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Extracellular chloride replacement by isethionate induces abnormal spontaneous release of transmitter at the frog neuromuscular junction

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1 Replacement of chloride by isethionate in Ringer solution bathing frog skeletal muscle fibres induces, after a delay of about 30 min, marked mechanical activity which was blocked by tubocurarine. This effect is reversed by washing out the isethionate.
2 Miniature end plate potentials (m.e.p.ps) and giant potentials (potentials $> 2 \times$ modal value) were recorded intracellularly in normal Ringer and isethionate Ringer solution.
3 The frequency of m.e.p.ps was unaltered by isethionate. The proportion of giant potentials increased from 3% in normal Ringer to 24.5% in isethionate Ringer after 90 min. This effect is usually reversible if the exposure to isethionate does not exceed 2 h.
4 The giant potentials were large enough to initiate trains of action potentials and still occurred in the presence of tetrodotoxin or Ca^{2+}-free Ringer. Isethionate produced no change in the τ_D of miniature endplate currents.
5 Chloride replacement by propionate produced no change in the proportion of giant potentials.
6 It is suggested that the isethionate anion can induce giant potentials and the possible mechanism of action is discussed.

Introduction

A number of monovalent organic anions including propionate, butyrate, gluconate and isethionate are used commonly as chloride ion substitutes. In a number of electrophysiological studies on frog skeletal muscle we have used the isethionate anion as a substitute for chloride for two reasons. Firstly it is likely that excitable membranes are generally more impermeant to isethionate than some other anions (see for example Woodbury & Miles, 1973). The anions of some weak acids can in fact cross membranes and even change the intracellular pH within several minutes. Isethionic acid is a strong acid and does not produce such effects (Sharp & Thomas, 1981). Secondly some organic anions, notably citrate, maleate and gluconate bind Ca^{2+} ions (see Christofferson & Skibsted, 1975). We have previously shown that isethionate has little effect on the contraction of the frog heart and conclude that the binding of Ca^{2+} by this anion is relatively unimportant (Wann, 1975).

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It is well known that the reduction of the extracellular chloride concentration produces a transient depolarization and thus contraction of frog skeletal muscle fibres (Hodgkin & Horowicz, 1959). We show in this study that replacement of extracellular chloride with isethionate in addition induces, after a delay, spontaneous mechanical activity of frog skeletal muscle fibres. This activity is generated by the abnormal discharge of transmitter from the presynaptic terminals. Some of these results have been communicated to the Physiological Society (Ashford & Wann, 1978).

Methods

The experiments were performed on sartorius muscle preparations from *Rana pipiens* or *Rana temporaria*. The muscle was carefully wrapped round a Perspex rod, stretched to not more than 1.5 times the *in situ* length, and transferred to a Perspex tissue bath (2.5 ml volume). This is a convenient method for repeated intracellular recording of action potentials (Stefani & Schmidt, 1972). The composition of the normal Ringer solution was (mM): NaCl 115, KCl

2.5, $CaCl_2$ 1.0 and Tris hydrochloride (tris (hydroxymethyl) aminomethane hydrochloride) 2.0; the pH was adjusted to 7.4 with HCl. In the chloride substitution experiments equivalent amounts of sodium isethionate or sodium propionate replaced the NaCl (thus 'chloride-free' solutions actually contained 6.5 mEq of chloride ions). In experiments performed in the absence of extracellular calcium, the $CaCl_2$ of the Ringer was replaced by 6 mM $MgCl_2$ and/or 1 mM EGTA (ethyleneglycoltetra-acetic acid).

Membrane potentials, miniature endplate potentials (m.e.p.ps), action potentials and membrane input resistance were recorded intracellularly with glass microelectrodes filled with 3M KCl. Electrode resistances were between 3 and 10 MΩ. Miniature endplate currents (m.e.p.cs) were recorded extracellularly using a glass electrode (filled with 1 M NaCl in 2.5% agar) with a tip diameter of 10–30 μm. Permanent records were obtained by recording data on a four channel FM tape recorder (Thermionic T3000). Membrane input resistance was measured by the two microelectrode technique. A known current was injected through a microelectrode (filled with 3 M KCl) in a muscle fibre and the resultant electrotonic potential was recorded with a second voltage sensing electrode. To ensure that solutions were exchanged,completely chloride-free and ouabain containing solutions were perfused through the tissue bath at a rate not less than 2 ml min^{-1} for 5 min. In the majority of experiments where m.e.p.ps were recorded, neostigmine methyl sulphate was added at a concentration of 3×10^{-6} M. All experiments were performed at room temperature (20–24°C). The compounds used were ouabain, neostigmine methyl sulphate, tetrodotoxin (TTX), sodium propionate (all Sigma) and sodium isethionate (Eastman Chemicals and Sigma). All values quoted in the text and tables represent means ± s.e.mean. The differences between means were analysed by an unpaired Students' *t* test ($P < 0.05$ taken as significant).

Results

Comparison between the isethionate and ouabain-induced contractures

On substitution of the $[Cl]_o$ (at a constant $[K]_o$) by isethionate, a transient withdrawal contracture of the muscle is produced (Hodgkin & Horowicz, 1959). There was then usually no mechanical activity for 20–30 min. Thereafter in our experiments spontaneous mechanical activity was observed and this continued for many hours. Direct observation of the muscles revealed that individual fibres contracted asynchronously. Isometric recordings of the mechanical activity showed that it increased progressively in intensity and frequency during the first 30 min to 1 h, and the contractions were usually longer than the individual twitch response to nerve stimulation. We thus term the mechanical events contractures. The contractures were blocked by tubocurarine (10^{-5} M) and augmented by neostigmine (3×10^{-6} M) indicating a synaptic mechanism. They could also be abolished by replacing the isethionate Ringer with normal or propionate Ringer.

The delayed onset and the sensitivity of these contractures to tubocurarine makes them similar to the spontaneous activity observed when frog skeletal muscle is exposed to the cardiac glycoside, ouabain (Birks & Cohen, 1968a,b). However, unlike the ouabain-induced activity (Birks & Cohen, 1968b) the duration of the delay of onset of isethionate-induced activity was not dependent upon the extracellular divalent cation concentration.

Isethionate Ringer solution does not alter m.e.p.p. frequency

It has previously been demonstrated that ouabain markedly increases the m.e.p.p. frequency and induces spontaneous endplate potentials during the period of muscle contraction (Birks & Cohen, 1968a). Therefore the actions of isethionate Ringer and ouabain-containing Ringer were compared on the spontaneous quantal release of transmitter. Ouabain (10^{-4} M) induced a large increase in the frequency of m.e.p.ps following a short delay, agreeing with the results of previous investigators (e.g. Birks & Cohen, 1968a, b; Baker & Crawford, 1975). However, the m.e.p.p. frequency remained unchanged in

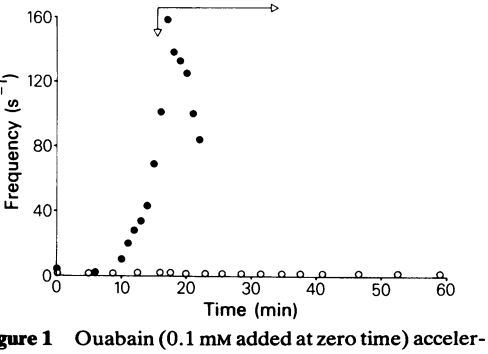

Figure 1 Ouabain (0.1 mM added at zero time) accelerates the spontaneous release of transmitter after an initial delay (●). The arrow indicates the time at which spontaneous contractures began during ouabain exposure. In comparison, isethionate produces no alteration in the frequency of m.e.p.ps (○) (although spontaneous contractions are observed after ~30 min exposure). The ouabain data are from a single experiment. The (○) are the means of eight experiments (s.d. bars are smaller than the symbols). Ordinate scale: m.e.p.p. frequency (s^{-1}), Abscissa scale: time (min).

Table 1 The average m.e.p.p. frequency in normal Ringer and isethionate Ringer solution

Time interval (min)	*m.e.p.p. frequency* (s^{-1}) *Normal Ringer*	*Isethionate Ringer*
5	1.48±0.19	1.56±0.27
15	1.71±0.19	1.69±0.17
30	1.62±0.24	1.37±0.06
60	—	1.20±0.09

The frequencies were measured over 30 s intervals. The values represent the means±s.e. from 8 separate experiments.

the presence of isethionate Ringer (Figure 1) even during the period of spontaneous contractures. In eight experiments there was no significant difference between the m.e.p.p. frequency in normal and isethionate Ringer (see Table 1).

Isethionate Ringer solution causes giant potentials

Isethionate Ringer produced a marked increase in the occurrence of giant potentials (i.e. potentials larger than twice the modal amplitude, Figure 2).

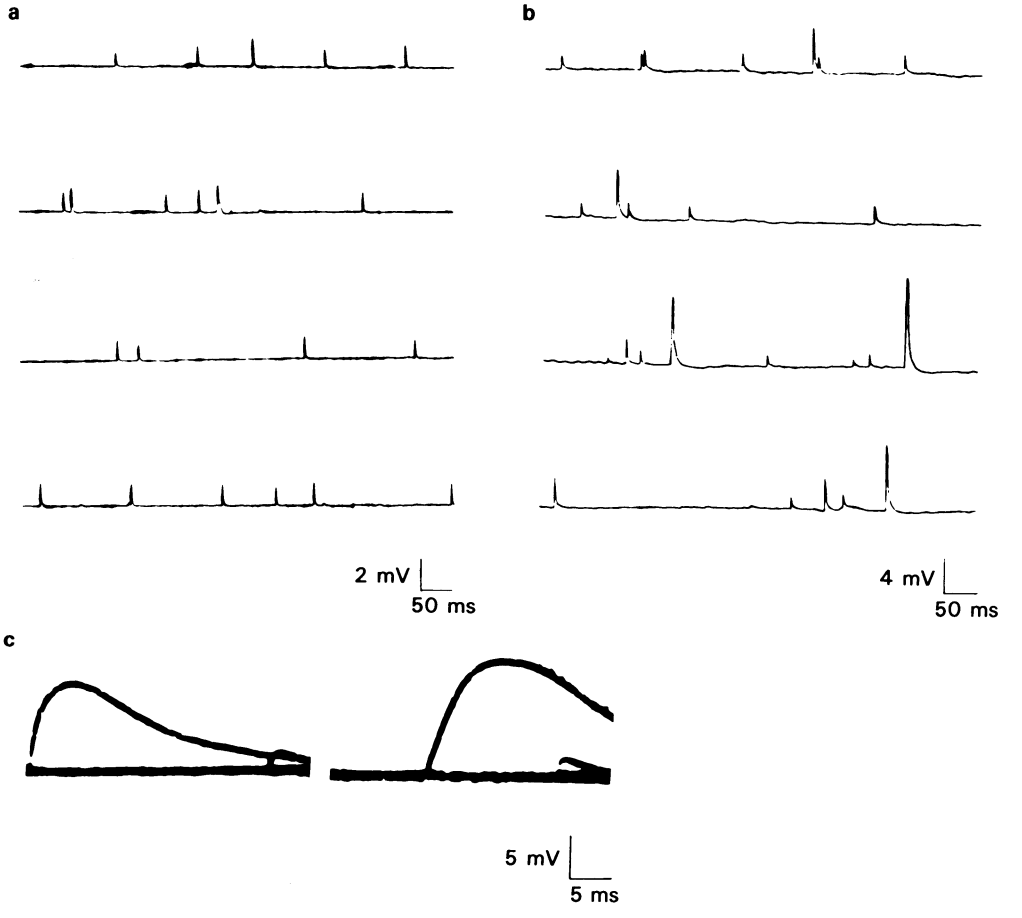

Figure 2 Examples of m.e.p.ps recorded in normal Ringer solution (a) and after one hour in isethionate Ringer (b). Note the occurrence of the large amplitude spontaneous potentials, and the different voltage calibration. (c) Two giant potentials recorded in isethionate Ringer. Note the slow rising phase in the case of the second potential.

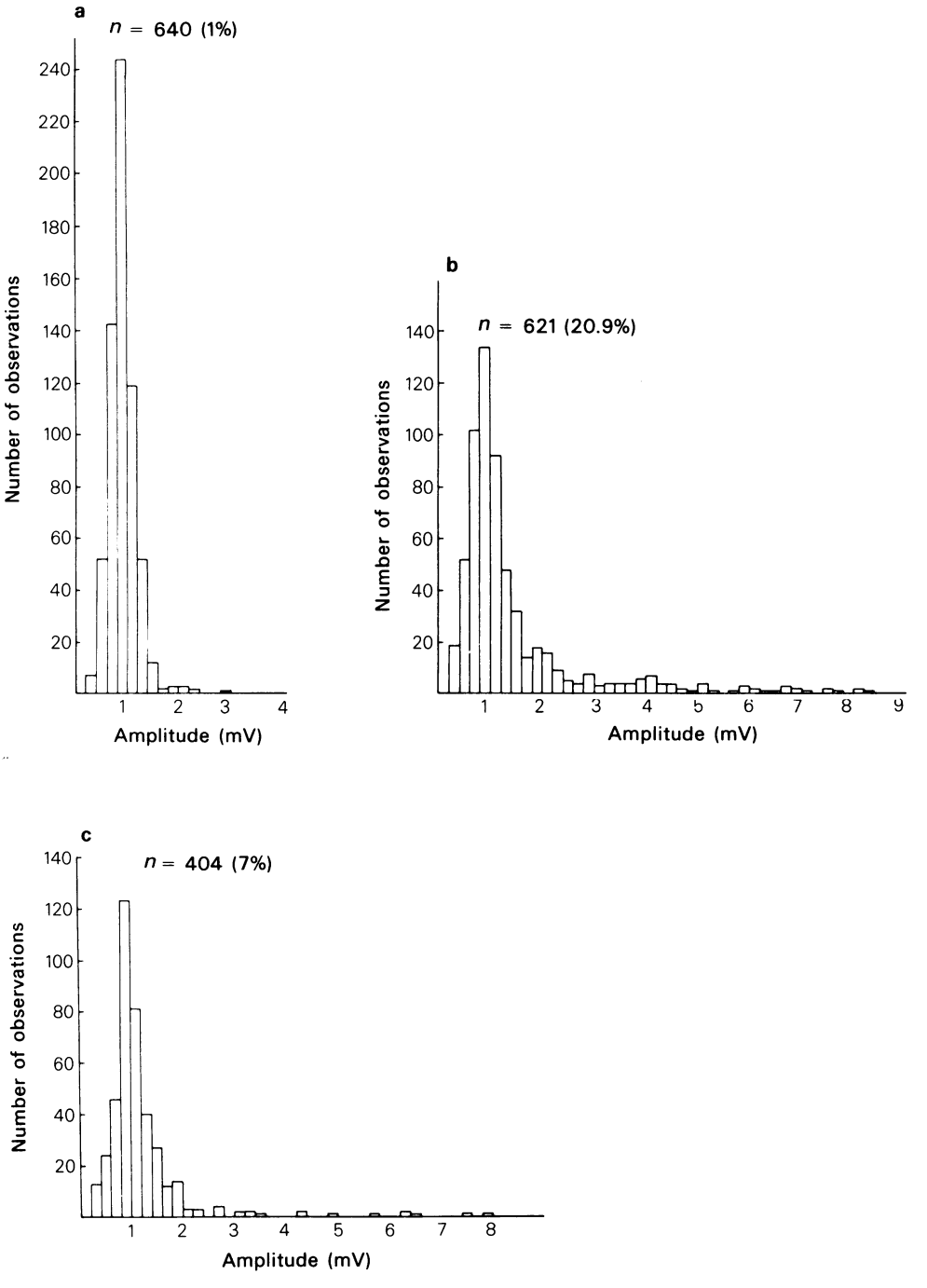

Figure 3 The amplitude distribution of spontaneous potentials recorded before, during and after exposure to isethionate Ringer solution. Ordinate scale: number of observations; abscissa scale: amplitude (mV). (a) Control in normal Ringer solution, (b) after 80 min of exposure to isethionate Ringer; (c) 10 min after returning to normal Ringer. *n* is the total number of observations and the % of giant potentials is given in parentheses.

Substitution of chloride by isethionate increased the input resistance from 0.64 ± 0.11 MΩ ($\bar{x} \pm 1 \times$ s.e.) to 1.35 ± 0.17 MΩ ($\bar{x} \pm$ s.e.). Such an increase would prolong the decay phase of the 'normal' m.e.p.p. with little effect on its amplitude or rise time (Gage, 1976). Indeed the normal m.e.p.p. decay phase was clearly prolonged in the isethionate Ringer compared to the normal Ringer (Figure 2).

The time course of the intracellularly recorded giant potentials usually did not differ significantly from that of the normal m.e.p.ps in the chloride-free Ringer. Thus the half decay time of the normal m.e.p.ps in isethionate Ringer was 9.59 ± 0.24 ms ($\bar{x} \pm$ s.e.; $n = 40$) and the half decay time of the giant potentials was 9.67 ± 0.21 ms ($\bar{x} \pm$ s.e.; $n = 40$). The rise times of both normal and giant m.e.p.ps in isethionate Ringer were usually ~3 ms. Occasionally however the larger giant potentials (> 6 mV) exhibited a slower rising phase (Figure 2c). Similar observations have been reported previously for giant potentials induced by other treatments (e.g. Liley, 1957; Pécot-Dechavassine, 1976).

Amplitude histograms of m.e.p.ps in the presence of isethionate Ringer are characterized by a skewed distribution which results from the presence of potentials many times the modal value of the control (c.f. Figure 3a and b). These giant potentials occur after about 20 min in the isethionate Ringer. The effect is usually reversible, and on replacement of the isethionate Ringer with normal Ringer the frequency of the giant potentials falls steadily (over a period of 30 min) towards control levels (Figure 3c). However, after long exposures to isethionate Ringer (> 2–3 h) the giant potentials sometimes persist for a considerable period of time after washing with normal Ringer (> 1 h).

The proportion of giant potentials (relative to the total number of spontaneous m.e.p.ps) increases with time. A typical experiment is illustrated in Figure 4. The percentage of giant potentials increases from

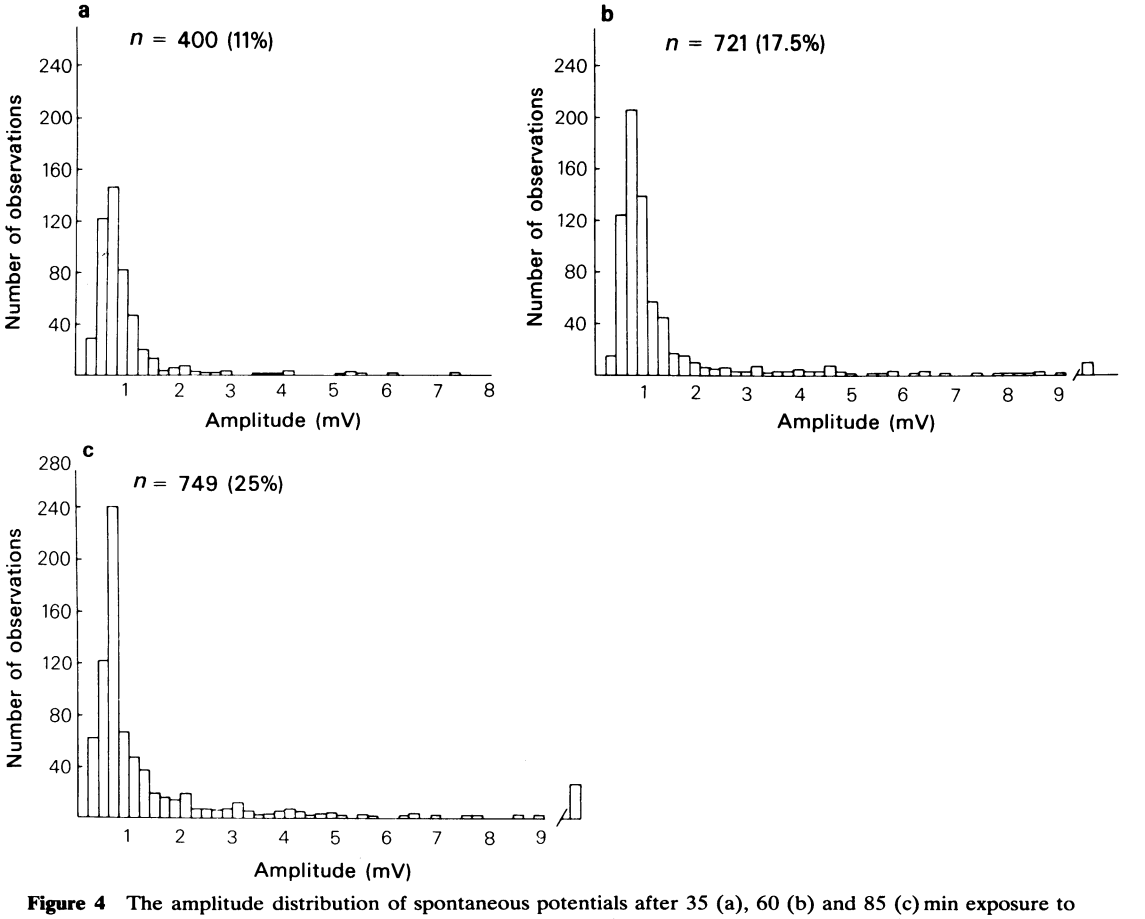

Figure 4 The amplitude distribution of spontaneous potentials after 35 (a), 60 (b) and 85 (c) min exposure to isethionate Ringer solution. Ordinate scale: number of observations; abscissa scale: amplitude (mV). Tetrodotoxin (1 μM) was present throughout. The % of giant potentials is stated on each graph.

11% after 35 min to 25% after 85 min in isethionate Ringer. In eight experiments the proportion of giant potentials increased from an average of 3% in normal Ringer to 24.5% after ~90 min in isethionate Ringer (range was 17.6% to 33.3%), and these levels are maintained during recordings over the next 60 to 90 min.

Several of our experiments were performed in the presence of TTX (see e.g. Figure 4). Giant potentials occurred in the presence of TTX there being no obvious difference in the frequency or time course of appearance. Moreover, as the muscle action potential was blocked, giant potentials exceeding 15 mV were observed. In some experiments the effects of high K^+ (10 mM) or hypertonic Ringer (165 mM Na^+) were tested on the frequency of the giant potentials once induced. The frequency of the giant potentials was unaltered unlike that of the normal m.e.p.p. frequency. Thus in one experiment the m.e.p.p. frequency (measured over 9×30 s bins) after 1.5 h in isethionate Ringer containing TTX was $1.87 \pm 0.10\,s^{-1}$: the frequency of giant potentials was $0.43 \pm 0.03\,s^{-1}$. Perfusion with hypertonic Ringer (165 mM Na^+) increased the m.e.p.p. frequency to $9.94 \pm 0.42\,s^{-1}$ and the frequency of giant potentials remained at $0.40 \pm 0.05\,s^{-1}$. In one other experiment with high K^+ isethionate Ringer (10 mM) the frequency of the giant potentials was again unchanged.

The amplitudes of the giant potentials induced by isethionate Ringer are capable of reaching values more than ten times the mean amplitude of the control m.e.p.ps and giant potentials larger than about 12 mV generated a muscle action potential

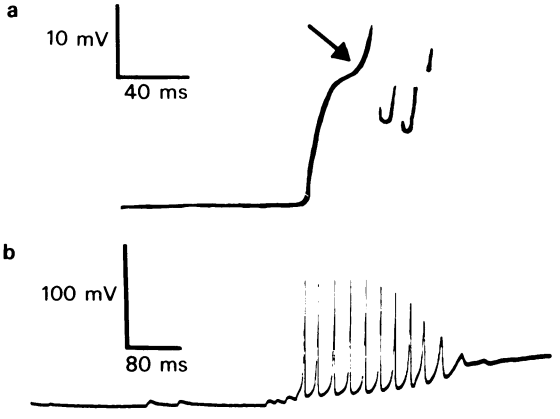

Figure 5 The generation of spontaneous action potentials in isethionate Ringer solution. (a) Example of a single spontaneous potential of sufficient amplitude to initiate a muscle action potential (at the arrow). Only the initial rapid upstroke of the first action potential is shown. (b) Large spontaneous potentials giving rise to a train of muscle action potentials. Eventually the strong contractions dislodged the recording electrode.

(Figure 5a). In most experiments the isethionate Ringer shifted the threshold for generation of an action potential by about 8 mV in an hyperpolarizing direction. In all recordings, a train of spikes, rather than a single muscle action potential was observed (Figure 5b). Such repetitive firing is the cause of the prolonged contraction of individual muscle fibres. Extracellular recording of the m.e.p.ps and giant potentials in isethionate Ringer showed that there was no significant difference in the time constant of decay (τ_D) of the underlying conductance change producing the potentials (Table 2). This indicates that the giant potentials are not due to an increase in the average open-time of endplate channels.

Effect of $[Ca^{2+}]_o$

Exposure to calcium-free isethionate Ringer (containing 1 mM EGTA and no magnesium) produced a smaller effect. The frequency of m.e.p.ps was very low in agreement with previous work (e.g. Miledi & Thies, 1971); giant potentials were observed in some but not all experiments and were much smaller and infrequent in comparison with experiments in which calcium was present. In Ringer solution containing no calcium, 6 mM $MgCl_2$ and 1 mM EGTA, isethionate still induced some giant potentials. Increasing the calcium concentration (to 0.4 mM or 1.0 mM) after an exposure to Ca^{2+}-free/Mg^{2+}-free isethionate Ringer produced a rapid increase in m.e.p.p. frequency to greater than control values and within 10–15 min a high proportion of giant potentials were recorded (10–20%).

$[Cl]_o$ replacement by propionate

In four experiments, the extracellular chloride was replaced with another common organic anion, propionate. No effect on the m.e.p.p. frequency or the m.e.p.p. amplitude distribution (Figure 6) was observed over a recording period of 1–2 h. Therefore, the induction of giant potentials seems to be due to the presence of the isethionate rather than the absence of chloride.

Discussion

Spontaneous giant potentials (more than twice the modal amplitude) occur at normal frog endplates with a very low frequency (e.g. Fatt & Katz, 1952; Durant & Marshall, 1980). High proportions of giant potentials can be induced experimentally at amphibian and mammalian neuromuscular junctions by a wide variety of treatments and agents and from their characteristic actions the treatments can be divided into three main categories.

Table 2 The time constants (τ_D) of decay of m.e.p.cs and giant currents recorded after 60–90 min exposure to isethionate Ringer

Experiment	*Normal m.e.p.cs* (ms)	*Giants* (ms)	n
1	2.23 ± 0.10	2.21 ± 0.06	25
2	3.22 ± 0.11	3.14 ± 0.12	15
3	2.38 ± 0.11	2.43 ± 0.15	20

Each value represents the mean ± s.e.mean of equal numbers (n) of m.e.p.cs and giants.

Firstly, there are treatments which induce giant potentials but only after a delay in normal Ringer following exposure to the treatment e.g. exposure for several hours to isotonic calcium chloride (Katz & Miledi, 1969; Heuser, Katz & Miledi, 1971), prolonged tetanic nerve stimulation (Heuser, 1974) and prolonged exposure to acidic or hypertonic conditions (Pécot-Dechavassine, 1970; Pécot-Dechavassine & Couteaux, 1971; 1972). Secondly, some chemical agents (mainly toxins) produce spontaneous giant potentials after a delay in the presence of the agent and also significantly alter m.e.p.p. frequency. Some examples are, vinblastine (Turkanis, 1973; Pécot-Dechavassine, 1976), taipoxin (Cull-Candy, Fohlman, Gustavsson, Lüllmann-Raud & Thesleff, 1976a) botulinum toxin (Cull-Candy, Lunah & Thesleff, 1976b) and crotoxin (Hawgood and Santana de Sa, 1979). Thirdly, other chemical agents induce giant potentials within minutes of application, but without affecting m.e.p.p. frequency. Examples of these have so far been confined to a group of related compounds, i.e. 3, 4 diaminopyridine and 4-aminoquinoline (Katz & Miledi, 1979; Durant & Marshall, 1980; Molgó & Thesleff, 1982).

Extracellular chloride replacement by isethionate in this study increases the proportion of spontaneous giant potentials in a manner similar to that of the amino compounds (category three, above), i.e. the giant potentials appear after a short delay during the exposure and without any concomitant change in m.e.p.p. frequency. There is no significant change in the modal value of the m.e.p.p. amplitude distributions and the decay time of extracellularly recorded m.e.p.cs is unaffected. Because a high proportion of giant potentials are observed with no overall change in m.e.p.p. frequency, it is extremely unlikely that their appearance is attributable to the simultaneous release of independent quanta. Additionally, there were no obvious discontinuities in the growth phases

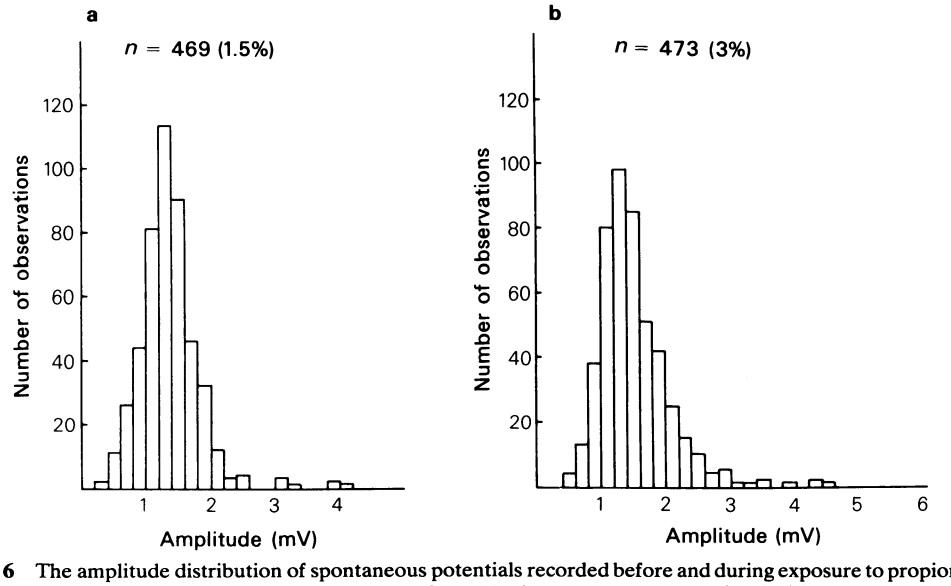

Figure 6 The amplitude distribution of spontaneous potentials recorded before and during exposure to propionate Ringer solution. Ordinate scale: number of observations; abscissa scale: amplitude (mV). (a) Control in normal Ringer solution; (b) after 90 min in propionate Ringer. Tetrodotoxin 1 μM was present throughout. The % of giant potentials is given in parentheses.

of extracellularly recorded giant m.e.p.ps. Giant potentials occur in the presence of TTX, the absence of calcium and at endplates blocked by high Mg^{2+}, therefore it is unlikely that any regenerative or local presynaptic depolarization at the nerve terminal is responsible for their appearance. Also stimulation of the m.e.p.p. frequency by high K^+, hypertonic or ouabain-containing solutions does not increase the frequency of the giant potentials (our results and see Molgó & Thesleff, 1982). This suggests that the release of the quanta producing giant potentials is not dependent on preterminal Ca^{2+} fluxes. In other experiments however (see above) we have shown a dependence on the external $[Ca^{2+}]$. The Ca^{2+}-dependence requires to be investigated in more detail in future experiments.

A significant number of the giant potentials exhibited a prolonged rising phase in comparison to both normal m.e.p.ps and other giants in the presence of isethionate. The reason for these slow-rising giant potentials is unknown. An anticholinesterase effect of the isethionate can be discounted because neostigmine is present in the majority of experiments, and there is no increase in the proportion of such giant potentials when the neostigmine is added to normal Ringer. Further, in the absence of neostigmine, not all the giant potentials are affected. The increased duration of the rising phase may be due to a local saturation of receptors hence producing activation of more distant receptors which also contribute to the time course (del Castillo & Katz, 1956).

Many of the above mentioned characteristics of the giant potentials induced by isethionate are very similar to those induced by other treatments (e.g. vinblastine, 3, 4-diaminopyridine and 4-aminoquinoline). We might therefore expect that the giant potentials have a common origin. Heuser (1974) suggested that the giant potentials originate from the release of cisternae which are involved in synaptic vesicle recycling. However this is unlikely to be the means by which isethionate induces giants because these types of giant potentials (e.g. induced by high frequency stimulation or exposure to acid or hypertonic conditions) are only detectable after a delay when the treatment has ended. There are other, more likely mechanisms for the appearance of giant potentials. Firstly, giant potentials could be due to release of multiquantal packets of acetylcholine derived from the fusion of synaptic vesicles (Pécot-Dechavassine & Couteaux, 1972; Pécot-Dechavassine, 1976) or secondly, to release from sites other than at the 'active zones' (Molgó & Thesleff, 1982). Thus the drug or treatment either induces the fusion of synaptic vesicles (perhaps an internal action?) or selectively stimulates non 'active zone' spontaneous release (an external site?). If we assume that isethionate does behave as a completely impermeant anion, then perhaps the latter mechanism is the more attractive. Further experiments are required in order to test systematically whether either of these proposed mechanisms can adequately account for the origin of the giant potentials, and the wide variety of drugs and treatments which increase their frequency of occurrence. Replacement of external chloride by isethionate could prove a useful tool in investigating the origin of such giant potentials.

The abnormal firing of repetitive action potentials which accompanied the spontaneous mechanical activity in our experiments is worth a comment. Chloride ions contribute a major share (68%) to the resting membrane conductance of frog skeletal muscle fibres (Hutter & Noble, 1960), and evidence suggests that replacement of the external chloride by a relatively impermeant substitute reduces the electrical stability of the muscle membrane (Adrian & Bryant, 1974). A consequence of this instability is the appearance of repetitive spike activity following the first muscle action potential. Similar instability is observed in the abnormal skeletal muscle membrane condition known as myotonia, one of the main features of which is a reduced membrane chloride conductance (see e.g. Bryant, 1979). Indeed normal muscle fibres can be made to exhibit all the characteristic features of myotonia by substituting an impermeant anion for the external chloride (Rüdel & Senges, 1972; Adrian & Bryant, 1974). However, the basis for the membrane instability associated with myotonia is not simply a reduced chloride conductance; potassium accumulation in the T-system (Adrian & Bryant, 1974) and a shift in the sodium activation kinetics (Adrian & Marshall, 1976; Bryant & Hershneck, 1977) may also be important. All of these factors may well contribute to the appearance of repetitive firing in a chloride-free environment following the generation of a single action potential by a giant potential.

In conclusion, it is clear that the spontaneous mechanical activity observed when frog skeletal muscle is exposed for a period of time to isethionate Ringer is due to the appearance of spontaneous giant potentials which occasionally are of sufficient amplitude to elicit a train of muscle action potentials.

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