EFFECT OF CAFFEINE ON THE NEUROMUSCULAR JUNCTION OF THE FROG, AND ITS RELATION TO EXTERNAL CALCIUM CONCENTRATION

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Summary The effect of caffeine on the neuromuscular transmission was investigated by recording the end-plate potential (e.p.p.) of the frog sartorius muscle using an intracellular microelectrode.

2. Caffeine increased the e.p.p. amplitude. The threshold concentration was about $0.5-1 \times 10^{-4}$ g/ml. The amplitude of e.p.p. was $158 \pm 16\%$ of control size in the presence of $3 \times 10^{-4}$ g/ml caffeine.

3. The quantum content was increased in the presence of $2 \times 10^{-4}$ g/ml and $2.5 \times 10^{-4}$ g/ml caffeine by factors of about 1.5 and 2, respectively.

4. The frequency of the miniature end-plate potential (m.e.p.p.) was increased by about 3-fold with $3 \times 10^{-4}$ g/ml caffeine without an appreciable increase of m.e.p.p. size.

5. The potentiating effect of caffeine became more marked when calcium concentration of the external medium was lowered, but the effect was usually transient when the calcium concentration was below 1/10 of the normal value.

6. After a train of stimuli at 50 Hz for 30 sec post-tetanic potentiation occurred in the presence of caffeine with the same time course as in the control Ringer's solution.

7. It is suggested that caffeine acts on the presynaptic nerve terminal and facilitates the release of calcium from the stored site within the nerve terminal.

Since Axelsson and Thesleff (1958) showed that the caffeine contracture was not mediated by changes in the membrane potential, caffeine has been used by many investigators to activate the excitation-contraction coupling (see Sandow, 1965). Low concentration of caffeine produced no contracture, but increased twitch tension. It has been shown that caffeine acts on the sarcoplasmic reticulum and frees the bound calcium (Bianchi, 1961; Herz and Weber, 1965;
ISAACSON and SANDOW, 1967; LÜTTGAU and OETLKER, 1968; WEBER and HERZ, 1968; EBASHI et al., 1969). On the other hand it is well known that calcium plays an important role in the release of neural transmitters (DEL CASTILLO and KATZ, 1954a,b,c; BOYD and MARTIN, 1956; JENKINSON, 1957; MARTIN, 1965; KATZ, 1966; KATZ and MILEDI, 1967, 1968). It may, therefore, be assumed that caffeine has some effect on the release of the transmitter through the action of calcium. ELMQVIST and FELDMAN (1965) showed that frequency of the miniature end-plate potential (m.e.p.p.) was increased when caffeine was applied in a Ca-free solution but was decreased when it was applied in the presence of EDTA. In the present experiments lower concentrations of caffeine, which induced no caffeine contraction, were applied to frog muscles and their effect on the neuromuscular junction was investigated and, further, an examination was made to see whether the external calcium had any influence on the caffeine effect.

METHODS

The sciatic nerve-sartorius muscle of the frog (Rana nigromaculata) was used. The nerve was stimulated with a pair of silver electrodes in a wet chamber. Micro-electrodes were filled with 3 M-KCl and had a resistance between 10 and 15 MΩ. Potential changes were recorded through a pre-amplifier with negative capacity. The composition of the normal Ringer's solution was NaCl, 115 mm; KCl, 2.5 mm; CaCl₂, 1.8 mm; NaHCO₃, 2.5 mm. The solution was maintained at 20–22°C by using the thermoelements (Coolnix, Komatsu Denshi Co.). Neuro-muscular transmission was blocked with 1–9 × 10⁻⁶ g/ml d-tubocurarine. Bath solution was continuously exchanged with a new solution throughout the experiments. Caffeine (C₈H₁₀O₂H₄·ExH₂O, Japanese Pharmacopoeia) was dehydrated by keeping at 42°C for one night and stocked as a solution of 1×10⁻² g/ml. The experiments were done mainly from January to May and some in July.

RESULTS

Effect on e.p.p.

When the muscle was soaked in a Ringer's solution containing approximately 1–3 × 10⁻⁴ g/ml caffeine, the amplitude of the end-plate potential (e.p.p.) increased and attained the final level in about 3 min. Upon removal of caffeine the amplitude of e.p.p. returned to the original level in about the same time (Fig. 1). Figure 1 also indicates that the electrotonic potentials produced by small depolarizing current pulses were almost constant, while the e.p.p. amplitude increased to about 1.7 times of the control in the presence of caffeine. Figure 2 shows the relationship between the increase of e.p.p. size and caffeine concentration. Threshold concentration of caffeine was about 0.5–1 × 10⁻⁴ g/ml. At 3 × 10⁻⁴ g/ml caffeine, the e.p.p. size was 158 ± 16% (mean ± S.D.) of the control. Concentrations above 4 × 10⁻⁴ g/ml were not tested, because at these high concentrations
Fig. 1. Effect of caffeine on the amplitude of e.p.p. (●) and the transverse membrane resistance (×). Caffeine was added to the bath solution during the period indicated. The bath solution was changed continuously.

Fig. 2. Relationship between the e.p.p. amplitude and caffeine concentration. Ordinate: Amplitude of e.p.p.s expressed as the percentage increase relative to the control before application of caffeine. (a) in normal Ca (1.8 mM). (b) in 1/10 Ca (0.18 mM). Bars indicate ± S.D.
Table 1. Effect of caffeine on quantum content of e.p.p. (a) Measured before caffeine treatment. (b) Measured after removal of caffeine. N: Number of impulses. F: Number of failures. m: Quantum content of e.p.p. calculated from F.

The outside medium contained 0.9 mm of Ca and 14 mm of Mg.

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Control</th>
<th>Caffeine, $2 \times 10^{-4}$g/ml</th>
<th>Quantum content ratio</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>N</td>
<td>F</td>
<td>m</td>
</tr>
<tr>
<td>1 a)</td>
<td>118</td>
<td>54</td>
<td>0.78</td>
</tr>
<tr>
<td>b)</td>
<td>91</td>
<td>45</td>
<td>0.70</td>
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<tr>
<td>2 a)</td>
<td>131</td>
<td>39</td>
<td>1.21</td>
</tr>
<tr>
<td>b)</td>
<td>50</td>
<td>14</td>
<td>1.27</td>
</tr>
<tr>
<td>3 a)</td>
<td>55</td>
<td>22</td>
<td>0.92</td>
</tr>
</tbody>
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Control | Caffeine, $2.5 \times 10^{-4}$g/ml | Control (m) | Caffeine (m) |
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<tr>
<td>4 a)</td>
<td>44</td>
<td>31</td>
<td>0.35</td>
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<tr>
<td>b)</td>
<td>44</td>
<td>9</td>
<td>1.4</td>
</tr>
</tbody>
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Caffeine contracture occurred and it was difficult to measure the action of caffeine precisely. Caffeine increased the e.p.p. amplitude more effectively in summer than in winter. The amount of the e.p.p. increase by caffeine in summer was about 1.5 times of that in winter. In Ringer's solution with low calcium, the slope of the dose-response curve of caffeine was steeper than that in the Ringer's solution with normal calcium (Fig. 2b). It is known that lowering the external calcium concentration decreases the quantum content of e.p.p. (DEL CASTILLO and KATZ, 1954a,b; see MARTIN, 1965). Addition of $3 \times 10^{-4}$ g/ml caffeine to Ringer's solution with 1/10 of the normal calcium (containing $1 \times 10^{-6}$ g/ml d-tubocurarine) increased the size of e.p.p.s up to 191 ± 33% (mean ± S.D.).

The quantum content was calculated from the number of failures of e.p.p. responses using Mg-blocked preparations (DEL CASTILLO and KATZ, 1954b), and the effects of caffeine on the quantum content was examined. Table 1 indicates that the quantum content was increased to about 150% by $2 \times 10^{-4}$ g/ml caffeine and to about 200% by $2.5 \times 10^{-4}$ g/ml caffeine.

Figure 3 shows that the addition of caffeine to the bathing solution gradually increased the frequency of the miniature end-plate potential (m.e.p.p.) from 0.57/sec to 1.6/sec in about 5 min. On returning to normal Ringer's solution the frequency decreased slowly to the control level in about 10 min. Caffeine at the concentration used had little or no effect on the amplitude of m.e.p.p.

**Effect of calcium**

In a physiological solution in which the calcium concentration was decreased to 1/10 of normal (the 1/10 Ca Ringer's solution), the effect of caffeine on the size of e.p.p. was more pronounced than in the normal Ringer's solution. As shown in Fig. 2, a and b, caffeine at $3 \times 10^{-4}$ g/ml was about 1.4 times more effective in
Fig. 3. Frequency of m.e.p.p.s. The number of m.e.p.p. was counted for every 30 sec. period.

Fig. 4. Effect of caffeine on the amplitude of e.p.p. in 1/10 Ca (0.18 mM Ca) Ringer's solution. (b) An example which showed a transient increase of e.p.p. by caffeine in 1/10 Ca Ringer's solution.
Fig. 5. Effect of caffeine on the amplitude of e.p.p. in 1/20 Ca (0.09 mm Ca) Ringer’s solution. Normal Ringer’s solution contained 6 × 10⁻⁶ g/ml d-tubocurarine and 1 × 10⁻⁶ g/ml d-tubocurarine in 1/20 Ca Ringer’s solution.

The 1/10 Ca Ringer’s solution than in the normal Ringer’s solution. Figure 4a shows an example in which 2 × 10⁻⁴ g/ml caffeine increased the amplitude of e.p.p. to about twice the control. In this particular experiment, the increased amplitude of the e.p.p. was maintained constant so long as the caffeine concentration was kept constant. However, in some other fibers, as shown in the example of Fig. 4b, the addition of caffeine to the bathing solution increased the e.p.p. size only transiently; the e.p.p. size was decreased to the control level in 2–3 min, although the same concentration of caffeine was maintained. This transient increase of e.p.p. size by the application of caffeine was often seen in the 1/10 Ca Ringer’s solution. At lower concentrations of Ca (down to 1/20) almost all fibers showed such a transient increase. Figure 5 shows an example obtained in the 1/20 Ca Ringer’s solution. In this experiment the concentration of d-tubocurarine used was 6 × 10⁻⁶ g/ml in normal solution and 1 × 10⁻⁶ g/ml in 1/20 Ca Ringer’s solution. Upon changing the calcium concentration from normal to its 1/20, the amplitude of e.p.p. decreased from 2.5 to 1 mV. Addition of 3 × 10⁻⁴ g/ml caffeine in low calcium solution almost doubled the e.p.p. size, but the size was soon decreased. The amplitude of the e.p.p. recovered reversibly by switching back to the normal solution. Although the microelectrode was kept inserted in the muscle throughout this experiment, injury to muscle by the microelectrode may be disregarded, because no appreciable change in the time course of e.p.p. was detected at the end of experiment.
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Figure 6 shows the relationship between calcium concentration in the external medium and relative change in amplitude of e.p.p. produced by $3 \times 10^{-4}$ g/ml caffeine. The points in Fig. 6 are the mean and the bars indicate ±S.D. When the number of samples was small (*) the bars show the range instead of S.D. In Fig. 6a, the amplitude was measured about 1–2 min after caffeine application. In Fig. 6b, the amplitude was measured at 5 min after the caffeine application, when the e.p.p. amplitude reached a stationary value. Filled circles are the mean values of e.p.p. size which showed only a transient increase. Although the value are rather variable, the potentiating effect seems more marked when the calcium concentration became low.

**Post-tetanic potentiation**

When repetitive stimulation was applied to the nerve at 50 Hz for 30 sec the post-tetanic potentiation appeared in the normal and the caffeine added Ringer’s
The present experiment showed that caffeine increased the amplitude of e.p.p. to about 160% at the concentration of $3 \times 10^{-4}$ g/ml. At this concentration of caffeine the membrane resistance of the frog muscle showed no appreciable change, although at higher concentrations the membrane resistance decreased.
slightly (Axelson and Thesleff, 1958). The frequency of m.e.p.p. was increased by the addition of caffeine without appreciable effect on the amplitude of m.e.p.p. These results suggest that the action of caffeine is mainly presynaptic in nature. This view was confirmed by the observation that the quantum content measured by the failure method was increased by caffeine (Table 1). Since, however, Mambrini and Benoit (1963) showed an increase in the acetylcholine sensitivity of the frog end-plate by the action of 1–5 × 10⁻⁴ g/ml caffeine and Shinnick and Jacobs (1971) suggested presynaptic and postsynaptic effects of theophylline in isolated cat tenuissimus muscle, the effect of caffeine may be partly post-synaptic.

According to the quantum hypothesis (Del Castillo and Katz, 1954b) the quantum content \( m \) is expressed as \( m = pN \), where \( p \) is the release probability and \( N \), available number of quanta. Thus, an increase in the quantum content is the result of an increase in either \( p \) or \( N \). The present experiment does not provide direct evidence to determine between these two possibilities. However, the effect of calcium concentration on the action of caffeine suggests that an increase in \( p \) is more probable.

In low calcium Ringer's solution, which contained, e.g., 1/10 of the normal calcium, the addition of caffeine produced a more marked increase of the e.p.p. size than that in normal calcium Ringer's solution (Fig. 2). If caffeine, in analogy with the effect on the sarcoplasmic reticulum (Bianchi, 1961; Weber and Herz, 1968) were to release calcium from the stored site in nerve terminal, the calcium thus released would increase the transmitter output, but the potentiation thereby produced would be more pronounced in the low calcium Ringer's solution, since a small increase in the quantum content would cause a marked increase in the e.p.p. size under such a condition. Although caffeine increased the amplitude of e.p.p. it did not fully compensate for the effect of low calcium (Fig. 5). Christensen and Martin (1970) showed that the effect of external calcium concentration on the mean quantum content is attributable to the change in the release probability and not to the number of quanta available for release. Caffeine might increase the probability of transmitter release, possibly through its effect on the mechanism which releases calcium ions from the stored site in the nerve terminal.

In a low calcium medium, the caffeine effect was often transient and after washing out caffeine the amplitude of e.p.p. became smaller than that before addition of caffeine. Endo et al. (1970) showed that the sarcoplasmic reticulum of the skinned fiber which once released calcium ion by caffeine could not take up enough calcium when the concentration of free calcium ion in medium was very low, and the degree of restoration depended upon the concentration of free calcium ion. The transient increase of e.p.p. in the low calcium Ringer's solution during the action of caffeine suggests that the amount of stored calcium or available calcium in nerve terminal may be small and depleted by prolonged application of caffeine. The calcium once released from the nerve terminal by caffeine may not be fully replenished in low calcium medium. In rat diaphragm pre-
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treated with EDTA, caffeine 5 mM caused only a short transient increase of m.e.p.p.
frequency (ELMOVIST and FELDMAN, 1965). From the experiment it was suggested
that stored calcium could be depleted by caffeine in mammalian diaphragm.

HOFMANN (1969) estimated the quantum content of e.p.p. by a train of stimuli
at 25 Hz in mammalian diaphragm. He found that caffeine protected the
depression of transmission and suggested that caffeine increased acetylcholine mobilization
and hastened the transmitter replenishment. Since in the frog muscle the
depression in the e.p.p.s size after stimulation is small compared with the mam-
malian muscle, such a run-down of the e.p.p.s amplitude as in the mammalian
end-plate is usually not observed and it is difficult to distinguish the two components
of facilitation, i.e. an increase in the release probability and an increase in the
number of quanta available for release (MALLART and MARTIN, 1967). In the
present experiment the post-tetanic potentiation was tested to determine the
effect of caffeine on the mobilization of transmitter. The time course of post-
tetanic potentiation was not changed by action of caffeine and the relative increase
of potentiation was smaller in the caffeine Ringer’s solution than in the normal
Ringer’s solution, although the absolute value of e.p.p. size was about 130%
greater in caffeine solution. When two shocks with an interval of 30 msec were
applied during the post-tetanic potentiation, the facilitation of second e.p.p. was
less remarkable in caffeine Ringer’s solution than in the normal solution. These
results might explain the fact that during the post-tetanic potentiation in caffeine
Ringer’s solution an increased transmitter output depleted the stored transmitter
more than that in the normal solution resulting a less marked facilitation of the
second e.p.p.

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