THE TEMPERATURE DEPENDENCE OF THE TIME COURSE OF GROWTH AND DECAY OF MINIATURE END-PLATE CURRENTS IN CARP EXTRAOCULAR MUSCLE FOLLOWING THERMAL ACCLIMATION

BY A. A. HARPER, J. R. SHELTON AND P. W. WATT

The Department of Anatomy and Physiology, The Old Medical School, The University, Dundee DD1 4HN, UK

Accepted 9 June 1989

Summary

1. The effect of temperature (5–35°C) on the decay and growth phases of miniature end-plate currents (MEPCs) was investigated in extraocular muscle from freshwater carp acclimated to either high (28°C) or low (8°C) temperature.

2. The temperature dependence of the time constant of decay ($T_D$) was found to follow an Arrhenius relationship; the relationship between log $T_D$ and reciprocal of absolute temperature ($1/K$) being linear in both groups. The $T_D$ of MEPCs recorded from cold-acclimated carp was not statistically significant from that of the warm group.

3. $T_D$ was moderately temperature-dependent. Regression gave a $Q_{10}$ of 1.78 for the warm-acclimated carp, corresponding to an activation energy, $E_a$, of 41.15 ± 2.17 kJ mol$^{-1}$. For the cold-acclimated carp, the $Q_{10}$ was 1.79, and $E_a$ was 41.43 ± 2.46 kJ mol$^{-1}$.

4. Growth time ($T_G$) was less susceptible than $T_D$ to temperature change. The relationship between growth time (taken as the time for MEPCs to rise from 20 to 80% of maximum) and temperature was linear for the cold-acclimated group, with a $Q_{10}$ of 1.34 and $E_a$ of 20.94 ± 4.75 kJ mol$^{-1}$. The data for the warm group were, in contrast, best fitted by two linear regressions meeting at 15.1°C. At temperatures below 15°C $Q_{10}$ was 3.16 and $E_a$ was 82.20 ± 15.47 kJ mol$^{-1}$; above 15°C, $Q_{10}$ was 1.22 and $E_a$ was 14.15 ± 12.24 kJ mol$^{-1}$.

5. The acetylcholinesterase inhibitor neostigmine increased $T_D$ by approximately twofold and raised $T_G$ to approximately 1.4 times control values. These effects were observed across the temperature range scanned for both groups.

6. The results are discussed with reference to the documented effects of temperature and temperature acclimation on membrane lipids and proteins.

Introduction

Few organisms live in a constant environment such as those provided experimentally, for example, in culture. It is generally accepted that living organisms have evolved a versatile physiology which permits them to optimize their

Key words: neuromuscular junction, miniature end-plate currents, thermal acclimation, carp extraocular muscle.
functioning in response to changes in the environment. This adaptive response is especially important for those animals experiencing seasonal variations.

When an organism alters aspects of its functioning in response to complex environmental factors it is said to have acclimatized. However, if the changes are because of a single laboratory-controlled factor the process is termed acclimation.

During the past several decades many investigators have studied the cellular basis of acclimation to a variety of environmental challenges. In this report we have examined temperature acclimation. It is generally accepted that poikilotherms, e.g. fish, adapt or acclimate to operate optimally at the prevailing environmental temperature. A general finding is that the process or activity of the system being studied is greater or faster in fish acclimated to cold temperatures than in their warm-acclimated counterparts. Such changes have been well documented; for example, for skeletal muscle biochemical characteristics (myofibrillar ATPase activity) and mechanical performance (Johnston et al. 1975; Heap et al. 1985).

However, the extent to which such compensatory adaptations of functions occur for excitable membranes has been comparatively little studied; few studies have examined, directly, the effects of thermal acclimation on the electrical characteristics of nerve or muscle cells. Changes in the electrophysiological characteristics of central neurones of the snail, Helix, have been reported by Langley (1979), who found that the rate of rise and fall of the cell body action potential decreased with increasing acclimation temperature.

The neuromuscular junction (NMJ) has been extensively used as a 'model synapse' to study the effects of thermodynamic and environmental variables, for example, temperature and hydrostatic pressure (Kordas & Zorec, 1984; Ashford et al. 1982). It offers the advantage of allowing the separate study of aspects of pre- and postsynaptic membrane function.

To our knowledge there are no studies that have examined, directly, the extent to which the neuromuscular junction acclimates with temperature. Relevant studies have been confined to comparative studies of species from distinct thermal environments; for example, Macdonald (1983) examined spontaneous miniature currents (MEPCs) in an amphibian, Bufo marinus, and a marine teleost, Trachurus novaezelandiae.

Here we report the effects of thermal acclimation on the neuromuscular junction of the freshwater carp (Cyprinus carpio). We have studied the effects of temperature on the time constant of decay and growth phase of miniature end-plate currents (MEPCs) recorded from the oblique extraocular muscle from carp acclimated to either warm (28°C) or cold (8°C) temperatures.

A preliminary report of these studies has been published (Harper et al. 1989).

Materials and methods

Thermal acclimation

Freshwater carp (Cyprinus carpio) were obtained from Newhay Fisheries,
Selby, Yorkshire, UK. They were maintained at 14°C in tanks of running aerated fresh tap water for 2–3 weeks before being acclimated to warm (28°C) or cold (8°C) temperatures. This was achieved by changing the temperature in small steps so that the target acclimation temperatures were attained after 5–7 days. Fish were then maintained at the acclimation temperatures for at least 3 weeks before being used for electrophysiological experiments. We elected to use these temperatures because these are the preferred temperatures for cold-acclimated and warm-acclimated carp when exposed to a thermal gradient (Penney & Goldspink, 1979).

During the maintenance and acclimation periods the fish were fed with a commercial pellet diet and kept under a 12 h:12 h photoperiod. Both groups were found to feed, both by observation and by examination of gut contents.

**Preparation**

Fish were stunned by a blow to the head, and killed by transection of the spinal cord and pithing, both rostrally and caudally. The dissection was then carried out on an ice-cold platform. The eye and associated muscles were isolated and removed from the orbit. The inferior oblique extraocular muscle was identified and cleared of associated connective and adipose tissues. The muscle was pinned out in a thermoregulated tissue chamber lined with Sylgard.

**Myofibrillar ATPase activity**

It was of paramount importance to determine whether the myofibrillar ATPase activity of these animals showed changes similar to those documented in previous studies (Johnston et al. 1975; Heap et al. 1985). A portion of the white epaxial muscle was removed from two fish from each acclimation group (masses 115 and 120 g for the cold-acclimated fish, 134 and 140 g for the warm ones). These samples were pooled and assayed for specific activity at 10, 20 and 30°C. The methods used for the assay of enzymic cleavage of ATP were as previously described (Heap et al. 1985).

**Electrophysiology**

The size and mass of fish from both groups used for the electrophysiological experimentation were closely matched, being 15·3 ± 0·72 cm and 105±12·8 g \((N = 6)\) for the cold group and 14·8 ± 0·68 cm and 113±11·2 g \((N = 6)\) for the warm group.

During isolation and subsequent experimentation the preparation was bathed in oxygenated Ringer's solution (adapted from Shuttleworth, 1972) comprising in (mmol l\(^{-1}\)); NaCl, 140; KCl, 2·7; CaCl\(_2\), 1·5; NaHCO\(_3\), 15; and Heps, 25; pH 7·5. Temperature in the experimental chamber was regulated by a recirculating thermostatic which circulated a water/ethylene glycol mixture through a jacket which surrounded the chamber. The temperature of the bathing solution was measured using a thermocouple probe (resolution 0·1°C).

Spontaneous miniature end-plate currents (MEPCs) were recorded using a focal extracellular electrode. The electrodes were fire-polished, had an internal tip
Diameter of 10–30 μm, were filled with 1 % (w/v) agar in 1 mol l⁻¹ NaCl solution, and typically tip resistance was 0.2–1 MΩ. Connection to the recording system was by Ag/AgCl pellets. The signal was fed to an electrometer (WPM701) amplified and filtered, bandwidth 0.5–10 kHz. MEPCs were captured on a digital storage oscilloscope using the pre-trigger facility. The signals were read from the oscilloscope memory to a chart recorder or dot matrix printer to give hard copies which were used for subsequent analyses.

**Analysis of MEPCs**

All MEPCs were analysed individually. The decay phase was analysed by a log-linear regression analysis of the current amplitude from its peak to 0.1–0.2 of this value. Plots of miniature currents with a correlation coefficient (r) greater than 0.98, indicating a single exponential decay, were accepted. This criterion was satisfied by 84 % of all currents sampled (N = 624). The time for the current to decay to 0.37 of its maximum was taken as the time constant of decay, T_D. Eight currents were sampled at each test temperature and the mean T_D used in subsequent analysis.

The growth time (T_G) of the MEPC was measured as the time for it to increase from 20 to 80 % of its peak value, as described by Gage & McBurney (1975).

**Statistical analysis**

Data are presented as mean ± one standard error. Number of samples, N, in brackets.

For the Arrhenius plots the data were analysed by a method to determine the best fit of a line and testing for the probability of a discontinuity or break and hence two separate linear regressions (Jones & Molitoris, 1984). All other data were compared using Student's t-test.

**Results**

First, it was necessary to assay the myofibrillar ATPase activity of skeletal muscle from the fish to ascertain if the fish had thermally acclimated. The white epaxial muscle was chosen for this measurement, both because it has been frequently used before and data for comparison are available in the literature and also because the extraocular muscles were too small to yield sufficient material for analyses.

Table 1 presents the results of the ATPase assay at 10, 20 and 30°C of muscle from warm-acclimated and cold-acclimated fish. The activity of the ATPase in muscle from cold-acclimated fish was higher than in their warm-acclimated counterparts at all three test temperatures. The absolute levels and differences in enzyme activity between the warm-acclimated and cold-acclimated groups are in good agreement with previous results (Heap et al. 1958). We therefore considered that the fish were acclimated, at least according to this criterion.

Miniature end-plate currents were recorded from the white band of the oblique
The effect of temperature on MEPC decay

The decay phase of MEPCs recorded from both groups of fish exhibited moderate temperature dependence, the \( T_D \) increasing with decreasing temperature. Individual currents used in the analysis of the decay phase for end-plates from cold-acclimated fish are shown in Fig. 1. The amplitude of the MEPCs captured was typically approximately 500 \( \mu \)V, in agreement with that recorded from other preparations (Gage, 1976). The amplitude of the MEPCs was subjectively considered to decrease with decreasing temperature. However, since the amplitude of extracellularly recorded MEPCs depends on the position and geometry of the recording electrode, analysis of this parameter was not performed.

The effect of temperature on \( T_D \) for warm-acclimated and cold-acclimated fish is presented as an Arrhenius plot (log \( T_D \) vs linear 1/K) in Fig. 2. The MEPCs from the cold group decayed on average 16% faster than those from the warm group over the temperature range scanned (5–35°C). However, this difference was not statistically significant (\( P > 0.05 \)). Linear regressions of log\( T_D \) and reciprocal absolute temperature were well correlated for each group: \( r = 0.94 \) cold group, \( r = 0.96 \) warm group. For the warm group this yielded a \( Q_{10} \) value of 1.78, corresponding to an activation of energy (\( E_a \)) of 41.15 ± 2.17 kJ mol\(^{-1}\). For the
cold group, $Q_{10}$ was 1.79 and $E_a$ was $41.43 \pm 2.46$ kJ mol$^{-1}$. The $T_D$ at 15°C was approximately 1.18 ms for the cold group and 1.37 ms for the warm group, in good agreement with the only comparable study at a similar temperature (0.903 ms; Macdonald, 1983).

**The growth phase $T_G$**

The growth phase, $T_G$, taken as the time for the current to rise from 20 to 80% of its maximum amplitude, was measured in MEPCs from both warm-acclimated and cold-acclimated muscles. The relationship between $T_G$ and temperature for
MEPCs showed a large variation (Fig. 3) characteristic of growth time measurements.

The data for the cold-acclimated group were fitted by a single linear regression

\[ \ln T_D = 24.037 - 41.43/R T \] (\( r = 0.94 \)), warm group \( \ln T_D = 23.770 - 41.15/R T \) (\( r = 0.96 \)), where \( R \) is the gas constant (8.314 J mol\(^{-1}\)), and \( T \) is absolute temperature (K). All data points are the mean of measurements from eight individual MEPCs.

Fig. 2. Comparison of MEPC decay time constants \( T_D \) for muscles from warm-acclimated and cold-acclimated fish (\( N = 6 \) in each group). The data are fitted by linear regression lines: cold group \( \ln T_D = 24.037 - 41.43/R T \) (\( r = 0.94 \)), warm group \( \ln T_D = 23.770 - 41.15/R T \) (\( r = 0.96 \)), where \( R \) is the gas constant (8.314 J mol\(^{-1}\)), and \( T \) is absolute temperature (K). All data points are the mean of measurements from eight individual MEPCs.

Fig. 3. The effect of temperature on the growth time \( T_G \) of MEPCs recorded from muscle from cold-acclimated and warm-acclimated fish. Each data point represents the mean of eight individual currents. The results for the cold group were fitted by a linear regression of \( T_G \) and temperature, \( E_a = 20.94 \pm 4.75 \) kJ mol\(^{-1}\). The data for the warm group were, however, best fitted by two separate regressions with a break point at 15.1°C (\( P < 0.01 \)), the \( E_a \) values at temperature above and below the break being 14.15 \( \pm \) 12.24 and 82.2 \( \pm \) 15.47 kJ mol\(^{-1}\), respectively.
(r = 0.59) with a Q₁₀ of 1.34 and $E_a$ of 20.94 kJ mol⁻¹. The data for the warm group, however, were best fitted by two linear regressions with a split or break at 15.1°C ($P < 0.01$). The Q₁₀ and activation energy at temperatures above the split, 15.1°C, were 1.22 and 14.15 kJ mol⁻¹, respectively, these measurements below the split being 3.16 and 8.20 kJ mol⁻¹. A similar split in the temperature dependence of $T_G$ and comparable activation energies have been reported for mouse diaphragm MEPCs by Robertson & Wann (1984).

Effects of cholinesterase inhibitors

A reduction of acetylcholinesterase activity at the neuromuscular junction will increase both the $T_D$ and $T_G$ of miniature currents by increasing the lifetime of transmitter in the cleft. To test whether the increases in $T_D$ and $T_G$ with decreased temperature were due to a partial inhibition of acetylcholinesterase activity, we performed several experiments in the presence of 5 μmol L⁻¹ neostigmine, a cholinesterase inhibitor. Neostigmine was applied to the preparation for at least 15 min before recording data.

Neostigmine increased $T_D$ approximately twofold across the temperature range 8–31°C in both warm and cold preparations (Fig. 4). The growth phase also increased, to approximately 1.4 times the control value.

Discussion

We have investigated the effect of temperature in the range 5–35°C, on extracellularly recorded spontaneous miniature end-plate currents in skeletal muscle from both warm-acclimated and cold-acclimated freshwater teleost fish (Cyprinus carpio). The mean time constant of the decay phase recorded from the cold group throughout the temperature range studied was not significantly different from that recorded from the warm group. The temperature dependence of the growth time of MEPCs of warm-acclimated fish displayed a break or split at 15°C, whereas a similar relationship for cold-acclimated material was best fitted by a simple linear regression. Experiments in the presence of a cholinesterase inhibitor indicate that these effects are not due to differential temperature sensitivity of acetylcholinesterase activity.

With presently available data the mechanisms underlying these changes in $T_D$ and $T_G$ must remain somewhat tentative.

The time course of MEPCs follows that of the underlying postsynaptic membrane conductance change (Gage, 1976): the amplitude and time course of decay determine the amount of charge transferred and thus the amplitude of the postsynaptic potential. The time course of decay of the MEPCs is thought to be due to either (i) the rate of dissociation of the transmitter–receptor complex or (ii) the rate of the conformational change of the receptor from an active to an inactive form. Thus, it reflects the lifetimes of receptor/channels opened in response to the release of a quantum of acetylcholine; $T_D$ being equated to the average open time of these channels (Magelby & Stevens, 1972).
Thermal acclimation of teleost end-plate

Fig. 4. (A) The effect of 5 μmol l⁻¹ neostigmine on currents recorded at 28°C from a cold-acclimated fish. (B) Action of neostigmine on $T_D$ of MEPCs recorded from muscles from cold-acclimated and warm-acclimated fish, one in each case. Neostigmine increased $T_D$ approximately twofold across the temperature range sampled in both warm-acclimated and cold-acclimated fish; each point is the mean of measurements from eight individual currents.

The time constant of decay is voltage-sensitive, decreasing with membrane depolarization and increasing with hyperpolarization. This has been demonstrated for many poikilothermic vertebrate species, for example, fish, amphibians and reptiles (Macdonald, 1983; Gage & McBurney, 1975; Dionne & Parsons, 1981). No measurements were made of the postsynaptic muscle fibre membrane potential in the present study. The membrane potential of the cold group would, however, have to be depolarized by approximately 6 mV compared with the warm-acclimated muscles to account for the difference in $T_D$ observed, using the data describing the voltage sensitivity of $T_D$ given by Macdonald (1983).

A difference in membrane potential has been observed in skeletal muscle fibres of the green sunfish, *Lepomis cyanellus*, having mean membrane potentials of 92 mV at the acclimation temperature of 25°C and 86 mV at 7°C (Klein & Prosser, 1985). No difference in membrane potential was recorded between groups, however, when recordings were made at the same intermediate temperature. The membrane potential of the white muscle of *Lepomis* displays a high temperature sensitivity, hyperpolarizing with increasing temperature. If this behaviour is
shared by carp extraocular muscle it will act to decrease the temperature dependence of $T_D$, which may underlie the low $Q_{10}$ of this parameter when compared with other preparations.

Studies are required which directly investigate the postsynaptic fibre membrane potential and voltage sensitivity of $T_D$.

The growth phase of extracellularly recorded MEPCs is not significantly affected by postsynaptic membrane potential (Gage, 1976). The durations of the growth phase in muscles from cold-acclimated and warm-acclimated fish were similar at temperatures above 15°C. At temperatures below 15°C the $E_a$ of the process determining $T_G$ for warm-acclimated MEPCs was much greater than that calculated for MEPCs from muscles from cold-acclimated fish: approximately four times that for the cold group.

Similar splits in the $T_G$-temperature relationship and activation energies have been reported for growth phase duration for MEPCs recorded in mouse diaphragm (Robertson & Wann, 1984), whilst the linear $T_G$-temperature relationship and magnitude of the $E_a$ for the cold group is similar to that reported for fish and toads (Macdonald, 1983; Gage & McBurney, 1975). Thus the temperature sensitivity of the MEPCs in skeletal muscle from cold-acclimated fish is akin to that of toad (i.e. linear), whereas that for MEPCs from warm-acclimated fish has a split and $E_a$ values rather like that reported for warm-blooded vertebrates, indicating, perhaps, some adaptive alteration for optimal performance at the higher temperature in the warm-acclimated group.

The growth time is so short that unless there are considerable changes in the parameter it will not change the charge displacement. An increase in $T_D$ will result in an increased amplitude and decay time of the postsynaptic miniature end-plate potential, if the cable properties of the muscle fibre remain unchanged. If these characteristics are also found in the physiologically evoked end-plate current it will result in more secure neuromuscular transmission.

What processes underlie the observed changes in $T_G$ and $T_D$?

The fast decay time ($T_D$) of a MEPCs measured in the present study is in agreement with that previously measured for a marine teleost by Macdonald (1983), who proposed that this correlated with the increased unsaturation and fluidity of nerve membrane lipids from fish.

It is probably instructive to compare the $T_D$ values of several poikilothermic vertebrates, which are available in the literature. Data are calculated from the temperature dependence of $T_D$ given in the report and are presented as the $T_D(18°C)/T_D$ (environmental temperature). Teleosts: *Cyprinus* 8°C-acclimated this study, 0.99 (18°C)/1.81 (8°C); *Cyprinus* 28°C-acclimated this study, 1.15 (18°C)/0.66 (28°C); *Trachurus*, marine teleost (Macdonald, 1983) 0.62 (18°C)/0.35 (23°C); *Bufo*: (Gage et al. 1975) 3.15 (18°C)/$T_D$ (environmental temperature).

Changes in $T_D$ may be due to a direct effect of temperature on the receptor protein or an indirect one on its lipid microenvironment. It has been proposed that
Thermal acclimation of teleost end-plate

Thermal acclimation of teleost end-plate

$T_D$ is affected by the physicochemical state of the protein's lipid surround. Thus alcohols ($> C_6$) and anaesthetic agents, which have been demonstrated to increase the fluidity, decrease $T_D$ (Gage & McBurney, 1975; Gage et al. 1978). Alternatively, a change in the temperature sensitivity of acetylcholinesterase activity may be responsible for the change in $T_D$ seen with temperature acclimation.

The range of growth phase, $T_G$, was extensive, extending over a four- to fivefold range at a given test temperature. This behaviour is well documented and is thought to be due to differences in the width and diffusion pathways out of the synaptic cleft (Gage, 1976); the low temperature sensitivity of the process observed in this study being similar to that expected where a physical process such as diffusion is rate-limiting.

The high $E_a$ calculated for $T_G$ at low temperatures for warm-acclimated muscle MEPCs indicates that a process other than diffusion is predominant. The presynaptic release function, altered cholinesterase activity or an alteration in the postsynaptic acetylcholine receptor, directly or indirectly mediated by a change in its lipid environment, are possible rate-limiting factors, as discussed by Robertson & Wann (1984).

That the growth phase was equally lengthened by neostigmine at low and high temperatures in fish acclimated to both high and low temperatures inclines us to the view that cholinesterase activity does not contribute to the differential temperature sensitivity of miniature currents. Indeed it is likely, as has been demonstrated in other species, that the cholinesterase enzyme also displays thermal adaptation (Hochachka, 1974), leading us to the view that some other mechanism is involved.

The physicochemical state of neuronal membrane lipid in teleosts has been demonstrated to change with thermal adaptation (Cossins, 1977). Thus, as predicted from homeoviscous theory, membranes from cold-acclimated goldfish were more fluid and had a decreased proportion of saturated fatty acids than those from warm-acclimated goldfish. This correlation was further extended between warm-acclimated fish and rats. Changes in fluorescence polarization consistent with partial homeoviscous adaptation have been demonstrated for synaptosome and myelin fractions from carp thermally acclimated under identical conditions to those used in the present study (A. A. Harper, P. W. Watt, N. A. Hancock & A. G. Macdonald, unpublished observations).

The results from this study indicate that the neuromuscular junction appears to show an alteration in function in response to thermal acclimation. The differential temperature sensitivity of the growth phase of MEPCs in muscles from warm-acclimated and cold-acclimated fish indicates that the dominant effect of temperature is likely to be presynaptic. Further studies of presynaptic functioning, e.g. miniature end-plate potential frequency and postsynaptic sensitivity to exogenously applied acetylcholine, are thus likely to prove of interest. The duration of the action potential invading the presynaptic terminal will change with temperature. The extent to which the functionally important evoked end-plate potential displays thermal acclimation requires direct study.
This work was supported by the University of Dundee. We thank Professor M. J. Rennie for his encouragement and support.

References


