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THERMAL ACCLIMATION, NEUROMUSCULAR SYNAPTIC DELAY AND MINIATURE END-PLATE CURRENT DECAY IN THE FROG *RANA TEMPORARIA*

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Summary

1. The effects of 1 or 2–3 weeks of acclimation to 4 °C and 24 °C of overwintering grass frogs (*Rana temporaria*) on the synaptic delay and on the time constant of the decay phase (τ) of miniature end-plate currents (MEPCs) in the neuromuscular junction of sartorius muscle were studied. In order to equalize the possible effects of differential starvation, the animals were usually cross-acclimated to the two temperatures.

2. Synaptic delay was not affected by temperature acclimation but was slightly prolonged by the more profound starvation at the higher temperature when the cross-acclimation procedure was not used. The average Q_{10} of synaptic delay between 4 and 24 °C was 2.60 and of minimum synaptic delays, 2.64. The corresponding values for apparent activation energy (E_a) were 65.79 and 66.48 kJ mol^{-1}.

3. The time constant of the decay phase of MEPCs was not affected by temperature acclimation. The average Q_{10} between 4 and 24 °C was 2.27. The corresponding E_a value was 56.02 kJ mol^{-1}.

4. The function of peripheral neuromuscular synapses is well regulated and changes in its time relationships do not appear to be involved in the thermal acclimation of frogs.

Introduction

Many life functions of ectothermic animals show some time-dependent compensation for the immediate effects of changes in environmental temperature. Such acclimation effects are evident in the functioning of nervous systems at the behavioural, systemic, neuronal and cell membrane level (Lagerspetz, 1974; Prosser and Nelson, 1981; Macdonald, 1990). They may also have a role to play in the endocrine system and metabolic seasonal acclimatization, e.g. in amphibians (Lagerspetz, 1977).

The effects of thermal acclimation on the functioning of the nervous system are probably adaptive for both the behaviour and the functional homeostasis (homeokinesis) (Prosser, 1982) of the animals concerned. Consequently, if the behaviour of the animal shows compensatory effects in response to temperature changes, their site of action should be found in some parts and functions of the controlling pathways.

Key words: temperature, thermal acclimation, neuromuscular synapse, end-plate, *Rana temporaria*, synaptic delay, end-plate current.

Synapses are the main integrative elements in the nervous system and are often considered to be thermally the most adaptive parts of it. Work in this area has largely been confined to neuromuscular synapses and concerns the shifts of heat- and cold-block temperatures and neuromuscular facilitation (Jensen, 1972; Harri and Florey, 1979; Jacobs and Atwood, 1981; White, 1983).

As far as we know, there are no records of attempts to measure the effect of temperature acclimation on the synaptic delay. However, Harper *et al.* (1989) have studied the effect of temperature acclimation on the miniature end-plate currents (MEPCs) of the extraocular muscle of the carp.

We therefore wanted to study whether the effects of temperature acclimation on the neuromuscular transmission are involved in the acclimation of nervous function. In our experiments, neuromuscular synapse did not show the expected acclimation effects.

Materials and methods

Animals and their thermal history

Frogs (*Rana temporaria* L.) were collected from their wintering places in Finland in October and November and stored in a cold room at 8–10 °C in a stainless-steel basin with running charcoal-filtered tap water and stones on the bottom. The animals were kept in continuous darkness and were not fed. Male and female frogs weighing on average 24 g (12–48 g) were used for the experiments. We did not find any sex- or size-dependent variation in our results.

For temperature acclimation, the frogs were transferred in groups of three to 3 l glass containers at 4 °C or 24 °C. The water at the bottom of the containers was changed three times each week. The frogs were kept at these temperatures for 14–25 days.

To determine whether acclimation at 24 °C caused a larger starvation effect than acclimation at 4 °C, some groups of animals were first kept for 7 or 14 days at one temperature and then for 7 or 14 days at the other. The frogs subjected to this double-acclimation or cross-acclimation procedure were then considered to be acclimated to the last prevailing environmental temperature.

In order to show that the frogs had become thermally acclimated by this procedure, we carried out pilot experiments in which we measured the conduction velocity in the sciatic nerve from animals kept at 24 °C and 4 °C, as described above. According to Meyer and Hegmann (1971), the conduction velocity in sciatic nerves of frogs (*Rana pipiens*), measured at high and intermediate temperatures, is increased by an acclimation to 23 °C for a period of 8 weeks compared with animals acclimated to 10 °C (inverse acclimation). We measured the conduction velocity from the difference between the peaks of compound action potentials recorded by two electrodes and found the conduction velocity at 20 °C to be 27.2±0.7 m s^{-1} (N=7) in the sciatic nerves of animals acclimated to 24 °C and 23.6±0.3 m s^{-1} (N=7) in animals acclimated to 4 °C. According to this criterion the animals used in our experiments were thermally acclimated.

Preparation

Frogs were decapitated and the spinal cord pithed. The sartorius muscle was separated

with the nerve attached. The nerve–muscle preparation was fixed to the Sylgard-coated bottom of a 4 cm Petri dish and cleaned from adhering muscle and connective tissue in normal frog Ringer's solution [NaCl, 111.2 mmol l^{-1}; KCl, 1.3 mmol l^{-1}; $CaCl_2$ (dry), 1.8 mmol l^{-1}; $NaHCO_3$, 1.2 mmol l^{-1}; pH 7.2–7.3].

The neuromuscular junctions were located under a compound microscope and a map of the probable sites was drawn. The Petri dish containing the preparation was then transferred into a chamber, the temperature of which could be controlled by external circulation from a thermocontrolled bath. The temperature was monitored by a 0.5 mm thick thermocouple kept close to the preparation in the Ringer's solution and an Ellab thermogalvanometer with an accuracy of 0.1 °C. The bathing solution was changed to Ca^{2+}-free EGTA–frog Ringer's solution (NaCl, 109 mmol l^{-1}; KCl, 1.9 mmol l^{-1}; $MgCl_2$, 5.5 mmol l^{-1}; Hepes, 10 mmol l^{-1}; EGTA, 1 mmol l^{-1}; pH 7.2–7.3).

Measurements of synaptic delay

Synaptic delay measurements were made at different temperatures, decreasing from 24 to 4 °C. The rate of temperature decrease in the bath was about 0.1–0.2 °C min^{-1}. The measurements were made at selected temperatures that were kept steady to within ±0.2 °C.

The classical Katz–Miledi technique (Katz and Miledi, 1965*a*,*b*) was used. A fire-polished glass capillary electrode containing 0.5 mol l^{-1} $CaCl_2$ was focally, but extracellularly, applied to the area of a neuromuscular junction. The low calcium concentration in the bathing solution ensured that neuromuscular transmission was blocked and muscle contractions during measurements were avoided. Transmission occurred only below the recording electrode, where a small amount of calcium diffused out from the electrode and raised the extracellular calcium concentration, thus allowing local transmitter release. The resistance of the electrodes was about 0.2 MΩ.

The nerve leading to the muscle was stimulated by hook electrodes and the measured signals were fed through a preamplifier to the oscilloscope. Delays were recorded either by photographing from the oscilloscope or by recording the signals from the amplifier with a data recorder. The recordings were analyzed either from enlarged photographs, or by a computer if data were recorded.

Synaptic delay was defined as the interval between the maximum inward current of the presynaptic action potential and the beginning of the inward current of the end-plate potential (Katz and Miledi, 1965*a*; Fig. 1 in the present study). For each temperature and frog, 10–15 measurements were taken. The mean for each frog at each temperature was calculated and later used in statistical analysis. In addition, the minimum delay values for each animal at each temperature were used in comparisons between the experimental groups.

Measurements of miniature end-plate current

Miniature end-plate currents were measured under the same conditions as the synaptic delays, but without nerve stimulation and in a normal frog Ringer's solution. The MEPCs were recorded as transient currents at the focal electrode (Fig. 2).

With extracellular electrodes, we could not measure the amplitudes of the MEPCs

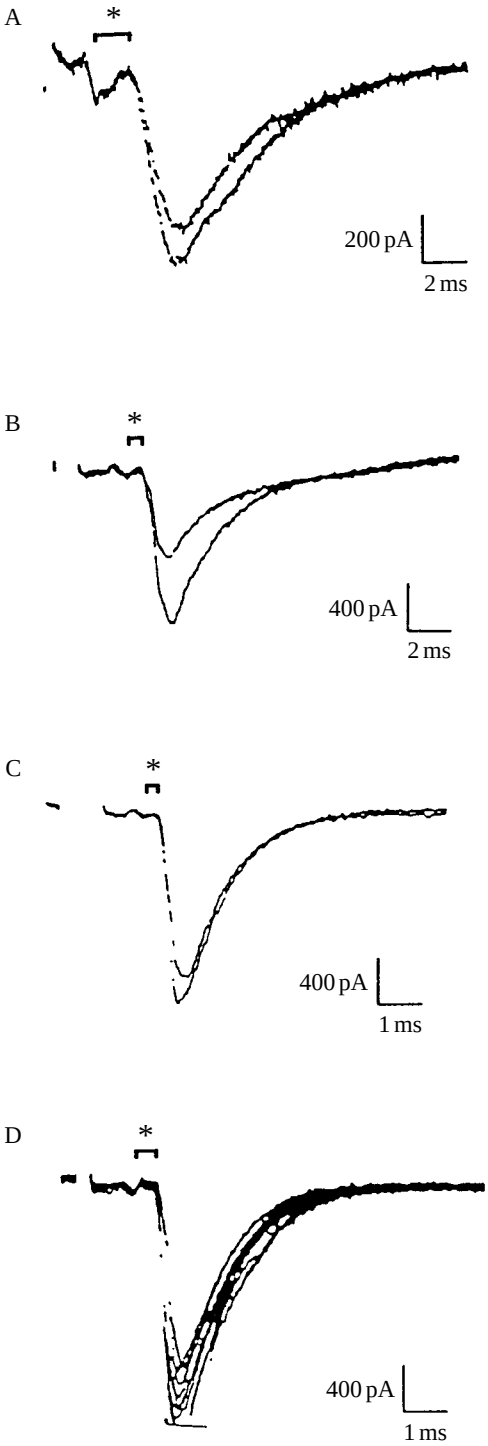

Fig. 1

Fig. 1. Examples of records of presynaptic excitatory currents and postsynaptic currents used to measure the synaptic delay at different experimental temperatures. The time and current scales are indicated beside each recording. (A) 4 ˚C, (B) 8 ˚C, (C) 20 ˚C. Each trace consists of two superimposed recordings. (D) Ten superimposed recordings taken at intervals of 1 s at 20 ˚C. The examples are from cold-acclimated animals. The synaptic delay is indicated by *.

reliably, and our experimental conditions did not allow the measurement of MEPC frequencies. For reasons given in the Results, of the possible variables of the MEPCs only the time constant of decay (τ) was calculated. It was defined as the time between the peak (100 %) and the value of 1/e (36.8 %) of the current. At each temperature and for each frog, 10–15 measurements were taken and averaged. The means were used in further analysis.

Statistical analysis

The statistical analysis was carried out using two-sample *t*-tests and analysis of variance. The means are usually given ± S.E.M. The Q_{10} values are given as $1/Q_{10}$. The values of the apparent activation energy (E_a) were calculated for the inverses of synaptic delays and of τ values. The Q_{10} and E_a values are for the temperature interval 4–24 ˚C.

Results

Synaptic delays

Examples of records of synaptic delays and postsynaptic currents at different temperatures are given in Fig. 1. The measurement of synaptic delay was made from single records, but a sample of ten superimposed successive records with stimulus intervals of 1 s is given in Fig. 1D. Although the amplitudes of extracellularly recorded postsynaptic currents vary, there is little variation in the synaptic delays.

The animals used in the first experiment (protocol 1) had been stored for about 3 months and then transferred to 4 ˚C for 2 weeks or to 24 ˚C for 1 week, and subsequently reacclimated to 24 ˚C for 1 week or to 4 ˚C for 2 weeks, respectively. The neuromuscular synaptic delays in preparations from animals in the two groups were measured at several

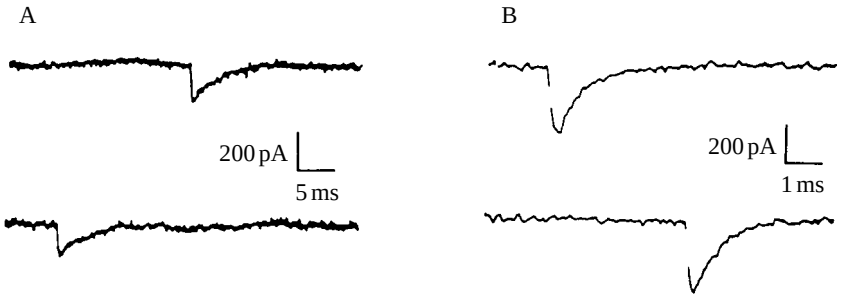

Fig. 2. Examples of miniature end-plate currents (MEPCs) at two temperatures: (A) 4 ˚C, (B) 24 ˚C. The time scales are different. The examples are from sets of consecutive recordings from one warm-acclimated animal.

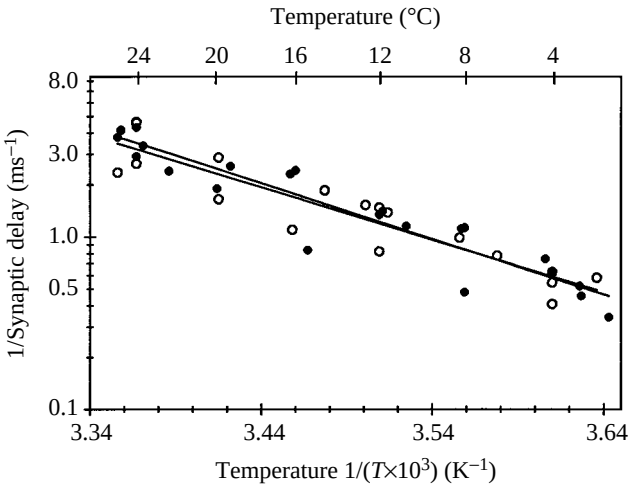

Fig. 3. Arrhenius plots of the inverse values of the mean synaptic delay obtained with protocol 1 (three animals in both acclimation groups). Final acclimation temperatures 4 ˚C (○) and 24 ˚C (●). For cold- and warm-acclimated animals r=0.92 and 0.95, respectively.

temperatures ranging from about 2 to 25 ˚C. The mean results are given in Fig. 3 as Arrhenius plots. The mean synaptic delays were about 0.3 ms at 24 ˚C and about 2 ms at 4 ˚C. The results for the two groups did not differ from one another. The mean Q_{10} value for cold-acclimated groups was 2.69 and for warm-acclimated animals 2.35. E_a values for the two groups were 73.06 and 69.21 kJ mol^{-1}, respectively.

The second experiment (protocol 2) was essentially similar to the first one, except that the animals had been stored for about 8 months and the acclimation and reacclimation times were 2 weeks at each temperature. The Arrhenius plot of the results (Fig. 4) is based on the mean values of all experimental animals, recorded at nine fixed temperatures. The means of the synaptic delays at the extreme temperatures do not differ from those found in the first experiment. The temperature dependence of the synaptic delays is similar in both acclimation groups and is also similar to that found in the first experiment. For this second experiment, the mean Q_{10} values for cold- and warm-acclimated groups were 2.55 and 2.61, respectively and E_a values for the two groups were 64.04 and 65.59 kJ mol^{-1}, respectively.

In the third experiment (protocol 3) the animals had been stored for 8 months, as in the second experiment, but then acclimated for 2–3 weeks either to 4 ˚C or to 24 ˚C without reacclimation. The results of this experiment are given in Fig. 5. The neuromuscular synaptic delays were measured at six fixed temperatures. Analysis of variance showed that the mean synaptic delays in preparations from animals acclimated to 24 ˚C were longer than those in animals acclimated to 4 ˚C (d.f.1=1, d.f.2=72, F=7.56, P<0.01). The Arrhenius plots given in Fig. 5 are similar to those from the first two experiments (Figs 3 and 4), although slightly further apart from each other. The mean Q_{10} values for cold- and warm-acclimated groups of the third experiment were 2.78 and 2.54, while E_a values were 69.87 and 61.33 kJ mol^{-1}, respectively.

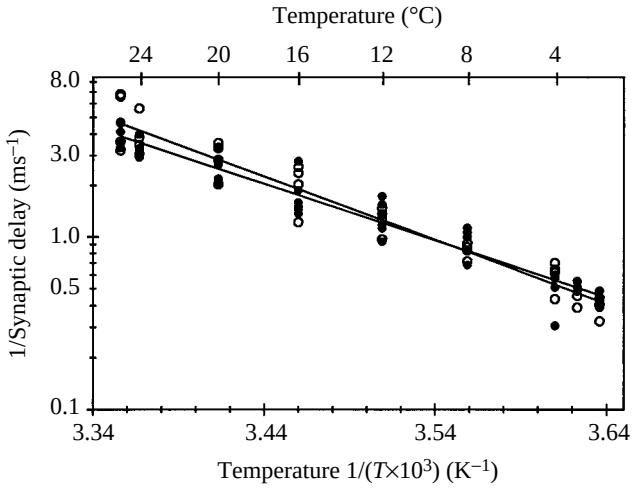

Fig. 4. Arrhenius plots of the inverse values of the mean synaptic delays obtained with protocol 2. Four animals acclimated to 4 ˚C (○), six animals acclimated to 24 ˚C (●). For both regression lines, r=0.97.

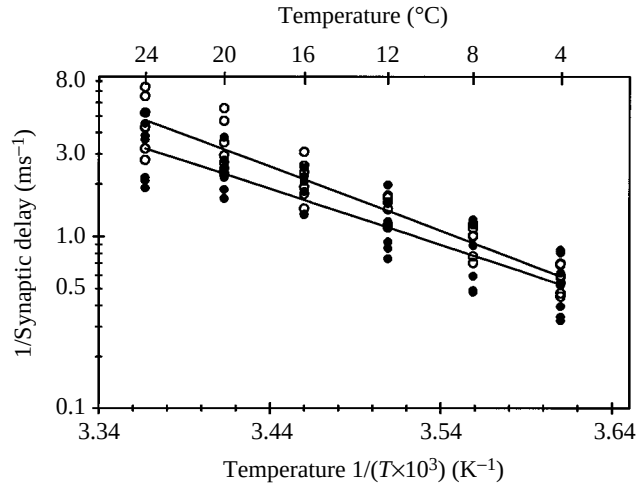

Fig. 5. Arrhenius plots of the inverse values of the mean synaptic delays obtained with protocol 3. Seven animals acclimated to 4 ˚C (○); seven acclimated to 24 ˚C (●). For cold- and warm-acclimated animals, r=0.95 and 0.88, respectively.

The minimum synaptic delay observed in the 10–15 individual values recorded for each animal at each experimental temperature was analyzed in the same way as the mean values. The results were similar to those for the mean delays. The temperature dependence of the groups did not differ from each other significantly, but in the third experiment, were slightly rotated in relation to one another. The mean minimum delays for all experimental animals were, at 4 ˚C, 1.50±0.10 ms (4 ˚C-acclimated, N=14) and

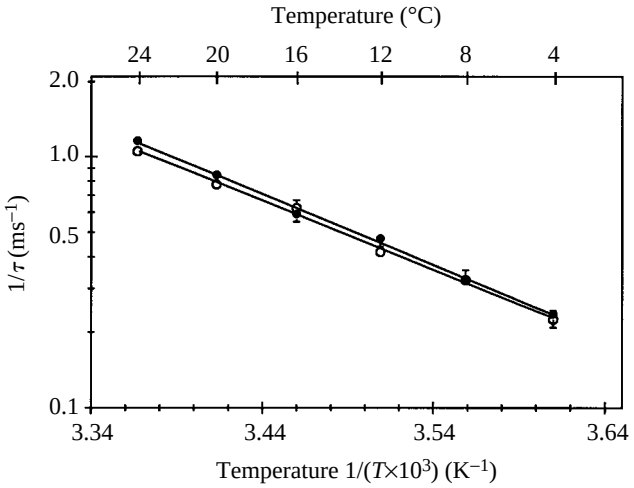

Fig. 6. Arrhenius plots of the inverse values of the mean time constants ($1/\tau$) of MEPC decay. Nine animals acclimated to 4 °C (○), 8–10 acclimated to 24 °C (●). Means are given with ±S.E.M. For both regression lines r=0.99.

1.57±0.14 ms (24 °C-acclimated, N=15), and, at 24 °C, 0.20±0.03 ms (4 °C-acclimated, N=14) and 0.24±0.03 ms (24 °C acclimated, N=16). The mean Q_{10} values of minimum synaptic delays for cold- and warm-acclimated groups were 2.75 and 2.54 while E_a values were 69.11 and 63.85 kJ mol^{-1}, respectively.

Miniature end-plate currents

Examples of MEPC records at two temperatures are given in Fig. 2. The growth phase of MEPCs was too rapid to allow for reliable measurements with the methods used. It is also much less sensitive to temperature than the decay phase (Gage and McBurney, 1975). These authors found the τ values and their temperature dependence in the sartorius muscle of toad *Bufo marinus* were similar, whether obtained with focal extracellular electrodes or with intracellular recording and voltage-clamping. The time constant of the decay phase (τ) was used, therefore, to characterize the MEPCs. The mean results of all measurements of τ were used for Fig. 6. No significant differences between the cold- and warm-acclimated animals were found. The τ values varied from about 0.9 ms (at 24 °C) to about 4.8 ms (at 4 °C). The mean Q_{10} values for cold- and warm-acclimated groups were 2.25 and 2.28 and the mean E_a values were 55.55 and 56.49 kJ mol^{-1}, respectively.

Discussion

Frogs feed largely on living animals, and they do not feed while overwintering. During late summer, food reserves are stored, especially as glycogen in the liver, and these stores are sufficient for normal wintering conditions and even supply the energy needed at the breeding season in spring (Pasanen and Koskela, 1974; Koskela and Pasanen, 1975; Lagerspetz, 1977). Starvation effects are therefore unlikely to occur in the overwintering

frogs. For instance, no difference in synaptic delay was found in animals kept for 3 or 8 months under winter conditions in the laboratory (in protocols 1 and 2, Figs 3 and 4).

However, if overwintering frogs are subjected to high temperatures for thermal acclimation, more of these energy reserves are consumed, and the possible starvation effect may interfere with acclimation. Therefore, in most of our experiments, the frogs were subjected to a double-acclimation or cross-acclimation procedure: the animals were kept first at one of the acclimation temperatures and then at the other for a corresponding period. This was done in order to ensure that both groups of frogs experienced the same degree of starvation. The animals were then considered to be acclimated to the second exposure temperature.

The acclimation periods ranged from 7 to 25 days, a period generally sufficient for the metabolic and neurochemical acclimation effects to be completed in the wintering frog (Lagerspetz, 1977). In our pilot experiments on the thermal acclimation of the conduction velocity in sciatic nerve, our results were similar to those of Meyer and Hegmann (1971) and it therefore seemed reasonable to regard our experimental animals as being acclimated to the temperatures to which they were exposed.

In the measurements of neuromuscular synaptic delay, we used the same animal preparation and method as Katz and Miledi (1965*a*). In the study of Katz and Miledi (1965*b*), the minimum delays were approximately 0.5 ms at 20 °C, 4 ms at 4 °C and about 5 ms (between 3.5 and 7 ms) at 2 °C. The average Q_{10} between 2 °C and 19 °C was 3.14. The mean minimum delays found by us varied from about 0.3 ms at 20 °C to about 1.5 ms at 4 °C with an average Q_{10} of 2.64 between 4 and 24 °C, and these were thus shorter and less temperature-dependent than those found by Katz and Miledi (1965*b*). We cannot at present explain this difference, but it may be due to differences in the collection and holding of animals and in the rate of temperature change experienced by the frogs.

The acclimation of frogs to 4 °C and 24 °C did not affect the neuromuscular synaptic delay and its temperature dependence (Figs 3 and 4). The effect of thermal acclimation on synaptic delay has not been studied before. The exocytosis of transmitter is considered to be the main temperature-sensitive part of synaptic transmission (Macdonald, 1988). Our results indicate that, at least in peripheral nerve terminals, the rate of exocytosis is not affected by thermal acclimation.

The small effect found in the third experiment (Fig. 5) may be attributed to the more profound starvation in the animals acclimated to the higher temperature. It would be interesting to know which molecules fail to be synthesized, thus causing this disturbance to an otherwise regulated function.

Magazanik *et al.* (1979) found the time constant of the decay phase of MEPC (τ) in the sartorius muscle of *Rana temporaria* to be on average 1.4 ms at about 20 °C, and Miledi and Uchitel (1981) found a mean τ value of 5 ms in the fast fibres of pyriformis muscle of the same species at about 6 °C. Our results agree with these, being 1.26±0.05 ms (N=19) at 20 °C and about 4 ms (interpolated value) at 6 °C. Our results also seem to agree well with the values of τ of J. A. Macdonald on the extraocular muscle of the goldfish (*Carassius auratus*) (Macdonald and Montgomery, 1986, p. 808, note 17), which are 1.3 ms at 20 °C and 3 ms at 10 °C.

Macdonald and Montgomery (1986) compared the value of τ for peripheral synaptic

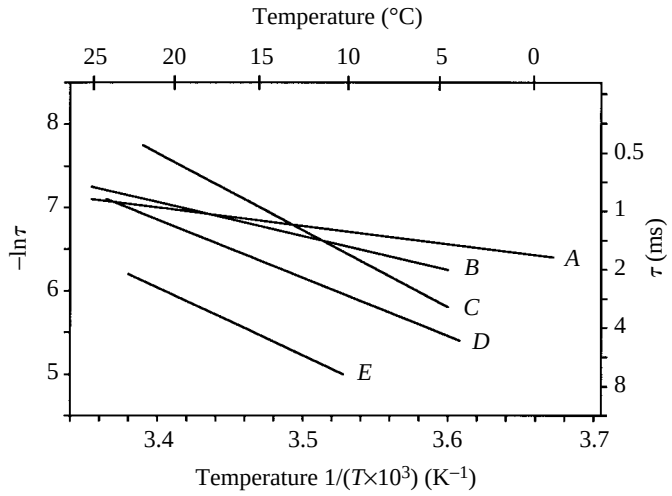

Fig. 7. Arrhenius plots of natural logarithms of the mean decay time constant ($-\ln\tau$) of MEPC decay in different fish and amphibian species. *A*, *Pagothenia borchgrevinki* (Macdonald and Montgomery, 1986); *B*, *Cyprinus carpio* (Harper *et al.* 1989); *C*, *Trachurus novaezelandiae* (Macdonald and Balnave, 1984); *D*, *Rana temporaria* (this study); *E*, *Bufo marinus* (Gage and McBurney, 1975).

function and its temperature dependence in the Antarctic fish *Pagothenia borchgrevinki* with that in a fish from a temperate climate (*Trachurus novaezelandiae*), studied by Macdonald (1983) and Macdonald and Balnave (1984), and found a marked difference in the temperature dependence. In Fig. 7 their results are combined with the results of Gage and McBurney (1975) on the tropical toad *Bufo marinus*, with those of Harper *et al.* (1989) on the temperate-cool climate carp (*Cyprinus carpio*) and with our results on the frog from a cool climate. The points for goldfish (Macdonald and Montgomery, 1986), a species originating from a warm climate, would be on line *D*, which depicts our results on the frog. The preparations used were the extraocular muscle in the fish and the sartorius muscle in the amphibians.

Macdonald (1983) and Macdonald and Balnave (1984) presented evidence that the MEPCs of teleost fish decay more rapidly than those of other vertebrates. However, together with other data from the literature, our results on the frog show that the values of τ measured at the acclimation or holding temperatures of the animals seem not to differ clearly, at least between fishes and amphibians. The climatic origin of the species studied seems to be more important in this respect.

The temperature dependence of τ is similar in the species from temperate and cool climates (excepting *Trachurus novaezelandiae*) and the temperature dependence of τ for the Antarctic species (*Pagothenia borchgrevinki*) is smaller than those of the other five species. This difference is probably due to evolutionary adaptation.

The time constant of the decay phase of MEPCs (τ) in the frog was not changed by temperature acclimation. Harper *et al.* (1989) obtained the same result with cold- and warm-acclimated carp. These authors also found a slight, but significant, thermal

acclimation effect in the duration of the growth phase of MEPCs recorded at low temperatures from the extraocular muscle of cold- and warm-acclimated carp. Recent evidence indicates that the amount of transmitter released at the motor terminals of frogs varies with the season (Lnenicka, 1993). For the reasons given above, we did not study the growth time of MEPCs or their frequency and amplitude.

The main part of the decay phase of a MEPC is apparently due to a change in the conformation of acetylcholine receptor-channel molecules: this closes the channel and decreases the inward synaptic current (Hille, 1992). Our results indicate that the rate constant of conformational changes of receptor-channel molecules is not affected by thermal acclimation.

Our general conclusion is that the time relationships of peripheral synaptic transmission are well regulated in frogs and not susceptible to the effects of long-lasting changes in the ambient temperature. The seasonal adaptive variation in the behaviour of frogs does not seem to depend on changes in the function of peripheral synapses.

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