order to arrive at an estimate of threshold in terms of transmitter release, we made an estimate of the number of quanta which would have to act to reach threshold (Wood and Slater, 1997). We first recorded threshold EPCs in muscle fibres partially blocked by D-tubocurarine. Direct comparison of the amplitudes of such threshold EPCs with the amplitudes of minEPCs, recorded in the absence of D-tubocurarine, is complicated by the fact that this drug may influence the time-course of synaptic currents (Katz and Miledi, 1973). We therefore calculated the net charge flowing across the membrane during threshold EPCs (EPC amplitude $\times$ exponential decay time constant of EPC), since the effect of the charge entering the cell during a current pulse which are brief (relative to the membrane time constant) is independent of the exact time course of that pulse. To convert this to quantal content, we divided it by the charge flowing during minEPCs, recorded in the absence of D-tubocurarine. For both soleus and EDL muscles, the threshold quantal content, estimated by the 'direct' method, was about 13.

This approach to determining the 'threshold quantal content' is subject to several complicating factors. The current and voltage electrodes must be placed close to the NMJ to minimise the effects of spatial decay of applied current and recorded voltage. In practice, this can be ensured either by direct observation of the NMJ with appropriate optics or by requiring that the rise time of the EPP be less than 1 ms (Betz et al., 1984). A second complicating factor in determining threshold is related to the properties of the VGSCs (Ruff et al., 1987). The membrane potential at which experiments are conducted has considerable impact on the proportion of VGSCs that are inactivated (see section 5.3.2). This can be controlled by holding the membrane potential at a standard potential before determining threshold. Finally, it is clear that the geometry and molecular properties of the postsynaptic membrane, and how it is activated by the nerve, make it important to determine threshold at the NMJ itself, rather than far from it. As indicated in Section 6., the importance of this latter factor is likely to depend on the species and degree of maturity of the NMJs being investigated.

4.4. Comparison of reported values of safety factor at rat NMJs

Although the concept of a safety factor for neuromuscular transmission was first discussed in detail more than 30 years ago (Paton and Waud, 1962), there have been remarkably few efforts to assess it quantitatively. Such values as exist have been obtained using a variety of methods and preparations (see Section 3.2) and are not, therefore, strictly comparable. Nonetheless, they provide a general consensus that at NMJs in most mammalian limb muscles, studied in vitro, the safety factor is between 2 and 10 (Table 1).

Some of the highest estimates of safety factor come from pharmacological experiments of receptor occlusion at mammalian NMJs (Paton and Waud, 1962; Chang et al., 1975). By blocking increasing fractions of AChRs with D-tubocurarine, it was found that 80–90% of the receptors could be blocked before there was any failure of indirectly evoked contraction. These studies suggest that, at low frequencies, 5–10 times more ACh-gated channels are normally opened than is required to generate an action potential. Such experiments also made it clear that there is a substantial excess of AChRs at the NMJ (Ginsborg and Jenkinson, 1976). However, the distributed nature of transmitter release and the short distance over which the ACh in a single quantum acts means that in spite of this nominal excess, most of the AChRs are out of range of the ACh released by any given nerve impulse. It has become clear that the ACh released by a single nerve impulse opens, at most, about 1–2% of all the AChRs in the postsynaptic membrane (based on 2,500 AChRs opened/quantum, 100 quanta/nerve impulse, 1.5×10^7 AChRs/NMJ) (Magleby, 1994).

Many studies have used intracellular recording techniques to estimate the safety factor. In their classic study of the EPP in mammalian muscle, Boyd and Martin (1956) estimated that the EPP, in the absence of an action potential, would be about 35–40 mV while a depolarisation of only 10–20 mV would be required to generate an action potential. Thus, a safety factor of 2–4 can be derived from their data. In other studies, the amplitude of the full-sized EPP was estimated from the product of the quantal content and the minEPP amplitude and then 'corrected' for non-linear summation (Kelly, 1978; Harris and Ribchester, 1979). In these studies, the quantal content was estimated from the variance of EPPs recorded at NMJs blocked with D-tubocurarine. Since the variance method substantially overestimates the quantal content (Section 4.2.2) this approach is likely to overestimate of EPP amplitude. In the same studies, the action potential threshold was measured by passing rectangular current pulses through membrane some distance from the NMJ. This is likely to overestimate the threshold (Section 4.3). Since both components of the safety factor ratio are overestimated, the errors generated would tend to cancel each other out.

In our own experiments, we have derived a value for safety factor which indicates how many more quanta are released per nerve impulse than are required to trigger an action potential. Using the methods described in Section 4.2 and 4.3, we have obtained values

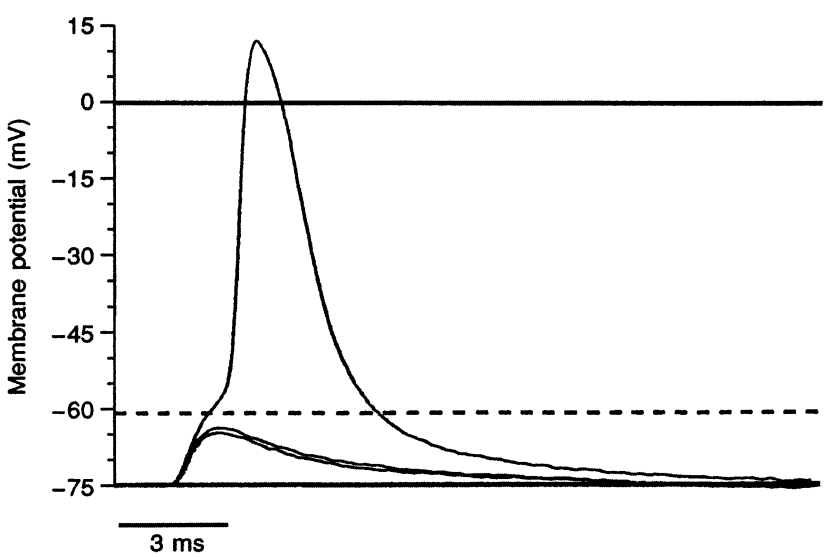

Fig. 11. Determination of the action potential threshold at the NMJ. The amplitude of the EPP in an isolated rat soleus nerve-muscle preparation has been reduced by blocking most of the AChRs with D-tubocurarine (2×10^{-7} M). Stimulating the nerve at 0.2 Hz evoked EPPs and an action potential in the same muscle fibre. The threshold, defined as the first point of inflection on the rising phase of the action potential, was -61 mV in this example (from Wood and Slater, 1995).

Table 1
The range of reported safety factor for neuromuscular transmission in different muscles[a]

Species	Muscle	Safety factor	Source
Rat	Soleus	1.8	Gertler and Robbins, 1978
		3.5	Wood and Slater, 1997
	EDL	2.0	Gertler and Robbins, 1978
		5.0	Wood and Slater, 1997
		6.0	
	Diaphragm	3.0–5.0	Chang et al., 1975
		2.0–3.6	Kelly, 1978
		1.7–2.8	Wareham et al., 1994
Mouse	Soleus	4.6–5.8	Banker et al., 1983
	EDL	2.4–2.8	Harris and Ribchester, 1979
		3.2–5.8	Banker et al., 1983
Human	Intercostal	2.0	Elmqvist et al., 1964
Cat	Tenuissimus	2.0–4.0	Boyd and Martin, 1956
	Tibialis	4.0–12.0	Paton and Waud, 1967
	Sartorius	4.0–12.0	Paton and Waud, 1967
Frog	Cutaneous pectoris	4.0	Grinnell and Herrera, 1980
	Sartorius	1.0	Grinnell and Herrera, 1980
		3.1–5.5	Adams, 1989

[a] In some cases the values are derived from data presented in the paper referred to. These values are not directly comparable with one another since different methods have been used to estimate safety factor (see Section 4) and different experimental conditions such as temperature, pattern of activity and age of animals may influence the reported value (see Section 6)

within the range observed by others (Wood and Slater, 1997). In rat soleus and EDL, the normal quantal content is 46.3 and 65.1 respectively while the corresponding threshold quantal contents are 13.3 and 13.0. Thus, the calculated safety factors are 3.5 for soleus and 5.0 for EDL.

Whatever approach is taken it seems that remarkably similar values are found for safety factor in vitro at normal mammalian NMJs stimulated at or near 1 Hz (Table 1). While some estimates appear to be based on more firm methodological foundations than others, all are likely to be imperfect estimates of the true situation in vivo. At a functional level, it is clear that these values of safety factor reflect a fully adequate reserve of transmitter release, since in normal circumstances, neuromuscular transmission does not fail. In subsequent sections, we consider some of the factors that are likely to determine the value of the safety factor in vivo.

5. Features of the NMJ that influence safety factor

The safety factor of neuromuscular transmission is influenced by many features of the pre- and postsynaptic components of the NMJ. Some of these features are structural and likely to be relatively stable on a time scale of minutes or hours. Others are more dynamic and reflect physiological changes within the cells on a much more rapid time scale.

5.1. Presynaptic features influencing transmitter release

5.1.1. Nerve terminal size

If the amount of ACh required to generate an action potential were constant, then one way of enhancing safety factor would be to increase transmitter release per nerve impulse. For many years it has been realised that the quantal content is related to the size of the motor nerve terminal (Kuno et al., 1971; Harris and Ribchester, 1979; Slater et al., 1992). Generally, large motor nerve terminals release more transmitter than small terminals (Fig. 12). This implies that the number of quanta released per unit area of presynaptic membrane is relatively constant ($0.15-0.30$ quanta/μm^2, stimulation at 1 Hz), even in motor nerves from species as diverse as frog, chicken, mouse, rat and man (Slater et al., 1992; Wood and Slater, 1997, 1998a). It is likely that this is related to the density of active zone particles and hence, probably, of VGCCs. In mouse and man, where the density of active zones has been measured, it is about 2.5 per μm^2 (Fukunaga et al., 1982, 1983). Taken together, these values imply that, for a single nerve impulse, vesicles are released from only 10% of the active zones. This suggests that there is a substantial potential for an increase in release per unit area. Whether this is ever fully utilised is not clear.

Changes in nerve terminal size are unlikely to occur on a moment to moment basis but such changes do occur throughout normal life (see Sections 6.4 and 6.5) and in pathological situations (see Section 7). There is also some evidence that changes in release per unit area occur over a time scale of days in response to changes in the efficacy of neuromuscular transmission (see Section 6.5.2). Whether this is associated with changes in the density of active zones or of the probability of release from each active zone is not yet known.

5.1.2. Pattern of activity

During normal activity, quantal content, and hence the safety factor, appear to vary considerably. The pattern of activation of motor units in vivo is very different from that used in most estimates of safety factor made in vitro (Section 4.4). The most detailed studies of motor unit activity in free living animals have been made in rats. 'Slow' motor units, such as are found in the rat soleus muscle, are generally active for

long periods of time at a continuous frequency of 10–20 Hz. 'Fast' units, as in the EDL, are active in short bursts (5–10 impulses) at much higher frequencies (50–100 Hz) (Hennig and Lömo, 1985). During normal activity, there are substantial variations in transmitter release from moment to moment. The high safety factor means that neuromuscular transmission remains reliable at all times.

Variations of quantal content are influenced by activity in at least two ways. Studies of quantal content during repetitive activity at 100 Hz in vitro show that there is an initial increase ('facilitation') followed by a decrease ('depression') (Magleby, 1994). The facilitation of transmitter release within the first few 10 s of ms after a nerve impulse appears to be due to the persistence of elevated Ca^{++} levels in the nerve terminal. It seems likely that following a single nerve impulse, the Ca^{++} that enters the nerve terminal diffuses rapidly away from the active zone and release falls rapidly to a near basal level (Stanley, 1997). Nonetheless, in the bulk of the terminal cytoplasm, the Ca^{++} level remains slightly increased for up to 50 ms (Magleby, 1994). During repetitive activation, it appears that this residual Ca^{++} accumulates to a level where a clear increase in both the frequency of spontaneous quantal release and evoked release can be seen (Van der Kloot and Molgo, 1994).

Opposing the potentiating effect of Ca^{++} accumulation is what appears to be a reduction of the number of synaptic vesicles that can be 'readily' released. Within the nerve terminal there are at least two pools of synaptic vesicles (Van der Kloot and Molgo, 1994). One is a readily releasable pool which probably corresponds to vesicles 'docked' at the active zones. At rat NMJs, this probably represents a few hundred vesicles, substantially less than 1% of the total vesicles in the terminal (Reid et al., 1999). The other pool consists of vesicles bound to components of the cytoskeleton by the protein synapsin (Hilfiker et al., 1999). In some experiments in which the nerve was stimulated at frequencies of 3–100 Hz, there was an initial rapid decline of the quantum content, within 10–20 impulses (Fig. 8) (Kamenskaya et al., 1975), although in other studies the decline was less (Glavinovic, 1979a). This 'run down' of quantal content has been interpreted as resulting from the partial emptying of a pool of 'readily releasable' vesicles (Kamenskaya et al., 1975; Reid et al., 1999). During a burst of activity typical of a fast motor unit (Kamenskaya et al., 1975), this run down might well result in the quantal content, and hence the safety factor, falling to as little as 50% of its resting value. On a slightly longer time scale, the activation of second messenger cascades by activity may lead to a 'mobilisa-

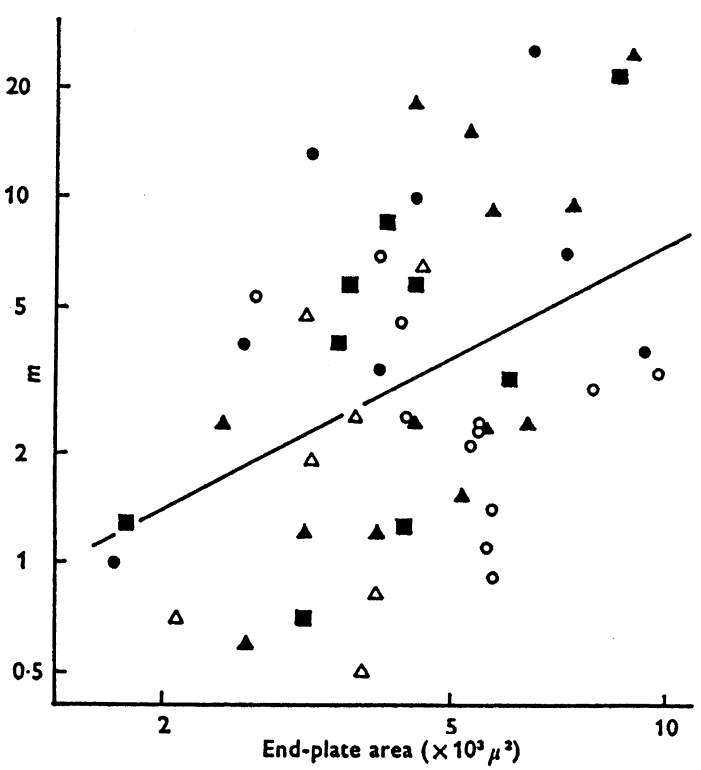

Fig. 12. Quantal content (m) is related to synaptic area in frog sartorius muscle. The quantal content, which was reduced by bathing the preparation in a solution with reduced Ca^{++} and increased Mg^{++}, was measured by both the direct and variance methods. The end-plate area was estimated from the same muscle fibres by measuring the area occupied by the product of a histchemical reaction for AChE. There is a weak, but significant ($p < 0.01$) correlation. Results are shown from five individual muscles, each with a different symbol (from Kuno et al., 1971).

tion' of the large number of vesicles previously bound to synapsin. In this way, the quantal content may be upregulated to match the pattern of activity.

5.1.3. Short term modulation of release

There is increasing evidence that quantal release from the motor nerve terminal can be modulated on a time scale of seconds or minutes by activation of a variety of receptors, located on the presynaptic terminal or Schwann cells. The role of cholinergic, purinergic and adrenergic receptors in modulating ACh release is described below. Clearly these presynaptic receptors interact under physiological conditions but the nature of such interactions has not been investigated. In addition to these receptors, a number of reports indicate that the modulation of release at the NMJ may be quite complex with nitric oxide (Oliver et al., 1996; Grozdanovic et al., 1997), serotonin receptors (Hirai and Koketsu, 1980; Das et al., 1989) and glutamate receptors (Waerhaug and Otterson, 1993; Berger et al., 1995; Fu et al., 1995) all suggested to be present. These may be localised pre or postsynaptically and to date there is no clear understanding of their role at the NMJ. What is most important to appreciate is that even when the structural features of the nerve terminal remain fixed, there may be substantial changes in safety factor because of altered transmitter release in response to variations in activity.

5.1.3.1. Cholinergic modulation. D-tubocurarine is usually described as a neuromuscular blocking agent because of its effects on postsynaptic nicotinic AChRs. However, numerous studies have now demonstrated that D-tubocurarine also has presynaptic actions (Bowman et al., 1988). For example, Glavinovic (1979b) has shown that the rundown of the EPC seen during repetitive stimulation is greatly increased in the presence of D-tubocurarine. It was proposed that a positive feedback autoreceptor, which ACh normally activated to maintain release during repetitive stimulation, was blocked by D-tubocurarine. However, it has also been suggested that activation of the nicotinic autoreceptors may decrease transmitter release (Miledi et al., 1978; Wilson, 1982; Ferry and Kelly, 1988). It is not clear how to reconcile these opposing views that activation of cholinergic autoreceptors can lead to either facilitation or depression of ACh release. However, part of the answer may be that there are multiple presynaptic AChRs that are activated at different frequencies of nerve stimulation (Tian et al., 1994). Thus at low frequencies (<1 Hz) neuronal type nicotinic AChRs would be activated which would inhibit further ACh release. At higher frequencies ACh release would be maintained by activation of a muscle type nicotinic receptor which enhances transmitter mobilisation and release. Recent evidence supports the presence of such a positive feedback muscle type nicotinic receptor, facilitating ACh release (Singh and Prior, 1998). In any situation where release is enhanced then the safety factor for neuromuscular transmission would be correspondingly increased.

5.1.3.2. Purinergic modulation. The role of purinoceptors in neuromuscular transmission has been reviewed in detail (Ribeiro et al., 1996; Henning, 1997). ACh release is inhibited by adenosine (Ginsborg and Hirst, 1972), adenosine diphosphate and adenosine triphosphate (ATP) (Ribeiro and Walker, 1975) at both rat and frog NMJs. The depression of both evoked and spontaneous release is thought to be mediated via P1 receptors, activated by adenosine, which is produced from the degradation of ATP (Ribeiro and Sebastiano, 1987; Hamilton and Smith, 1991; Smith and Lu, 1991b). This may be ATP released from motor nerve terminals (Redman and Silinsky, 1994; Silinsky et al., 1999) or from muscle on contraction (Smith, 1991a). At present it is unclear whether the P1 receptors responsible for this effect are present on the motor nerve terminal or on the surrounding Schwann cells (Robitaille, 1998).

In addition to an inhibitory effect on ACh release, adenosine in high concentrations may cause a facilitation of release by activation of a subtype of P1 receptors (Silinsky et al., 1989). More recently it has been demonstrated that ATP itself can depress transmitter release via P2 purinoceptors (Giniatullin and Sokolova, 1998; Hong and Chang, 1998) and that ATP can have a direct action on AChRs (Igusa, 1988; Mozrzymas and Ruzzier, 1992). Under physiological conditions the predominant effect of activation of purinoceptors is to reduce transmitter release. During periods of repetitive stimulation this effect would reduce the safety factor but would allow sustained transmitter release, albeit at lower levels.

5.1.3.3. Adrenergic modulation. Adrenaline and noradrenaline have long been known to cause a potentiation of neuromuscular transmission. This effect underlies the use of the adrenoceptor agonist ephedrine in the treatment of myasthenia gravis (Felice and Relva, 1996), (see Section 7.2.1). Ephedrine has been shown to have both pre and postsynaptic effects on neuromuscular transmission (Sieb and Engel, 1993). Postsynaptically ephedrine can enhance AChR conductance (Sieb and Engel, 1993) although it has also been shown to block AChRs (Bouzat, 1996). Presynaptically, adrenaline and noradrenaline increase quantal release by activation of both α and β adrenoceptors (Wessler and Anscheutz, 1988; Vizi, 1991). More recently, it has been found that noradrenaline synchronises evoked release through its activation of β-adrenergic receptors (Bukchareva et al., 1999). This allows a greater and more rapid peak

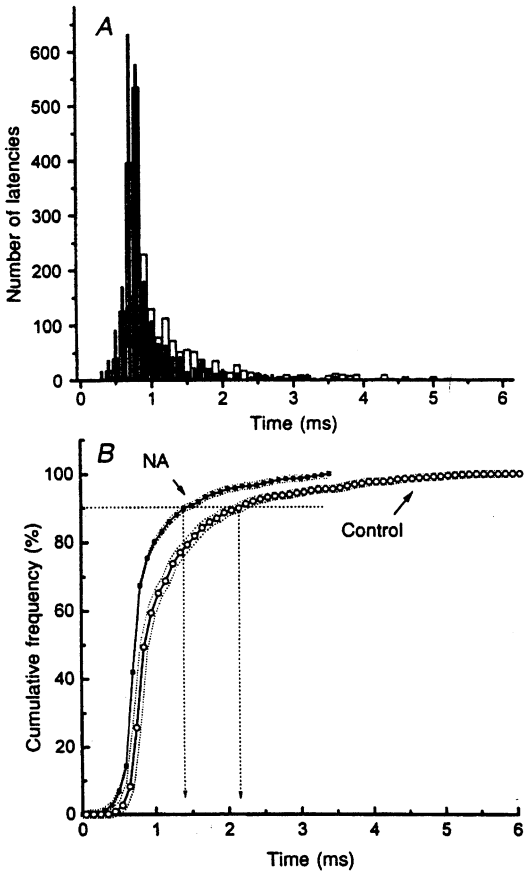

Fig. 13. Noradrenaline synchronises nerve-evoked quantal release. An isolated frog *cutaneous pectoris* nerve-muscle preparation, bathed in a low Ca^{++}-high Mg^{++} solution, was stimulated at 0.5 Hz. Extracellular recordings were made of the nerve terminal action potential and a total of 2268 evoked quantal events. The cumulative frequency of these latencies is plotted. 1×10^{-5} M noradrenaline reduced the latency of quantal release and its variation. The vertical dotted lines indicate the times when 90% of the quanta have been released (from Bukchareva et al., 1999).

depolarisation to be achieved from the same number of quanta than would otherwise occur (Fig. 13). Consequently, the effects of catecholamines would be to increase the safety factor, and hence the reliability, of neuromuscular transmission at times of stress. At present, the mechanism of this adrenergic effect is not known.

5.2. Postsynaptic features influencing the response to transmitter

5.2.1. AChE

Before it can bind to an AChR, each ACh molecule must traverse the AChE-rich basal lamina. Following vesicle exocytosis, this happens sufficiently rapidly that most ACh molecules are bound to AChRs before they are hydrolysed. Once bound, the ACh molecules are protected from the AChE, but as they dissociate, they are rapidly cleaved (Katz and Miledi, 1973). The activity of AChE terminates ACh action on a time scale that is fast with respect to the mean open time of the ACh-gated channels (1–1.5 ms in adult mammalian muscle). It thus prevents most ACh molecules from rebinding to a second AChR, possibly further from the site of release. Thus AChE activity restricts both the spatial and temporal extent of the action of a single quantum (Fig. 14), allowing for high frequency activation of muscle (Hartzell et al., 1975). Normally, AChE is present in very considerable excess, so modest changes in AChE density would be expected to have little impact on safety factor (Anglister et al., 1994). When AChE activity is substantially reduced in pathological conditions, the safety factor is initially enhanced, but secondary disruption of the postsynaptic apparatus may ensue (see Sections 7.2.4 and 7.3).

5.2.2. Density and distribution of AChRs

The effect of a quantum of ACh released from the nerve is influenced by the number of AChRs it can activate. The concentration of AChRs in the membrane opposite the presynaptic release sites is about 10 000 per μm^2 (Salpeter, 1987). At this high density the AChRs form a paracrystalline array and it is unlikely that there can be any further increase in their density. The binding of ACh to the AChRs is sufficiently rapid that most of

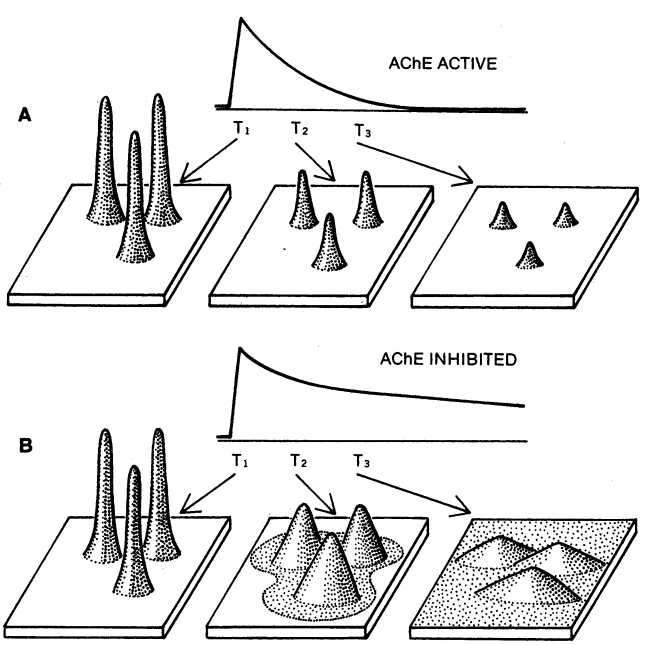

Fig. 14. The action of AChE at the NMJ. In the presence of normal AChE activity (A), the ACh molecules released as a 'quantum' from a given site on the nerve terminal are digested before they have spread into the 'territory' of another quantum. Thus each quantum acts independently of the others. When AChE activity is blocked (B), ACh diffuses much further and the territories of adjacent quanta overlap. As a result, the ACh concentration rises in a much larger region of the synaptic cleft, and more AChR are activated (from Hartzell et al., 1975).

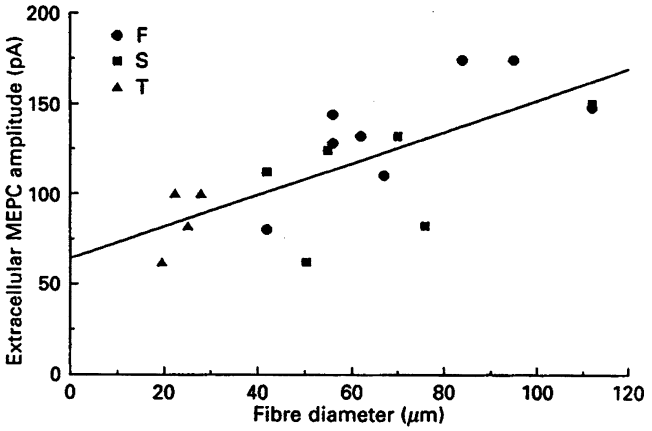

Fig. 15. minEPC amplitude is related to muscle fibre diameter in a snake muscle. The amplitude of extracellularly recorded minEPCs varies according to fibre diameter and hence, 'input resistance' in fast (F) slow (S) and tonic (T) muscle fibres in a garter snake muscle. This suggests that the efficacy of individual quanta released in this muscle is regulated such that larger muscle fibres receive larger synaptic currents (from Wilkinson et al., 1992).

the ACh molecules in a quantum are bound within 0.3–0.5 μm of the site of release, saturating the AChRs in that region. This results in the opening of about 2000–2500 channels in a 'saturated disc' of postsynaptic membrane about 1 μm across (Salpeter, 1987).

It is generally assumed that the density of AChRs is similar at all NMJs. One interesting study suggests that this may not be so (Wilkinson et al., 1992). In garter snakes, it was found that mEPC amplitude is greater in large, low resistance muscle fibres than in small, high resistance fibres (Fig. 15). One possible explanation for this is that there is a systematic variation in AChR density, though this has not been shown explicitly.

Changes in the density and distribution of AChRs are known to occur in a number of pathological situations (Akaaboune et al., 1999) (see Section 7.2.2). In almost all cases, these lead to a reduction of safety factor and impairment of neuromuscular transmission.

5.2.3. VGSCs/ Folds

In addition to AChRs, the postsynaptic membrane contains a high density of VGSCs (see Section 2.3, Fig. 6). These have been detected using both electrophysiological (Betz et al., 1984; Beam et al., 1985) and ligand labelling methods (Haimovich et al., 1984; Flucher and Daniels, 1989; Boudier et al., 1992; Wood and Slater, 1998b). Immunolabelling at the EM level has shown that the highest density of VGSCs is in the depths of the postsynaptic folds, where they may be 10 times as concentrated as in the membrane far from the NMJ (Flucher and Daniels, 1989). If many VGSCs are present in a given area, a smaller fraction of these need to be opened to generate an action potential than if few channels are present. This, in turn, means that less depolarisation is required to generate an action potential; i.e. the threshold is lower. Well-known examples of this principle at work in the nervous system are the nodes of Ranvier and the axon hillock of many neurons. In both cases, the density of VGSCs is very high and the threshold depolarisation accordingly low (Catterall, 1992). At the NMJ, the presence of such a high density of VGSCs adjacent to cation channels opened by ACh substantially lowers the threshold for action potential generation. This, in turn, means that fewer cation channels need to be opened to reach threshold. For a given number of quanta released, this effectively amplifies the transmitter effect and thereby increases the safety factor for neuromuscular transmission.

The effective depolarisation of the membrane containing the high density of VGSCs is augmented by the geometry of the postsynaptic folds. The narrow interfold space forms a high resistance path to current flow (Vautrin and Mambrini, 1989). Thus the flow of current induced by opening ACh-gated channels has its greatest depolarising effect on the membrane of the folds where VGSCs are concentrated at high density. A detailed consideration of the quantitative aspects of this effect has recently been given (Martin, 1994). The opening of sodium channels resulting from this depolarisation would be expected to amplify the effect of the transmitter released from the nerve and reduce the threshold for action potential generation (Martin, 1994; Wood and Slater, 1997), thereby enhancing safety factor.

Since the classic studies of Hodgkin and Huxley (1952), it has been clear that an important effect of membrane depolarisation is the inactivation of the VGSCs. For the VGSCs of the squid axon, repolarisation to the resting potential reactivates the channels within a few milliseconds. In addition to fast inactivation of VGSCs, mammalian skeletal muscle exhibit a much slower form of inactivation acting over several minutes (Ruff et al., 1987). While the rapid inactivation of the channels terminates the action potential, this slower inactivation plays a role in the excitability of muscle. The observation that slow inactivation is a more prominent form of VGSC inactivation in fast twitch muscle fibres than in slow twitch muscle fibres (Ruff et al., 1987; Ruff and Whittlesey, 1992) has important consequences for action potential generation (Wood and Slater, 1995). At the same resting membrane potential, a larger proportion of VGSCs will be inactivated in fast twitch muscles than in slow twitch muscles, making the fast twitch muscle relatively inexcitable. Indeed we found that in recordings from rat EDL muscle fibres it was necessary to hold the membrane potential at -90 mV for several minutes in order to eliminate this slow inactivation and to allow an action potential to be generated (Wood and Slater, 1995).

Furthermore, not all VGSCs in skeletal muscle are the same. Indeed it has been suggested that there may be transcripts of as many as 7 or 8 sodium channel genes expressed in skeletal muscle (Schaller et al., 1992). Two types of functionally significant skeletal muscle VGSCs (SkM1 and SkM2) have been described, distinguished by their relative tetrodotoxin (TTX) sensitivity and their developmental appearance (Catterall, 1992). The TTX-resistant (TTX-R) SkM2 channels predominate in immature muscles whereas the TTX-sensitive (TTX-S) SkM1 channels predominate in mature muscles (see Section 6.3.2). The SkM2 channels have slower kinetics, smaller single-channel conductance and a more positive current–voltage relation than the SkM1 channels (Yoshida, 1994). Relatively little is known about how the changing proportions and distribution of these two forms of VGSC during development influences the generation of muscle fibre action potentials.

5.2.4. Muscle fibre diameter and R_{in}

During development and variations of normal usage, the diameter of muscle fibres changes significantly and this influences the postsynaptic effect of transmitter. The depolarisation caused by the flow of current into a muscle fibre is proportional to the 'input resistance' of the cell. This, in turn, is directly related to the resistance per unit area of surface membrane (closely related to permeability) and inversely related to the (fibre diameter)$^{3/2}$ (Katz and Thesleff, 1957). Thus, if the number of ACh molecules per quantum was constant, then the minEPP amplitude should be greater for a small muscle fibre than for a large one. This is in fact what is observed (Fig. 16). One result of this relationship is that small muscle fibres require fewer quanta to reach threshold than large ones. One might therefore expect that small muscle fibres would have a higher safety factor than large ones, but there is little evidence that that is the case. This is presumably because of the ability of the nerve terminal to adapt its size, and hence quantal content, to match fibre diameter (Sections 5.1.1 and 6.5).

6. Biological variation and safety factor

Normal vertebrate neuromuscular junctions vary greatly in their size and form. Consistent variations exist between NMJs in different species, in muscles of different function in the same species, and in different stages of development. In each case, the observed structural variations are likely to have an important impact on the safety factor.

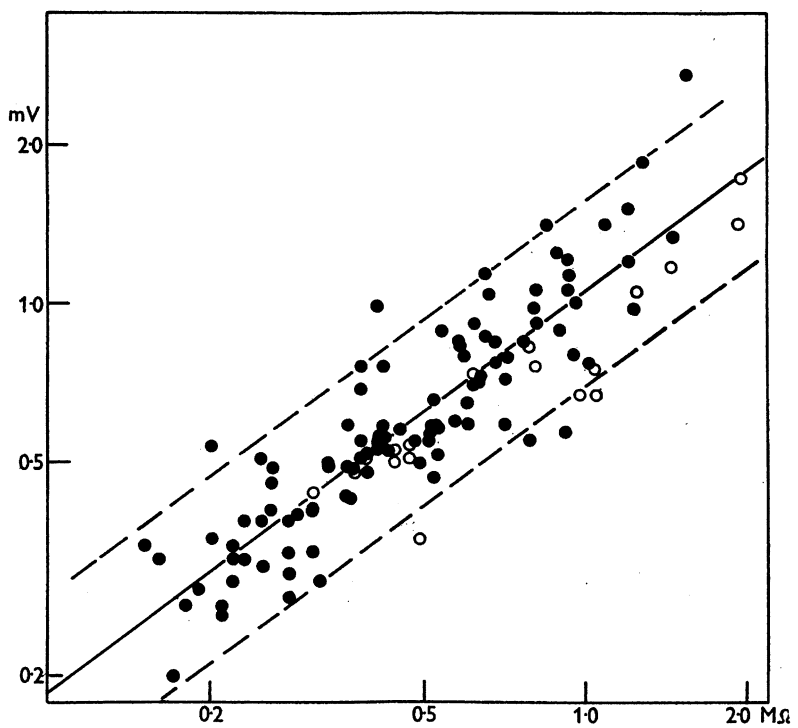

Fig. 16. minEPP amplitude (vertical axis) is proportional to muscle fibre 'input resistance' in two frog muscles (open circles, sartorius; filled circles, extensor digitorum longus IV). MinEPPs recorded from small muscle fibres, which have high input resistance, are larger than those recorded from large muscle fibres which have relatively low input resistance (from Katz and Thesleff, 1957).

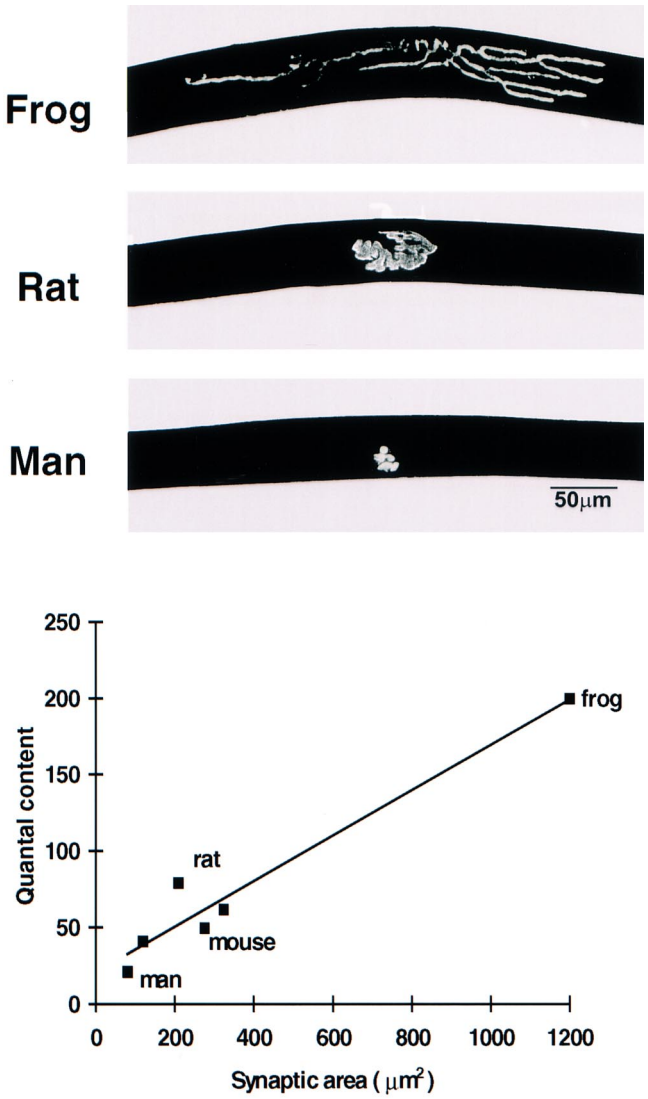

Fig. 17. The quantal content is related to the size of NMJs in different species. Upper panels represent individual muscle fibres in (from above) frog (*cutaneous pectoris*), rat (*soleus*) and man (*vastus lateralis*). The NMJs have been visualised in these fluorescence micrographs by labelling the AChRs with a fluorescent conjugate of α-BgTx. The graph shows that the quantal content in these muscles and in a mouse muscle (*epitrochleoanconeus*) is approximately proportional to the synaptic area. The line is the best-fit determined by least squares ($r = 0.973$, $p < 0.001$).

6.1. Variation between species

Studies of NMJs from different species indicate that they vary in size, quantal content and ultrastructure. Detailed analysis of human NMJs reveals that their area is only 50% of that in rat/mouse and only 20% of that in frog (Fig. 17) (Slater et al., 1992). Electrophysiological studies of these species show that the quantal content is directly related to the area of the NMJ (Fig. 17) (Slater et al., 1992). A consequence of this relationship is that for muscle fibres of approximately the same diameter and input resistance, many fewer quanta are released in man than in frogs.

Other things being equal, the relatively low quantal content of human NMJs would be expected to lead to a low safety factor for neuromuscular transmission. Indeed, when the safety factor for neuromuscular transmission in man is calculated using the classical approach, based on values of action potential threshold derived from current passing studies, it is little greater than 1, even at a low frequency of stimulation. Yet common experience, as well as detailed EMG studies (Bigland-Ritchie et al., 1978, 1982), indicate that neuromuscular transmission rarely fails in man.

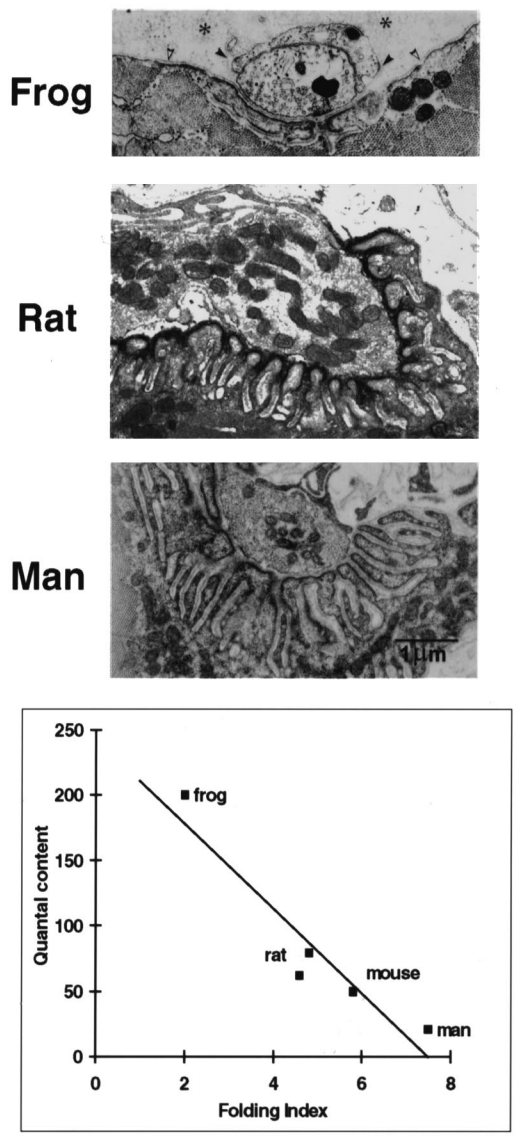

Fig. 18. The extent of postsynaptic folding is inversely related to quantal content in NMJs from a number of species. There is much more folding at human NMJs than at those in frog. This is consistent with the hypothesis that the folds, and the sodium channels within them, serve to amplify the effect of the transmitter released from the nerve. The line is the best-fit determined by least squares ($r = 0.945$, $p < 0.005$).

As is so often the case in biology, 'other things' are not equal. Ultrastructural studies show that there is a very substantial increase in postsynaptic folding that accompanies the decrease in quantal content (Fig. 18). In humans the postsynaptic area is increased about 8 times by the extensive infoldings of the postsynaptic membrane whereas in frogs the postsynaptic area is only doubled. If, as has been argued (Section 5.2.3), the postsynaptic folds and the VGSCs harboured within them amplify the effect of ACh released from the nerve terminal, then the low quantal content at human NMJs may be effectively counterbalanced by the more extensive folding. Unfortunately, careful estimates of safety factor have not yet been made in frog or man. While anecdotal evidence suggests that the safety factor may be generally lower in man than in mice, there is little solid evidence for this view.

An interesting exception to the reciprocal relationship between nerve terminal size and folding is presented by NMJs in chickens. While the quantal content (Wood and Slater, 1998a), and the size of the nerve terminals in chickens is similar to that in rats, postsynaptic folds are virtually absent (Wood et al., 1996). This suggests that the safety factor itself may be low in chickens. Although this has not yet been rigorously established, the generally unathletic nature of domestic chickens is not inconsistent with a safety factor substantially lower than that in rats.

In summary, these observations of a reciprocal relationship between nerve terminal size and the extent of postsynaptic folding suggest that different vertebrate species use different strategies to achieve an adequate safety factor of neuromuscular transmission. In the lower vertebrates studied, folding is minimal and the nerve terminals are relatively large. In man, the nerve terminals are small, and this is apparently compensated for by the very extensive postsynaptic folds. In between are rats/mice, intermediate in both the size of the nerve terminal and the extent of folding. Why different species should have evolved different approaches to the problem of ensuring an adequate safety factor is unknown. One possibility is that as animals increase in size, each motor neuron has to innervate more and more muscle fibres. Considerations of economy may have demanded that the total area of presynaptic contact for a given motor neuron remained within limits, and thus favoured the evolution of postsynaptic mechanisms to compensate for this.

6.2. Variation within species

Even within the same species, there are systematic differences between NMJs which are related to variations in muscle fibre properties. The matching of nerve terminal size and quantal content to muscle fibre diameter has already been discussed (Sections 5.1.1 and 5.2.4). Another component of the variation within species is that muscle fibres differ in their functional properties. Individual motor neurons have distinctive patterns of activity and the muscle fibres they innervate-comprising the motor unit-are all similarly adapted to the functional requirements of the motor unit to which they belong.

A number of studies have found consistent differences in the efficacy of transmission in the NMJs associated with motor unit type (Gertler and Robbins, 1978; Lev-Tov, 1987; Wood and Slater, 1997). In the rat, soleus muscles have predominantly 'slow' motor units which contract relatively slowly and are activated more or less continuously at a frequency of about 10–20 Hz (see Section 5.1.2). In contrast, EDL muscles contain predominantly 'fast' motor units which are active in short, high frequency (up to 100 Hz) bursts. The structure and function of NMJs in both muscles have been studied in detail, and both pre- and postsynaptic differences have been reported.

The terminals of soleus motor neurons are larger and less varicose than those of EDL motor neurons (Waerhaug, 1992b; Wood and Slater, 1997) yet they release fewer quanta per unit area than those of fast nerve terminals (Gertler and Robbins, 1978; Wood and Slater, 1997; Reid et al., 1999). Contrary to the general trend (Section 5.1.1), the quantal content at soleus NMJs stimulated at 1 Hz is only 70–85% of that at EDL junctions (Wood and Slater, 1997; Reid et al., 1999). Thus the quantal content per unit area at soleus NMJs is less than 50% of that in EDL. As a result, at low frequency, the safety factor at soleus NMJs is less than that in EDL (Table 1) (Wood and Slater, 1997). During continuous activity at higher frequencies of stimulation, similar to those characteristic of soleus motor units in vivo, the quantal content declines in NMJs in both muscles. After 10 min of stimulation at 20 Hz, the quantal content in the soleus is 2.5 times that in EDL indicating that the soleus is better than the EDL at maintaining its quantal content (Fig. 19) (Gertler and Robbins, 1978; Reid et al., 1999). This ability to maintain quantal release, and hence safety factor, appears to be related to the release of a smaller fraction of the total vesicle pool per impulse in soleus than in EDL (Reid et al., 1999).

It has also been suggested that the postsynaptic components of the NMJ differ in fast and slow muscle fibres. In particular, it has been reported that the extent of postsynaptic folding is greater at NMJs in fast fibres than at those in slow ones (Padykula and Gauthier, 1970; Ellisman et al., 1976; Ogata, 1988), although this has not been confirmed in all studies (Wood and Slater, 1997). In addition, the density of VGSCs is greater, and increases more at the NMJ, in fast twitch fibres than in slow (Milton et al., 1992; Ruff, 1992; Milton and Behforouz, 1995). It thus appears that there are both

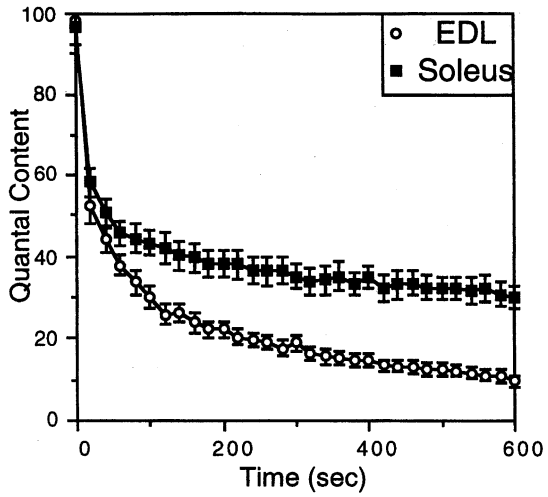

Fig. 19. Quantal release is better maintained during repetitive activity at NMJs in slow than in fast rat muscles. The quantal content was determined in isolated nerve-muscle preparations from fast (EDL) and slow (Soleus) rat muscles. The nerves were stimulated at 20 Hz for 10 min (from Reid et al., 1999).

pre- and postsynaptic specialisations of the NMJs in differing motor unit types which presumably help to match their efficacy to their normal patterns of activity.

6.3. Development of the NMJ

Extensive reviews of the development of the vertebrate NMJ can be found elsewhere (Sanes and Lichtman, 1999). Here, we will discuss the implications of the changes on safety factor, basing our account on results in rats and mice where the most detailed studies have been made. NMJs form at a very early stage in muscle development, when the muscle fibres themselves are very small. During the first few weeks after birth, there are changes in both pre- and postsynaptic components of the NMJ which result in increased effectiveness of neuromuscular transmission. This is closely correlated with the greater use of the muscles that underlies the development of the animal's motor behaviour during this period.

6.3.1. Presynaptic features

The increasing safety factor as the NMJ matures (Kelly, 1978; Wareham et al., 1994) is paralleled by a dramatic increase in the size of the motor nerve terminal (Slater, 1982; Balice-Gordon et al., 1990) (Fig. 20). Each muscle fibre is initially innervated by several motor axons. This polyneuronal innervation is resolved into the adult pattern of innervation by a single motor axon by 2–3 weeks after birth (Jansen and Fladby, 1990). As this takes place, the increase in size of the surviving axon terminal is paralleled by increased release of transmitter (see Section 5.1.1) (Diamond and Miledi, 1962; Dennis et al., 1981; Colman et al., 1997). This probably accounts for much of the observed increase in safety factor (Kelly, 1978; Wareham et al., 1994; Colman et al., 1997). There are developmental changes in the types of Ca^{++} channels present in the motor nerve terminals, and these may also contribute to the increases in transmitter release (Rosato Siri and Uchitel, 1999).

6.3.2. Postsynaptic features

The maturation of the presynaptic terminal is paralleled by several important postsynaptic changes. At the immature NMJs of mammals, the isoform of the AChR present differs from that in adults; a γ-subunit is

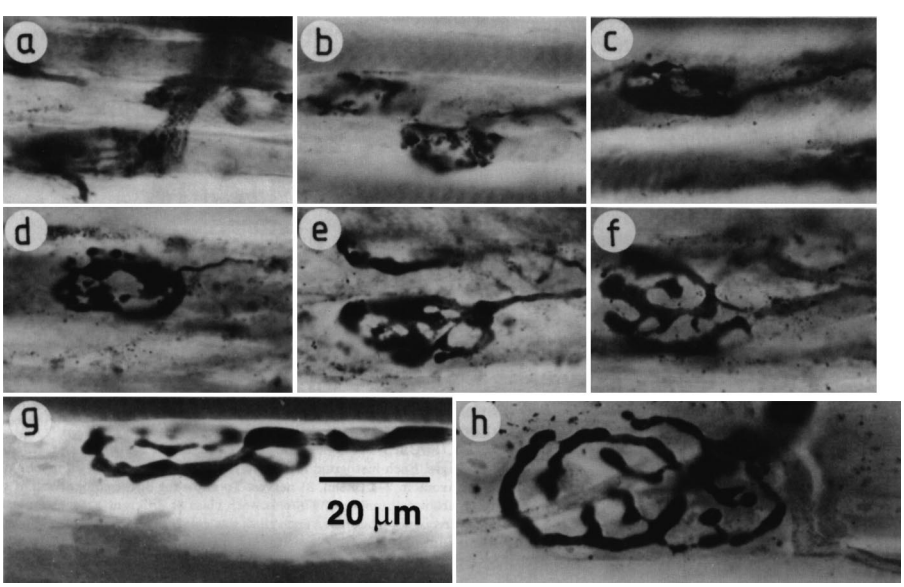

Fig. 20. Motor nerve terminals increase in size during maturation of the NMJ. Mouse EDL muscles were stained with zinc-iodide to reveal the motor nerve terminals. (a) newborn, (b) 1 week postnatal, (c–f) 2 weeks, (g) 3 weeks and (h) adult (from Slater, 1982).

present at the immature NMJ in place of the ε-subunit of the adult (Sanes and Lichtman, 1999). Functionally, while the single channel conductance of the immature AChR is less than in the adult the mean open time is longer. As a result, the presence of the foetal isoform means that for each opening of the channel, more cations enter the muscle fibre and more depolarisation occurs per ACh molecule, than in the adult. This effect enhances the safety factor and tends to counteract the effect of the low quantal content at immature NMJs.

The enhanced ability of individual ACh molecules to cause depolarisation at the immature NMJ is achieved at the expense of speed; the relatively long mean open time places one of several limits on the upper frequency at which the immature NMJ can be activated. In addition to the change in AChR isoform, there is a marked increase in AChR density in the postsynaptic membrane during embryonic development (Bevan and Steinbach, 1977; Matthews-Bellinger and Salpeter, 1983; Ziskind-Conhaim et al., 1984). By birth, however, the local density is similar to that in the adult.

Another important postsynaptic change to arise during development is the elaboration of the postsynaptic folds. In rats and mice, this occurs primarily during the first 3–4 weeks after birth (Fig. 21) (Kelly and Zacks, 1969; Korneliussen and Jansen, 1976; Matthews-Bellinger and Salpeter, 1983; Bewick et al., 1996), while in humans, it begins at about 18 weeks of gestation and continues for some years after birth (Arizono et al., 1984; Hesselmans et al., 1993). This is accompanied by important changes in the distribution of VGSCs (Lupa et al., 1993; Wood et al., 1998; Stocksley and Slater, 1999). In rats, these are first clearly detectable by immunolabelling at birth, in a zone 100–200 μm wide surrounding each NMJ. Only 1–2 weeks later, as the folds form, does the characteristic accumulation of VGSCs at the NMJ itself take place (Lupa et al., 1993; Wood et al., 1998). It is likely that the process of VGSC accumulation at the NMJ contributes to the increasingly high safety factor (Section 5.2.3) (Wareham et al., 1994).

As the postsynaptic folds are forming, the VGSCs undergo an isoform switch analogous to that of the AChRs. The embryonic, or SkM2 form, of VGSCs differs in several ways from the SkM1 form present in the adult (see Section 5.2.3). Of most impact on the safety factor of neuromuscular transmission is the fact that its opening is activated at membrane potentials considerably more negative than the SkM1 (Weiss and Horn, 1986). This has the effect of reducing the ACh-induced depolarisation required to trigger an action potential, and thus increasing the safety factor for transmission at the immature NMJ where the quantal content is very low. The small diameter of immature muscle fibres and the high input resistance resulting from it enhance this effect. As a result, even the current

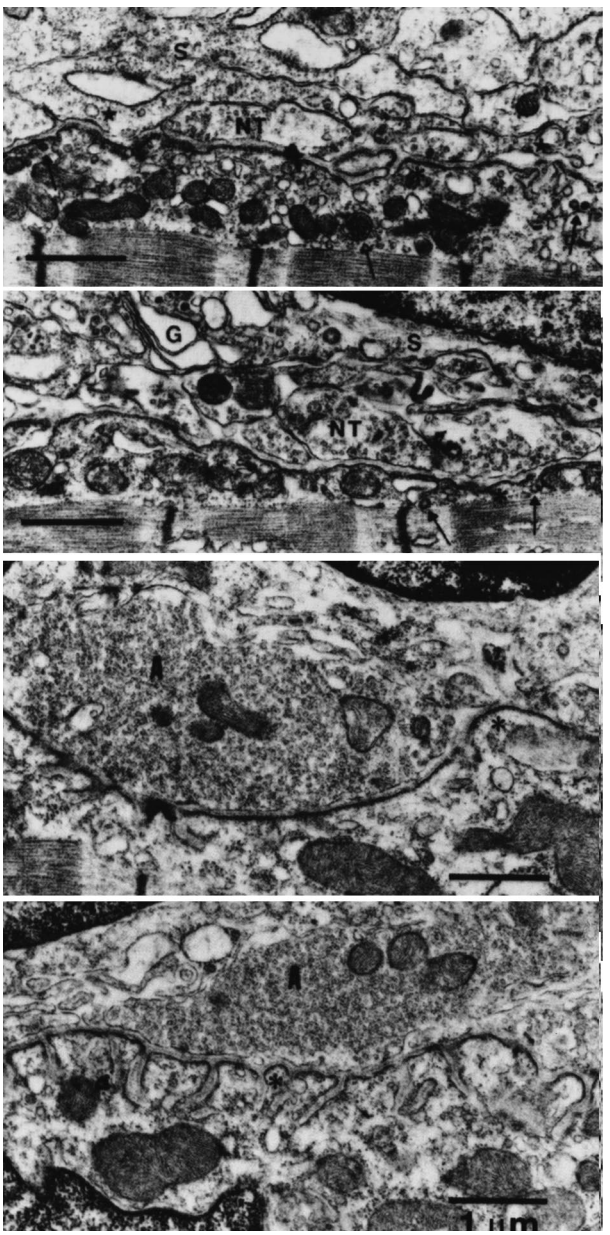

Fig. 21. Postsynaptic folds form soon after birth in mice. Electronmicrographs of NMJs in mouse EDL muscle at (from the top) birth, 3 days postnatal and 2 weeks postnatal (bottom two panels). Few folds are present at birth. Two weeks after birth, the extent of folding is much greater but is still highly variable. Scale bars represent 1 μm (from Matthews-Bellinger and Salpeter, 1983. Copyright 1983 by the Society for Neuroscience).

caused by the action of a single spontaneously released quantum may cause enough depolarisation to evoke an action potential in the muscle fibre (Jaramillo et al., 1988). Taken together, these effects result in an adequate safety factor of neuromuscular transmission even at immature NMJs where the quantal content is very low.

In summary, the many properties of the NMJ that determine the safety factor in the adult arise as part of a complicated developmental sequence which may last

several weeks. In the immature mammal, the small size of the muscle fibres and the specific properties of the AChRs and VGSCs ensure that neuromuscular transmission is effective even though relatively little transmitter is released from individual nerve terminals. This effectiveness may be seen to be 'bought' at the expense of speed. As the animal and its muscle fibres increase in size during development, the need to retain effective transmission at increased speeds is met, in part by increasing the size of the junction, and with it the amount of transmitter released. In addition, the effectiveness of the transmitter is increased by the elaboration of postsynaptic folds and by the expression of adult isoforms of AChRs and VGSCs. These changes permit the muscle to respond effectively to the high frequency activation that is required to move full-sized body parts at the required speed.

6.4. Aging

Once the mature NMJ has formed, there is evidence that remodelling occurs into old age (Barker and Ip, 1966; Wernig and Herrera, 1986; Andonian and Fahim, 1989; Grinnell, 1995), though at a slow pace (Lichtman et al., 1987). This process appears to involve both the pre- and postsynaptic components. In light of the discussion in earlier sections of this review, it seems likely that many of the changes in NMJ structure seen during aging would have an impact on the safety factor of neuromuscular transmission. However, there have been relatively few direct studies of neuromuscular transmission, much less safety factor, as a function of aging.

Anatomical studies in both laboratory mammals and man suggest continuing changes in NMJ structure with age (Barker and Ip, 1966; Arizono et al., 1984; Cardasis and Lafontaine, 1987; Oda, 1984; Wokke et al., 1990). In most cases, these result in more highly branched synaptic regions which may (Waerhaug, 1992a) or may not (Fahim and Robbins, 1982; Fahim et al., 1983) be associated with an overall change in their total area. On the presynaptic side, some studies have found an increase in the degree of preterminal branching (Oda, 1984; Andonian and Fahim, 1989; Prakash and Sieck, 1998), though this has not been seen in all such studies (Wokke et al., 1990). On the postsynaptic side, a number of studies have reported an increase in the complexity and branching of the region of specialised postsynaptic membrane. In addition, there is an increasing amount of specialised postsynaptic membrane that is not in contact with the nerve terminal, suggesting a partial withdrawal of the nerve (Arizono et al., 1984; Cardasis and Lafontaine, 1987).

These structural changes are accompanied by changes in the safety factor. In rat diaphragm, the safety factor was reported to increase steadily from about 4 to 10 during the first 9 months of life and then to decline (Fig. 22) (Kelly, 1978). The changes in safety factor were accompanied by similar changes in quantal content (though the values given are very high owing to the use of the 'variance' method (Section 4.2.2) to determine them). On the basis of existing evidence, it seems likely that the changes in quantal content are a major determinant of the changes in the safety factor during aging.

All these observations suggest that a gradual process of NMJ remodelling occurs throughout life. Recent studies have shown that the terminal Schwann cells play an important part in the induction of nerve terminal sprouting following partial denervation (Son and Thompson, 1996) and it is likely that they also influence the remodelling of the presynaptic terminal nerve during aging. So long as this process maintains the total extent of the nerve terminals, it is likely that the safety factor would be maintained. Eventually, however, the presence of 'unoccupied' postsynaptic membrane suggests that this endeavour fails. While the increasing complexity of the postsynaptic surface may represent an effort to enhance the effect of a diminishing quantal content, the overall effect seems to be a decline of safety factor in old age.

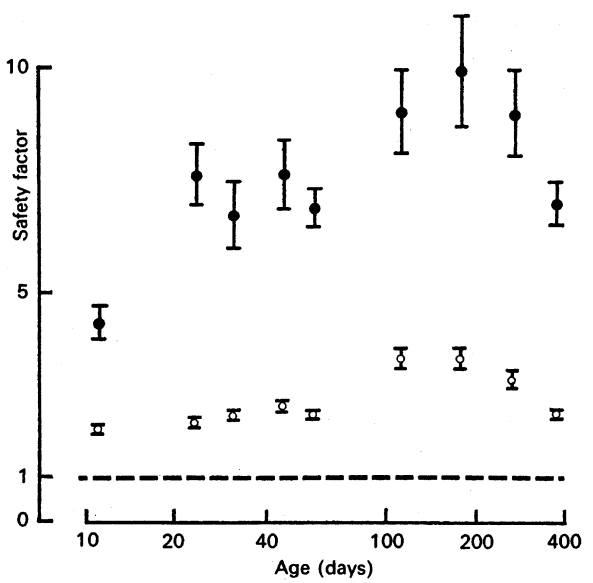

Fig. 22. The safety factor of neuromuscular transmission changes throughout life in rats. The safety factor was determined in isolated, partially curarised, rat phrenic nerve-diaphragm nerve-muscle preparations from rats of different ages. Filled circles show the safety factor determined from the first EPP of trains of stimuli at 10 Hz and the open circles show the safety factor determined from EPPs during the subsequent plateau phase of the train. The safety factor increases rapidly during early development (note logarithmic age scale), reaches a plateau at 200 days and then declines in later life (from Kelly, 1978).

6.5. Interactions that might contribute to the development of a high safety factor

It is clear from much of what we have discussed so far that mechanisms must exist within the cells of the NMJ which account for the long-term matching of pre- and postsynaptic features that underlie the maintenance of an adequate safety factor. Although little is known about these processes, a number of interesting experimental studies have provided some glimpses of the likely complexity of that regulation.

6.5.1. Control of nerve terminal morphology by activity

The nerve terminals of 'fast' (EDL) and 'slow' (SOL) motor neurons in rats show consistent pre- and postsynaptic differences (Section 6.2). How are these features of the NMJ regulated? Do they reflect inherent, autonomous properties of the individual motor neurons themselves or are they acquired characteristics, reflecting differences in the imposed patterns of activity and the properties of the target muscle fibres?

In some circumstances, the form and size of the nerve terminal appears to be determined by the motor neuron rather than by the muscle fibre it innervates. Thus, when fast nerves in adult rats are made to innervate slow soleus muscles at ectopic sites, the NMJs they form are larger and more varicose than those made by slow nerves (Waerhaug and Lømo, 1994). The possibility that these differences might be mediated by the pattern of activity in the nerve were tested in the same study by stimulating the fast nerve with a pattern activity characteristic of slow motor units. This had only a modest effect on nerve terminal size and form, suggesting that other features of the two types of motor neuron determine the form of the terminals and, presumably, the efficacy of transmission.

There have been several studies which have reported modest effects of voluntary exercise on NMJ morphology (Rosenheimer, 1985; Andonian and Fahim, 1987; Herscovich and Gershon, 1987; Deschenes et al., 1993; Panenic and Gardiner, 1998). These studies report differing effects that depend on muscle type and age of animal. In general, they reveal that exercise can cause an increase in the area of the NMJ, or can counteract senescence-related changes in NMJ morphology (Deschenes et al., 1993). This was particularly true in 'fast' muscles of the rat (Andonian and Fahim, 1987). Assuming the validity of the general 'rule' that quantal content is approximately related to synaptic area (see Section 6.1), these effects of activity would be expected to increase the safety factor, and thus be seen as an adaptive response to the increase in activity.

6.5.2. Control of transmitter release

While we have so far emphasised that the number of transmitter quanta released per unit area of nerve terminal is quite constant in a number of situations, there is also evidence that this feature of the NMJ is plastic, and can be modified in situations that require an increase in safety factor. Release per unit area can be modulated in the short term by a number of pharmacologically distinct mechanisms (see Section 5.1.3). Interestingly, a longer term modulation, whose mechanism is less clear, has also been demonstrated. When some of the postsynaptic AChRs in rats are permanently blocked by chronic application of α-bungarotoxin, there is an increase in quantal content over a period of days which appears not to be associated with any increase in the area of synaptic contact (Plomp et al., 1992). A similar increase is seen in myasthenia gravis, a disease in which the density of AChRs is reduced as a result of an autoimmune response (see Section 7.2.1).

Neither the nature of the feedback from muscle to nerve that controls this upregulation of transmitter output nor the cellular and molecular processes on which that feedback operates are well-understood. There is some evidence that it may involve activity of Ca^{++}-calmodulin-dependent protein kinase II in the motor nerve terminal (Plomp and Molenaar, 1996), but the relevant substrates for this enzyme are unknown. Nonetheless, the observations attest to a degree of plasticity of the NMJ that has generally been unappreciated in the past.

6.5.3. Muscle fibre size: use-dependent changes

The diameter of muscle fibres changes in response to varying patterns of use. Prolonged periods of disuse, such as follow joint and bone injury or denervation, lead to marked atrophy of most muscle fibres. This, in turn, results in an increase in the electrical input resistance of the fibres and hence in the depolarisation induced by each AChR channel opening. In situations where nerve damage and regeneration has occurred, this effect tends to increase the safety factor and thus favours effective muscle excitation by a functionally immature regenerating nerve terminal.

Any increase in muscle fibre diameter, as results from intense isometric training, would be expected to reduce the input resistance of the muscle fibre and hence lower the amplitude of the EPP (see Section 5.2.4). This, in turn should lower the safety factor of neuromuscular transmission. As discussed above (Section 6.5.1) there is some evidence that exercise leads to an enlargement of the motor nerve terminal (Panenic and Gardiner, 1998) and this may help to compensate for the effect of muscle fibre hypertrophy.

7. Pathology of the safety factor

The complexity of neuromuscular transmission means that there are many points at which the safety

factor can be compromised. Numerous diseases have been described, each quite rare, which are characterised by an impairment of neuromuscular transmission resulting in a weakness of voluntary contraction. These are known collectively as myasthenias or myasthenic syndromes. Some myasthenias are acquired autoimmune conditions in which antibodies are produced which interact with synaptic molecules (Whitney and McNamara, 2000). Others are inherited conditions, usually referred to collectively as congenital myasthenic syndromes which involve mutations in key synaptic molecules (Vincent et al., 1997; Beeson et al., 1998; Engel et al., 1999). In some of these, the mutated genes have been identified (see below). In most myasthenias, neuromuscular transmission can function adequately at low levels of activity, but begins to fail during intense or sustained activity, resulting in clinical weakness. This situation reflects a reduction of the safety factor which may result from one of a wide variety of cellular or molecular defects.

Here we describe briefly some of the best described conditions in which neuromuscular transmission and its safety factor are impaired. These are of interest because they reveal the wide range of cellular and molecular events that influence transmission. In addition, they reveal the considerable ability of the NMJ to respond to defects in some of its components by compensatory enhancement of others. We have not included in our discussion any detailed account of the effects of the wide variety of natural toxins which impair neuromuscular transmission. The properties and actions of these fascinating compounds have been well reviewed elsewhere (Senanayake and Roman, 1992).

7.1. Pathophysiology of transmitter release

The evoked release of transmitter quanta from the motor nerve terminal is a highly complex process (Section 2.1). It involves the synthesis and packaging of ACh into vesicles, the opening of ion channels regulating the influx of Ca^{++} into the terminal, and the subsequent activation of a multi-protein complex leading to exocytosis of ACh-filled vesicles. Diseases are known which interfere with each of these aspects.

7.1.1. Congenital myasthenic syndrome with episodic apnea

A number of patients have been described with an inherited defect of neuromuscular transmission that appears to be associated with impaired synthesis and or packaging of ACh. The best known of these conditions was initially described as 'familial infantile myasthenia' (FIM) (Engel, 1994a) but is now better referred to as 'congenital myasthenic syndrome with episodic apnea' (CMS with episodic apnea). Patients with CMS with episodic apnea show a gradual decline in force during continued exertion or repetitive stimulation, associated with a decline of the compound muscle action potential. The few in vitro electrophysiological studies of muscle biopsies from such patients showed that repetitive stimulation for prolonged periods (10 Hz for 10 min) caused a gradual decline in minEPP amplitude and neuromuscular transmission (Engel et al., 1981). This functional deficit was paralleled by a decrease in the diameter of the synaptic vesicles (Mora et al., 1987), although it is not clear that these two observations are causally related.

These observations suggest that in patients with CMS with episodic apnea, there is a defect of ACh metabolism or packaging which results in a gradual decline in the number of ACh molecules per quantum. These findings are similar to those induced by the drug hemicholinium, an inhibitor of choline uptake into the motor nerve terminal (Elmqvist and Quastel, 1965). The reduction of minEPP amplitude in patients with CMS with episodic apnea would mean that more quanta would have to be released to reach threshold. In the absence of such an increase, the safety factor would get smaller, leading to a failure of transmission during attempts at maintained exertion. As there are so few detailed studies of CMS with episodic apnea, any certainty about the nature of the abnormal cellular and molecular processes must await further study.

7.1.2. Lambert-Eaton myasthenic syndrome

The Lambert-Eaton myasthenic syndrome (LEMS) is an acquired condition resulting from the elaboration of autoantibodies against VGCCs in the motor nerve terminal (Eaton and Lambert, 1957; Lang et al., 1981; Vincent et al., 1989). It is characterised by muscle weakness and abnormal fatigability on exertion. The underlying autoimmune response is frequently associated with a small cell carcinoma of the lung in which the tumour cells have VGCCs which resemble those on the nerve terminal (Vincent et al., 1989). In general, the autoantibodies do not have a direct pharmacological blocking effect on the VGCCs. Rather, they bind to the VGCCs on the nerve terminal, leading to their down-regulation by endocytosis. In addition, they lead to a reduction in the number and size of active zones (Fig. 23; Fukunaga et al., 1982). The net effect is that fewer Ca^{++} ions enter the terminal during each nerve impulse, so fewer quanta of transmitter are released. This, in turn, leads to as reduction in safety factor that may result in transmission failure.

An interesting, and at first glance paradoxical, feature of LEMS is that during sustained activation of a muscle, there is a dramatic, but transient, increase in strength from an initially very low level (Vincent et al., 1989; Engel, 1994a). This 'post exercise facilitation' seems likely to be due to the temporary build up of Ca^{++} in the nerve terminal which results in a striking

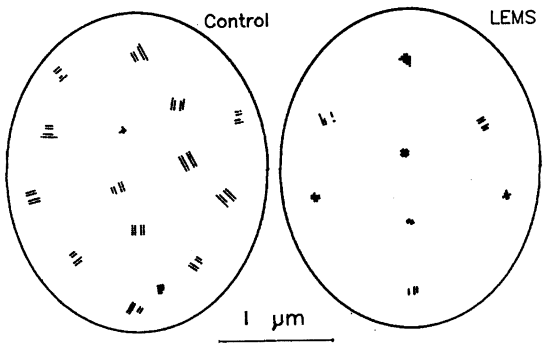

Fig. 23. Active zones decrease in number and size in LEMS. Stereometric reconstruction of freeze-fractured presynaptic membrane P face in control and LEMS muscle. The active zones are smaller and clusters of intramembranous particles are bigger in LEMS muscle (from "Paucity and disorganization of presynaptic membrane active zones in the Lambert–Eaton myasthenic syndrome", Fukunaga et al., 1982. Muscle and Nerve, 1982. Copyright © 1982, John Wiley & Sons, Inc. Reprinted with permission of John Wiley & Sons, Inc.).

potentiation of release, temporarily increasing the safety factor. A qualitatively similar potentiation occurs at normal NMJs (section 5.1.4) where its effect is generally undetectable, since the high safety factor normally ensures fully effective transmission even at resting NMJs. The most effective treatment of LEMS involves administration of 3,4-diaminopyridine (Lundh et al., 1990; Engel, 1994a). This drug blocks VGKCs in the nerve terminals, thereby prolonging the action potential. This enhances the entry of Ca^{++} into the terminal and thereby increases the quantal content and the safety factor.

7.1.3. Acquired neuromyotonia

Neuromyotonia is a disease characterised by hyperexcitability of motor axons (Newsom-Davis, 1997). As in all myotonias, its most characteristic feature is impaired muscle relaxation. Like LEMS, it is an acquired autoimmune condition. In this case, however, the targets of the antibodies are the VGKCs present in the motor axons and, possibly, in the nerve terminals themselves (Vincent et al., 1998). This results in a delayed repolarisation of the axon after each action potential, similar to that seen in the presence of 3,4-diaminopyridine (see Section 7.1.2). This would be expected to prolong the period of transmitter release from the terminal and the resulting depolarisation of the muscle fibre membrane, thus contributing to the generation of repeated action potentials in response to each nerve impulse. As yet there have been no detailed electrophysiological studies of neuromuscular transmission at NMJs from patients with neuromyotonia.

7.1.4. Miller–Fischer Syndrome

Miller-Fisher syndrome (MFS) is an acute paralytic disorder which is usually associated with IgG antibodies against the ganglioside GQ1b, an abundant component of the neuronal surface (Willison et al., 1993). These antibodies are usually elaborated as part of the response to a bacterial infection, particularly that induced by the gut bacterium *Campylobacter jejuni*. At least one effect of many MFS sera appears to be to block neuromuscular transmission (Roberts et al., 1994). The nature of the transmission block is currently being investigated (Buchwald et al., 1995; Plomp et al., 1999).

In vitro studies in which isolated nerve-muscle preparations are bathed in the test sera show a gradual increase in spontaneous minEPP frequency to a very high level and eventual failure of transmission (Roberts et al., 1994). These findings are consistent with either a depolarising block of the nerve terminal or an effect similar to that of α-latrotoxin (a component of the venom of the Black widow spider). α-latrotoxin causes a massive increase in minEPP frequency, apparently by forming cation channels in the nerve terminal membrane, leading ultimately to transmission block (Scheer et al., 1984).

Other studies, using rapid local application of MFS sera, have revealed a rapid and reversible decrease in evoked quantal release and a reduction in minEPP amplitude and ACh sensitivity of the muscle fibre membrane (Buchwald et al., 1998). It is likely that the differing results reflect the different experimental approaches used. In either case, it seems that MFS sera lead to a failure of neuromuscular transmission which is caused, at least in part, by interference with evoked quantal release and, hence, a decrease in safety factor.

7.1.5. Botulism

Perhaps the best known of all defects of transmitter release is that caused by toxins of the bacterium *Clostridium botulinum*. These highly potent toxins include at least 7 different protein components, each composed of a heavy chain responsible for selective binding of the toxin and a light chain with proteolytic activity (Montecucco et al., 1996). Botulinum toxins bind to the presynaptic terminal and are then internalised. Once inside the nerve terminal the toxins catalyse the cleavage and inactivation of one of a number of essential components of the vesicle release system (Montecucco et al., 1996). This results in complete block of depolarisation-induced quantal release and a reduction of the safety factor to zero.

In mammals, including man, the action of the botulinum toxins lasts for weeks. It appears that once within the nerve terminal, the toxin becomes immobilised and its activity persists, blocking transmission but not otherwise interfering with the integrity of the nerve terminal. Recovery occurs only when the nerve terminals grow new sprouts which 'escape' the influence of the toxin and form new synaptic contacts with the

muscle fibre (Montecucco et al., 1996). This process can take weeks, during which time the patient must be maintained on a ventilator.

In recent years, the ability of botulinum toxin to cause a localised, long-lasting decrease of safety factor has been used to control maladaptive contractions of muscle (Jankovic and Hallett, 1994). It has been particularly beneficial in the treatment of localised dystonias (e.g. blepharospasm, strabismus). Increasingly, it is being used to treat spasticity resulting from cerebral palsy (Rose and McGill, 1998). At present, however, very little is known about the long-term effects of botulinum toxin treatment on the motor system.

7.2. Pathophysiology of transmitter action

The postsynaptic response to transmitter consists mainly of the activation of two sets of ion channels; AChRs and VGSCs. Any situation that results in a reduction of the number of channels of either type will have the effect of reducing the safety factor and, ultimately, causing transmission failure. Several diseases that affect AChRs are well known. There is less evidence for conditions that affect the VGSCs.

7.2.1. Myasthenia gravis

Myasthenia gravis (MG), like LEMS and neuromyotonia (Section 7.1), is an autoimmune disease. In MG, the auto-antibodies are directed against the extracellular portion of the AChR (Boonyapisit et al., 1999). This aberrant immune response is probably triggered by the AChRs on the myoid cells of the thymus. As in LEMS, most of these antibodies bind and cross-link the AChRs, rather than causing a pharmacological block of their function (Boonyapisit et al., 1999). This cross-linking leads to complement mediated breakdown of the postsynaptic membrane and a marked decrease in the number of AChRs and the extent of postsynaptic folding (Engel, 1980). As a result, fewer AChRs are opened by each quantum of ACh and the amplitude of the minEPPs is reduced accordingly. This primary effect, together with the secondary loss of postsynaptic folds and the VGSCs in them (Ruff and Lennon, 1998), leads to a profound reduction of the safety factor and transmission block.

Recently, it has become clear that the deleterious effects of autoantibody attack on the AChRs may trigger a compensating reaction of the NMJ. In situations where there is a reduction of functional AChRs, there is a prominent increase in transmitter output from the nerve terminal in the absence of any obvious increase in the extent of synaptic contact (Section 6.5.2) (Plomp et al., 1995). This effect partially compensates for the loss of AChRs and helps to prevent the safety factor from falling still further.

7.2.2. AChR deficiencies

A number of forms of inherited myasthenic syndrome exist in which an important feature is a deficiency of AChRs. In these patients, the number of AChRs is drastically reduced, to levels that may be even lower than those encountered in myasthenia gravis (Vincent et al., 1981; Slater et al., 1997). As a result, there is a corresponding reduction in the amplitude of minEPPs and EPPs leading to a reduction of the safety factor and impairment of neuromuscular transmission. Recent molecular genetic studies indicate that many of these AChR deficiencies, like the 'slow channel syndromes' discussed below, are caused by mutations in the genes coding for the subunits of the AChR, particularly the ε-subunit which can be partially replaced by the immature γ-subunit (Beeson et al., 1998; Engel et al., 1999).

There is evidence that two forms of compensatory response are triggered by the deficit in AChRs in these conditions. One is a sprouting of the nerve terminal and the formation of new regions of synaptic contact over an abnormally long length of the muscle fibre (Vincent et al., 1981; Slater et al., 1997). The other is the abnormally increased expression of the γ-subunit of the AChR, characteristic of the immature NMJ (Beeson et al., 1998). It is likely that both responses are mainly triggered by the loss of muscle activity resulting from the disease. Whatever their cause, both responses should lead to at least a partial restoration of the safety factor and help to ensure some degree of functional innervation of muscle. As yet, no increase in quantal content analogous to that seen in MG has been reported in cases of inherited AChR deficiency, though few accurate values have been determined (Engel et al., 1990; Slater et al., 1997).

7.2.3. The slow channel syndrome

It has been recognised for some years that inherited conditions exist in which the kinetics of the EPP are abnormal. In the best known example, the 'slow-channel syndrome' (SCS), muscle weakness and disrupted structure of the postsynaptic region of the NMJ were found to be associated with a very prolonged decay phase of the minEPPs and EPPs (Engel et al., 1982). It was subsequently established that this is due to the prolonged opening of the ACh-gated ion channel following ACh binding (Engel et al., 1999). Most cases of SCS result from mutations in the ε-subunit of the AChR molecule. These mutated AChR molecules, when expressed in cultured cells, duplicate the kinetic abnormalities seen in biopsy samples from the patients studied (Engel et al., 1999).

Why does a prolongation of transmitter action lead to a reduction of the safety factor when it would be expected to do the opposite? Here the key observation is a disruption of the structure of the postsynaptic

region and an associated reduction of minEPP and EPP amplitude (the quantal content is normal) (Engel et al., 1982). The most likely underlying cause of this is suggested by studies showing that the ion channels opened by ACh are somewhat permeable to Ca^{++} (Takeuchi, 1963). Prolonged opening of these channels would thus allow an influx of Ca^{++} into the postsynaptic region of the muscle fibre. It is likely that this activates Ca^{++}-sensitive lytic enzymes in the muscle fibre, leading to the 'excitotoxic' disruption of the postsynaptic apparatus that characterises this condition (Engel, 1994a). As in MG, the loss of the folds and the AChRs and VGSCs associated with them could account for both a reduction in quantal effect and a loss of the amplifying power of the postsynaptic apparatus. Both of these effects would reduce the safety factor of transmission

A newly developed approach to therapy specifically related to these conditions is based on efforts to shorten the 'open time' of the AChR. Quinidine, a long-lived open-channel AChR blocker, normalises the prolonged channel opening events in channels from patients with SCS (Fukudome et al., 1998). It is currently being evaluated as a therapy for these patients.

7.2.4. AChE deficiency

Patients with an inherited loss of junctional AChE were identified some years ago (Engel et al., 1981). The immediate effect of a loss of AChE activity would be an increase in the safety factor and an enhancement of transmission (Section 5.2.1). However, in the longer term, these patients suffer from a structural breakdown of the postsynaptic region of the NMJ, most particularly a loss of well organised folds. As in SCS and MG, AChRs and VGSCs are lost, the safety factor falls and, eventually, transmission may fail.

It has recently been found that the mutations that give rise to AChE deficiency are located in the region of the gene encoding the long collagen-like tail that anchors AChR to the basal lamina in the synaptic cleft (Ohno et al., 1998).

7.2.5. Diseases of unknown molecular basis that affect NMJ structure

A number of other forms of inherited myasthenic syndrome have been identified in which a common feature is the reduction in the extent of postsynaptic folding (Slater et al., 1991). In some cases this has been sufficiently prominent as to suggest the existence of a distinct 'congenital paucity of secondary synaptic folds' syndrome, associated with reduce minEPP amplitudes and a deficiency of AChRs (Smit et al., 1988). However. the diversity of circumstances in which such a reduction of folds is seen is such as to question whether there is really any specific condition that affects the folds directly. Very little is known about the events that lead to the formation of the folds. In recent years, a reduction of folding has been seen in a number of mutant mice in which structural proteins are missing. These proteins include laminin $\beta 2$, nerve cell-adhesion molecule (NCAM), dystrophin, and utrophin (Sanes et al., 1998). A modest reduction of folding has been seen in all cases, suggesting that many proteins contribute to the stability of the folds.

Whatever the pathogenesis of these conditions, current thinking about the role of the folds (see Section 5.2.3) suggests that their reduction might cause a parallel decrease in the safety factor of neuromuscular transmission. While AChRs are concentrated at the crests of the folds, they also extend part way into the folds themselves. Thus a reduction of folding of four-fold, as seen in some patients, would be likely to reduce significantly the number of AChRs available to a given quantum of ACh. An even greater reduction of VGSCs, normally concentrated in the depths of the folds, would be expected, thus reducing the amplifying effect of the postsynaptic apparatus. It has not so far been possible to obtain accurate values for the threshold in biopsy samples from such patients. However, it seems likely from our work on the rat that the safety factor might well be reduced to one-half its normal value in these patients (Wood and Slater, 1997).

7.2.6. Channelopathies

A number of conditions are now known in which mutations of ion channels in the muscle fibre membrane give rise to altered excitability (Cannon, 1996). Many of these 'channelopathies' result in increased excitability which is manifested clinically as myotonia; impaired relaxation of the muscles. At a molecular level, this increased excitability results from delayed inactivation of VGSCs or decreased chloride conductance.

We are unaware of any detailed studies of neuromuscular transmission in cases of myotonia. However, it seems likely that hyperexcitability of the muscle fibre membrane would reduce the threshold for action potential generation and might therefore increase the safety factor. In this case, given the adaptive potential of the NMJ already referred to, it seems likely that some 'down-regulation' of neuromuscular transmission might well ensue. Studies aimed at revealing such adaptive changes of the NMJ in channelopathies might therefore be of considerable interest.

7.3. Therapy

As understanding of the events that determine the safety factor of neuromuscular transmission has developed, it has suggested a variety of ways of treating patients in whom transmission is abnormal. However, there are still only a few effective approaches to therapy in common use. We, the authors, are not clinicians, and

cannot offer any detailed guidance to those trying to devise suitable treatments for individual patients. Nonetheless, some brief comments based on the present review of the safety factor seem worth setting out.

Most known conditions affecting neuromuscular transmission are characterised by muscle weakness that is aggravated by use. This suggests that although the essential cellular components required for transmission are present, the safety factor is reduced. In approaching therapy, there are two main ways to try to increase the safety factor. The first is to try to increase the amount of transmitter released by each nerve impulse. The second is to try to increase the effect of the transmitter released on the muscle fibre membrane, generally by blocking AChE activity. Common sense might suggest that the former should be tried in cases where the primary pathogenic effect is an impairment of release (e.g. LEMS) and the latter in cases where it is on transmitter action (e.g. MG). In practice, however, either approach may be beneficial in both pre- and postsynaptic defects. Regardless of the primary defect, increasing the amount of transmitter released or its postsynaptic effectiveness may help to overcome the reduction in safety factor. In addition, combinations of both approaches may be particularly effective (Fawcett et al., 1995).

An important distinction between different forms of myasthenia is the nature of the primary pathogenic process. In many forms, this is an impairment of transmitter release or action, leading directly to a reduction of safety factor. In others, such as SCS and AChE deficiency, the primary effect is an enhancement of transmission that leads ultimately to structural damage and with it, loss of function. The appropriate treatment is likely to be very different in the two situations. Indeed, it may involve reducing the safety factor in cases where transmitter action is abnormally prolonged.

7.3.1. Conditions with a primary reduction of safety factor

In these conditions, any enhancement of transmitter release or action may increase the safety factor. The most successful way of increasing release has proven to be by blocking VGKCs in the nerve terminal, thus prolonging the depolarising phase of the nerve impulse and extending the period of transmitter release. The most satisfactory drug for this has proven to be 3,4-diaminopyridine (Lundh et al., 1990).

The most common approach to increasing transmitter action has been to prevent ACh breakdown by blocking AChE activity. This has the effect of allowing the ACh molecules in a single quantum to diffuse to AChR molecules that are further from the site of release than those normally activated. This at least partially compensates for the reduced local density of AChRs.

7.3.2. Conditions with a primary increase of safety factor

Therapeutic approaches that are effective in increasing the safety factor are likely to be ineffective or even deleterious in conditions where the underlying effect is an increase in safety factor. In the various forms of SCS, enhancing quantal release or blocking AChE activity would therefore be unsuitable. As already mentioned, quinidine reduces the mean open time of the abnormal AChR channels (Fukudome et al., 1998), making a potential candidate for treatment of these conditions.

There is at present no suitable treatment for AChE deficiencies. Clearly, anticholinesterases are ineffective; indeed the lack of effect of these drugs is one of the diagnostic criteria for the conditions. While marginally beneficial effects of a few other drugs have been noted (Engel, 1994a) there is at present no rational approach to the treatment of these conditions.

8. Conclusions

In this review of the safety factor at the neuromuscular junction, we have intentionally concentrated on observations made at vertebrate, mostly mammalian, NMJs. Not only is there more detailed information about the normal structure and function of these NMJs than in most other species but there is also a growing body of information about how mammalian NMJs develop and how they may be affected by pathological conditions. Consideration of this information suggests a number of conclusions concerning the normal interactions that may give rise to NMJs with an adequate safety factor.

(1) There is a substantial safety factor, of the order of 5 during low frequency (0.1–1 Hz) activity, at many vertebrate NMJs. At high frequencies, such as are encountered during normal use, the safety factor is reduced but is still normally adequate to ensure faithful neuromuscular transmission.

(2) The way in which NMJs in different species achieve such a safety factor varies, with some relying on high quantal content and others on extensive postsynaptic amplification provided by the folds and the VGSCs within them.

(3) The developmental mechanisms by which an adequate safety factor is established and subsequently maintained are not well understood. However, it is clear that a variety of interactions between the motor nerve terminal, the muscle fibre and the Schwann cell all play a part in ensuring an adequate safety factor.

(4) A reduction of the safety factor is associated with a number of pathological conditions. This may result from reduced transmitter release or impaired transmitter action. In many pathological circumstances, an ap-

parently adaptive response of the main cells of the NMJ leads to a minimisation of the functional deficit in neuromuscular transmission. This implies that, as in development, a rich variety of nerve-muscle interactions persist in the adult and that these help to maintain the high safety factor of neuromuscular transmission that is so essential for normal life.

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