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STATISTICS OF NEUROMUSCULAR TRANSMITTER RELEASE IN YOUNG AND OLD MOUSE MUSCLE

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SUMMARY

1. It was reported previously that in limb muscles of old (27–30 months) CBF-1 mice, quantal content (m) of evoked transmitter release was increased compared to that in young (9–12 months) mice. In diaphragm muscles there was no change with age. The object of the present study was to determine whether the age-related increase in transmitter release was due to increase in the binomial parameter n or the parameter p. The analysis also involved consideration of goodness-of-fit between observed and expected binomial distribution of the data.

2. Spontaneous miniature end-plate potentials (m.e.p.p.s) and evoked end-plate potentials (e.p.p.s) were recorded with intracellular techniques from soleus and diaphragm muscles bathed in low-Ca high-Mg medium. The goodness-of-fit between the observed e.p.p amplitude distribution and that expected from a binomial distribution was evaluated by χ^2 test.

3. In different muscles and at different ages, the percentage of fibres with binomial e.p.p. distributions varied from 17 to 44 %, even though in all fibres there was a similar proportionality between direct quantal content and the reciprocal of the square of the coefficient of variation of e.p.p. amplitudes. In addition, apparent graphical agreement between observed and theoretical binomial e.p.p. distributions was often not substantiated by the χ^2 criterion.

4. In soleus muscles from young mice, lowering the stimulus frequency from 10 to 0·5 Hz and shortening the train length from 250 to 100 pulses increased the prevalence of binomial e.p.p. distributions, but the same result was not obtained in diaphragm or soleus muscles from old mice. If the mean amplitude of groups of 10 e.p.p.s in any train showed any drift (> 10 %) then that train was excluded from the results. Thus, in order to make valid age comparisons, only fibres with binomial e.p.p. distributions were analysed further.

5. There was no change with age in m, n or p in diaphragm muscles, but in soleus muscles from old animals a nearly 2-fold increase in n entirely accounted for the increase in m.

6. If, as proposed by others, n represents the number of release sites, then the ageing soleus neuromuscular junction may have increased numbers or length of active zones or associated membrane components.

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INTRODUCTION

Neuromuscular transmitter release is greater in limb muscles of old than young CBF-1 mice (Banker, Kelly & Robbins, 1983), and similar changes with age have been reported in rat diaphragm (Smith, 1984). By recording from mouse extensor digitorum longus or soleus muscles in low-Ca high-Mg saline, it was found that the value of the quantum content (m), calculated directly, was two or three times greater in old than in young animals (Kelly & Robbins, 1983). In mouse soleus muscle the age-related increase in transmitter release is not accompanied by any increase in nerve terminal length or area, whereas in extensor digitorum longus the increase in terminal size is small compared to the relative change in release (Robbins & Fahim, 1985). Thus, some mechanism other than simple growth of the synapse must account for the increased transmitter release in aged mouse limb muscle.

The primary purpose of this study was to elucidate the mechanism by which the quantum content is increased in old mice. For this purpose, a statistical model describing quantal release of transmitter from presynaptic nerve terminals (del Castillo & Katz, 1954) was applied to data obtained in our experiments. This model assumes a number of units (n), each of which has an independent probability (p) of being activated when the nerve terminal is stimulated. Thus, in this binomial model the mean number of quanta (m) released by a series of nerve stimuli will be the product of n and p. The exact anatomical correlates for the parameters n and p are not yet certain, although it is likely that n represents the number of activated or activatable release sites rather than the number of vesicles in the nerve terminal. By determining how these binomial parameters change with age and comparing them with changes in other physiological or anatomical parameters it may be possible to obtain a better idea of what p and n really represent in the nerve terminal.

Statistical analysis of evoked release in response to successive stimuli, assuming a binomial distribution, yields the parameters n and p (e.g. Johnson & Wernig, 1971; Miyamoto, 1975). Whereas n and p both vary when external Ca concentration is changed, changes in n with constant p have been found after the initial stages of development and regeneration, as the number of release sites increases in parallel with synaptic growth (reviewed in McLachlan, 1978). In a non-growing synapse a similar result could come about if the density of release sites were to increase. Therefore, it was of interest to determine whether a similar increase in n alone (as in late development) also accounted for the age-related increase in m, or whether a different mechanism involving the parameter p was involved. However, it was not certain *a priori* that evoked release at either young or aged neuromuscular junctions would conform to a binomial distribution in Mg-blocked preparations (e.g. Wernig & Carmody, 1977) and so it was necessary to ensure that at each end-plate release did conform to binomial statistics before values of n and p could be used. For this reason, we first checked the goodness-of-fit between observed and expected distributions and analysed the conditions under which this fit was maximized in young and old mouse muscle.

METHODS

Young adult (12 months) or old (30 months) male CBF-1 mice (Charles River Laboratory) were anaesthetized with methoxyflurane (Pitman-Moore). Soleus and diaphragm muscles were removed and pinned onto a Sylgard (Dow Corning) surface in a modified Krebs saline (pH 7·2) of the following

composition (mM): NaCl, 135; KCl, 5; $NaHCO_3$, 15; Ca gluconate, 2·5; $MgSo_4$, 1; Na_2HPO_4, 1; (+)-glucose, 11. A gas mixture of 95 % O_2 and 5 % CO_2 was bubbled through the saline which was maintained at 30·0 ± 0·5 °C for all experiments. Conventional glass capillary micro-electrode techniques were used to make intracellular recordings of spontaneous miniature end-plate potentials (m.e.p.p.s) and evoked end-plate potentials (e.p.p.s), and only focal m.e.p.p.s and e.p.p.s with rise times of less than 1 ms were accepted. In order to record evoked e.p.p.s, a low-Ca (0·4 or 0·6 mM)–high-Mg (2·75 mM) Krebs saline was used and the nerve was stimulated supramaximally at 20, 10, 1, or 0·5 Hz. At least 1 min was allowed to elapse between successive trains. The higher (0·6 mM) Ca concentration was used only for the 0·5 Hz stimulation frequency because at this frequency (at which facilitation is negligible compared to that at 20 Hz), the number of failures at the lower Ca concentration was very large. 50–100 m.e.p.p.s and 100–950 e.p.p.s, depending on the particular experiment (see Results) were recorded at the same end-plates. In some experiments carried out to test the applicability of the computer calculations, 'cut-fibre' preparations were used in Krebs solution with 2·5 mM-Ca and 1·0 mM-Mg (cf. Banker *et al.* 1983).

M.e.p.p.s and e.p.p.s were digitized on-line via an analog-to-digital conversion board (10 kHz sample rate) in a Northstar Horizon microcomputer, which provided values for amplitude, frequency, rise time and half-decay time. Peak amplitudes were corrected to a standard resting membrane potential (r.m.p.) of −80 mV (Kelly, 1978). Individual m.e.p.p.s with amplitudes greater than twice the mean value in each muscle fibre were classed as 'giants' and not included in further calculations. Correction for non-linear summation was applied to all potentials (Martin, 1955), although for the m.e.p.p.s and Mg-blocked e.p.p.s the correction was very small. In the case of cut-fibre preparations, the correction factor was multiplied by 0·7 as the standard correction is believed to over-correct large e.p.p. amplitudes (McLachlan & Martin, 1981). Data were used only from end-plates at which the distribution of m.e.p.p. amplitudes was unimodal (Kelly & Robbins, 1984). The mean quantum content (m) of groups of plateau e.p.p.s (i.e. all e.p.p.s of a train after the first 10) was calculated directly from the ratio of the mean e.p.p. amplitude to the mean m.e.p.p. amplitude.

Data obtained from the Horizon microcomputer were later transferred to a PDP 11/03 minicomputer for calculation of the binomial parameters n and p and for comparison of expected and observed e.p.p. amplitude distributions using the χ^2 test. The equations used to generate the expected e.p.p. distributions (Miyamoto, 1975) involved the generation and addition of distributions of amplitudes around the number of quantal units using the measured m.e.p.p. amplitude and its variance. In order to validate the computer program, calculations were performed on data derived from previously published work by Miyamoto (1975; his Fig. 4 *A*, *B* and *C*), and Glavinovic (1979; his Fig. 1 *A* and *B*). Both sets of data yielded a binomial distribution, with values of n and p in good agreement with those published. We also used the program to analyse our own data obtained from cut-fibre mouse diaphragm preparations and found that eleven of fifteen (76 %) fibres examined fitted binomial distributions. Values obtained for m, n and p were 32·0 ± 5·3, 47·6 ± 8·5, and 0·734 ± 0·064 (mean ± 1 S.E., $N = 11$) respectively, and these values are comparable to those obtained by Glavinovic (1979) and Wernig (1975). Of the four which did not fit, two showed double peaks in the e.p.p. amplitude histogram, indicating possible nerve branch block. In order to validate the program with data in Mg-blocked preparations (i.e. low n and low p), data obtained from frog sartorius preparations of del Castillo & Katz (1954; their Figs. 7 and 9) were analysed. The program showed that e.p.p.s in their Fig. 7 ($m = 0·58$, $n = 15$, $p = 0·039$) fitted both binomial and Poisson distributions, whereas e.p.p.s from their Fig. 9 ($m = 37·2$, $n = 71$, $p = 0·524$) fitted binomial but not Poisson distributions.

Unless otherwise stated, the Mann–Whitney non-parametric test was used to determine the significance of differences between groups of data, with a significance level of 5 % or less. All values are expressed as mean ± 1 S.E. of mean. The χ^2 test was used to ascertain the goodness-of-fit between observed and binomial or Poisson distributions.

RESULTS

Criteria and conditions for binomial distribution of e.p.p.s

A stimulus frequency of 20 Hz was used in the initial experiments on Mg-blocked preparations because previous work had shown an increased quantum content (m) at this frequency in old compared to young soleus muscles (Kelly & Robbins, 1983).

However, it was found that under these conditions a large proportion of e.p.p. amplitude distributions (trains of 240 e.p.p.s) did not fit either binomial or Poisson distributions, although the e.p.p. amplitudes had reached a steady plateau value after the first 10 stimuli and there was no amplitude drift. This lack of fit would not have been obvious from a conventional plot of m obtained by the direct method *vs.* m calculated by the method of failures or from 1/(coefficient of variation)^2, both of

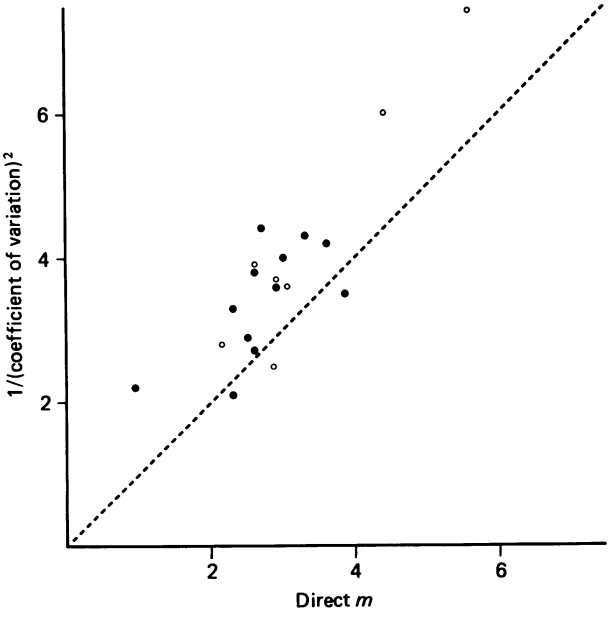

Fig. 1. Graph of 1/(coefficient of variation)^2 against directly calculated m in soleus muscles from old mice. The dashed line indicates the equality which would be expected if the release process followed Poisson statistics. Most points lie above the line, indicating that p is not negligibly small. Circles represent data from fibres in which the distribution of e.p.p. amplitudes did (○) or did not (●) conform to binomial statistics.

which rely on the assumption of a Poisson distribution. On closer examination, however, variance-derived values of m were consistently larger than directly calculated values of m (Fig. 1), as expected if p was not negligibly small. Furthermore, the correlation was the same for all data, regardless of the degree of fit with a binomial distribution. Therefore, the correspondence of direct and Poisson m could not be used as a criterion for acceptance of data for calculation of p and n.

The goodness-of-fit between observed e.p.p. amplitude distributions and those expected in a binomial distribution was often not reliably ascertainable by visual inspection (compare Fig. 2*A* and *B* with 2*C*). In addition, the significance level of the χ^2 value was not affected by changes in bin width over a reasonable range. Finally, in some cases the calculated value of p was negative, so no theoretical binomial distribution could be computed, and in these cases a theoretical Poisson distribution also did not fit. Fitting data to more complex binomial models, such as those which assume different values for p within one junction (Jack, Redman & Kwong, 1981)

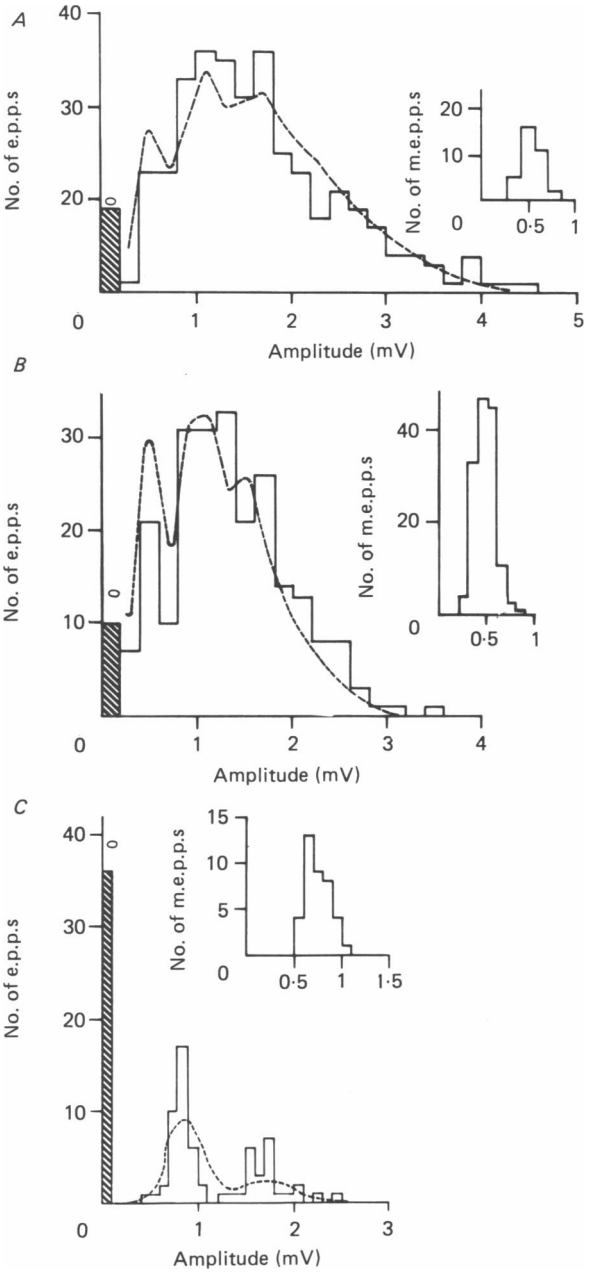

Fig. 2. Histograms of e.p.p. amplitudes in soleus muscles. E.p.p.s were elicited at 20 Hz (*A* and *B*; 239 e.p.p.s) or 0·5 Hz (*C*; 100 e.p.p.s) and all experiments were in 0·4 mM-Ca and 2·75 mM-Mg Krebs solution. The hatched block and the open circles represent observed and predicted failures, respectively, and the dashed line represents the binomial prediction. Inset is the corresponding m.e.p.p. amplitude histogram. *A*, observed and predicted values agree well (χ^2 values are 15·9 and 14·1 for Poisson and binomial, respectively, with d.f. = 14); $m = 3{\cdot}03$, $n = 20$, $p = 0{\cdot}15$. *B*, observed and predicted values do not agree (χ^2 values are 24·0 and 27·0 for Poisson and binomial, respectively, with d.f. = 11); $m = 2{\cdot}63$. *C*, observed and predicted values are more obviously different than in *A* or *B* (χ^2 values are 23 and 18 for Poisson and binomial, respectively, with d.f. = 5); $m = 0{\cdot}87$.

was not attempted because such calculation would be based upon so many assumptions that young and old comparison of any binomial parameters thus obtained would not be meaningful.

The proportion of e.p.p. amplitude distributions which fitted binomial or Poisson statistics or both varied between muscles (Table 1). In soleus there was no clear-cut age dependence of this proportion, whereas in diaphragm fewer data from old animals fitted either theoretical distribution. This observation was not merely a peculiarity of mouse muscle: a similar result was found in rat diaphragm, in which 43% of fourteen end-plates studied conformed to binomial statistics (see Wernig & Carmody, 1977, for similar findings in the frog).

TABLE 1. Percentage of muscle fibres in which the distribution of amplitudes of e.p.p.s evoked at 20 Hz in 0·4 mM-Ca and 2·75 mM-Mg conformed to binomial or Poisson statistics or both

	Age group (%)	
Muscle	Young	Old
Soleus	44	36
Diaphragm	62	17

The reasons for the lack of conformity to a binomial distribution varied from one end-plate to another. For example, in some fibres a combination of more failures and more e.p.p.s of higher quantum contents than predicted from binomial statistics might indicate nerve terminal block. In other cases, there was a population of e.p.p. amplitudes smaller than might be expected from the m.e.p.p. amplitude distribution, possibly because of the emergence of 'small mode' e.p.p.s during stimulation (Kelly & Robbins, 1984). Occasionally the observed amplitude distribution was more tightly grouped around the peaks than predicted by a binomial distribution (e.g. Fig. 2*C*). In such cases, although care was taken to exclude 'giant' m.e.p.p.s from calculations (see Methods), the variance of evoked quanta was less than the measured variance of m.e.p.p.s. Even if the release process followed binomial statistics (cf. Wernig, 1975), it was not felt justified to compare young and old binomial parameters from muscle fibres in which spontaneously released and evoked quanta were apparently from different populations. A frequent observation in non-fitting distributions was a paucity of values below and an excess of values above the major mode. Indeed, to a lesser extent this observation was also true even of distributions (e.g. Fig. 2*A*) which did conform to the binomial (according to the χ^2 test).

The purpose of this study was to compare values of n and p from young and old mice, and therefore we sought those conditions under which a greater proportion of e.p.p. amplitude distributions would conform to a binomial distribution. One possibility was that 240 e.p.p.s may not have been a sufficiently large sample, and so trains of 950 e.p.p.s were elicited from end-plates in soleus muscles (from young mice). Of five end-plates examined, none showed a fit to binomial statistics when all 950 e.p.p.s were used for the calculations. However, when successive groups of 200 e.p.p.s out of the total 950 e.p.p.s were examined, three out of five fibres had at least one subgroup which corresponded well to a binomial distribution. If only the first 100 e.p.p.s from a train of 950 (omitting the first 10) were used, then four of the five

TABLE 2. Percentage of muscle fibres in which the distribution of amplitudes of e.p.p.s evoked at 0·5 Hz in 0·6 mM-Ca and 2·75 mM-Mg conformed to binomial or Poisson statistics or both

	Age group (%)	
Muscle	Young	Old
Soleus	73	28
Diaphragm	29	42

TABLE 3. Binomial statistical parameters calculated from e.p.p.s elicited at a frequency of 0·5 Hz in 0·6 mM-Ca and 2·75 mM-Mg. Values are given as mean ± 1 S.E. of mean with the number of observations in parentheses. The asterisk indicates a significant difference between values from young and old animals. m_T is the mean quantum content of all end-plates in the sample, whether or not the e.p.p. distribution fitted a binomial distribution, whereas m, n and p were calculated only after the χ^2 criterion of goodness-of-fit was satisfied

	Soleus		Diaphragm	
Parameter	Young	Old	Young	Old
M.e.p.p. amplitude	0·53 ± 0·03 (15)	0·58 ± 0·03 (18)	0·94 ± 0·06 (17)	1·01 ± 0·1 (19)
m_T	0·70 ± 0·07 (15) *	1·64 ± 0·22 (18)	0·77 ± 0·11 (17)	0·82 ± 0·14 (19)
m	0·65 ± 0·08 (11) *	2·13 ± 0·52 (5)	0·57 ± 0·17 (5)	0·99 ± 0·32 (8)
n	7·82 ± 2·22 (11) *	14·59 ± 2·16 (5)	4·52 ± 1·51 (5)	7·44 ± 1·64 (8)
p	0·14 ± 0·03 (11)	0·15 ± 0·04 (5)	0·14 ± 0·03 (5)	0·14 ± 0·03 (8)

fibres conformed to binomial statistics. Thus, during long trains, there may be non-stationarity of n or p or both, due perhaps to variable facilitation or some other interaction between successive releases at 20 Hz, even in the absence of over-all drift. To investigate further the effect of frequency, 110 e.p.p.s were elicited at 1 and 10 Hz in the same fibres of soleus muscles from young mice, and the last 100 of these were used for calculations and testing against theoretical binomial distributions. Eight fibres, in which the value of m was 0·54–3·84 (1 Hz) and 0·62–4·11 (10 Hz), were examined in this way and no significant difference was found in the values of m at the two frequencies (Wilcoxon paired test). Of these eight fibres, five (63 %) conformed to the binomial distribution at 1 Hz, but only one (13 %) at 10 Hz, possibly due to interaction between successive releases at the higher frequency.

Statistics of transmitter release from muscles in young and old mice

From the above observations, it would seem that the optimum fit of observed e.p.p. distributions to binomial statistics would be attained with 110 e.p.p.s at 0·5 Hz. Even these conditions increased the proportion of fits to the binomial distribution only in the young soleus (Table 2), on which the optimizing procedures were developed. However, from the fibres in which calculation of n and p was valid, it could be seen that the increase in m with age in soleus muscles was due entirely to an increase in n rather than to a change in p, which remained constant with age (Table 3). As expected from previous work (Kelly & Robbins, 1984), in the diaphragm there was no significant difference (at the 5 % level) between old and young mice in the values of m, n or p.

DISCUSSION

Goodness-of-fit of observed and theoretical distributions

Although there was a relationship between 1/(coefficient of variation)^2 and directly calculated m, as predicted by both binomial and Poisson statistics, a proportion of e.p.p. amplitude distributions did not conform to binomial predictions when the χ^2 test was applied. Many previous studies in which there was good agreement between observed e.p.p. amplitude distributions and binomial predictions were in preparations with high values of m, p and n, e.g. cut-fibre preparations (Glavinovic, 1979) or glycerol-treated muscles (Miyamoto, 1975). In the present experiments using low-Ca preparations, m, n and p were low. Thus, it is possible that as p and n increase then the observed distribution becomes binomial, or alternatively that the χ^2 test becomes less sensitive in determining deviations from binomial statistics. Computer modelling (in a case with $n = 50$ and $m = 10$) suggested the latter possibility, because considerable deviation from binomial statistics (e.g. non-stationarity of n or p) could occur and yet not be detected by the χ^2 test (Brown, Perkel & Feldman, 1976). It is not possible to determine from the study of Brown *et al.* (1976) whether a decrease in n would increase the discrepancy between observed and theoretical distributions.

Wernig & Carmody (1977) blocked neuromuscular transmission in frog muscle with high-Mg solutions and found that over half of the cells tested had e.p.p. amplitude distributions which deviated significantly from binomial predictions, even though the value of m was about 15 in those experiments. Although their results were attributed to occasional failure of one or more nerve terminal branches, a similar explanation does not seem applicable to most of our non-binomial data. Rather, the negative and very low values of p frequently calculated from our results indicates temporal non-stationarity of n or p or both (Brown *et al.* 1976; McLachlan, 1978). Consistent with this interpretation was the observation that in long trains of 950 e.p.p.s small groups of 100–200 e.p.p.s did conform to binomial statistics, whereas the group as a whole did not. Facilitation could be another mechanism producing temporal non-stationarity, accounting for the observation (in young soleus muscles) that a greater proportion of observed distributions conformed to binomial predictions at 0·1 Hz than at 10 Hz. Indeed, any interaction between successive e.p.p.s would be incompatible with a basic assumption of the binomial distribution. Thus, it seems that only those end-plates at which n and p remained relatively unchanged throughout the recording period produced e.p.p. amplitude distributions which conformed to binomial statistics, and these were the ones used to compare young and old muscles.

We attempted to maximize the percentage of cells which would give usable data, but conditions which succeeded for young soleus muscle were not better for the other muscles. Therefore, it seems that only rigorous application of the χ^2 test allows one to select cells that conform to binomial statistics. Further, it may be that as n and p increase, even the χ^2 test may become relatively insensitive to deviations from the expected binomial distribution (Brown *et al.* 1976).

Comparison of young and old animals

The basic finding reported here is that n, but not p, is increased at the neuromuscular junction of old soleus muscles. Therefore, it is useful to consider what

biochemical or structural entities determine the magnitude of n. It has been suggested that n reflects cytoplasmic Ca concentration (McLachlan, 1978). However, certain indicators of increased cytoplasmic Ca, such as increases in m.e.p.p. frequency or facilitation, were not found at the ageing soleus neuromuscular junction (Banker *et al.* 1983).

Another suggested correlate of n is the number of transmitter release sites (McLachlan, 1978), and this assertion is based upon at least two observations. First, at both crayfish and frog neuromuscular junctions, n is of a similar magnitude to the number of active zones (Zucker, 1973; Wernig, 1975). Secondly, in development or regeneration of the neuromuscular junction, the main component of increased quantal content after the initial outgrowth stages is the parameter, n, since p rapidly approaches a value of 1 in these circumstances (Bennett & Florin, 1974; Bennett & Raftos, 1977). In the course of growth of terminals there is addition of new active zones as the terminal elongates, and in regeneration a lengthening of maturing active zones (Ko, 1984) is correlated with an increase in transmitter release.

At the ageing neuromuscular junction, release sites could increase in at least two ways: by an increase in the total length or area of the motor nerve terminals, or by an increased density or length of active zones with no change in terminal area. However, in the CBF-1 mouse soleus neither nerve terminal length nor area change after age about 12 months (Robbins & Fahim, 1985), and in a different strain of mouse, which does show senility changes after age about 1·5 years, there was no increase in nerve terminal length in diaphragm or gluteus muscle after age about 100 days (Hopkins, Brown & Keynes, 1985). Thus the increase in m (or n) cannot depend on a mechanism involving an increase in nerve terminal size. Therefore, we suggest that at the old soleus neuromuscular junction a greater density or length of active zones may account for the increase in n and m. This is a testable hypothesis, as morphometry of active zones is feasible (e.g. Fukunaga, Engel, Osame & Lambert, 1982).

Although the quantum content in the old soleus was more than double that in the young, there was no significant difference between young and old diaphragm. An increase in m may not occur at all or may only be delayed in the diaphragm, but in either case the continual respiratory activity or the particular pattern of activation of the diaphragm may protect it from changes taking place in other muscles. The diaphragm may also be different from limb muscles because of its relatively protected internal location and so both blood supply and temperature could be maintained at a higher level.

In conclusion, the results of these experiments show that before using parameters derived from the assumption of binomial statistics it is essential to ensure that the observed distributions do indeed conform to binomial distributions. This is because the assumptions involved in the calculations (e.g. stationarity of n and p or non-interaction between successive stimuli) may not be true at many neuromuscular junctions, especially in Mg-blocked preparations. By considering only those results which conformed to binomial statistics it was found that the increase in quantum content with age in soleus muscle was due entirely to an increase in the binomial parameter, n. This parameter is unlikely to represent vesicles in the nerve terminal, as the number of vesicles decreases with age (Banker *et al.* 1983), but probably represents activated or activatable presynaptic release sites.

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