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

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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 15·1°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 endplate 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 $15{\cdot}3 \pm 0{\cdot}72$ cm and $105 \pm 12{\cdot}8$ g ($N = 6$) for the cold group and $14{\cdot}8 \pm 0{\cdot}68$ cm and $113 \pm 11{\cdot}2$ 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 Hepes, 25; pH 7·5. Temperature in the experimental chamber was regulated by a recirculating thermochiller 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 (WPI M701) 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

Table 1. *Alterations in myofibrillar ATPase activity of carp white myotomal muscle*

Reaction temperature (°C)	Myofibrillar ATPase activity (μmol Pi min^{-1} mg $protein^{-1}$) Cold-acclimated	Warm-acclimated
10	1·159	0·636
20	2·119	1·796
30	3·459	2·619

The data are from a pooled myofibril preparation from two fish in each acclimation group.

extraocular muscle *in vitro* over the temperature range 5–35°C. Since teleost motor innervation is distributed along the length of the fibre, end-plates could not be discriminated visually but were located by sweeping the electrode across the muscle surface, as described by Macdonald (1983), and listening to the output of an audiometer. Temperature was changed in steps of approximately 6°C, and the preparation was allowed to equilibrate for 10–15 min before recording. No significant difference was found in the T_D between individual end-plates; however, in most instances the data were obtained from a single end-plate region. The focal electrode was removed from the end-plate region to prevent mechanical trauma to the tissue between recording periods. The bathing solution was renewed with fresh oxygenated Ringer between temperature changes. At least three test temperatures were recorded in each preparation.

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 μV, 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 ($P > 0{\cdot}05$). Linear regressions of logT_D and reciprocal absolute temperature were well correlated for each group: $r = 0{\cdot}94$ cold group, [illegible]6 warm group. For the warm group this yielded a Q_{10} value of 1·78, corresponding to an activation of energy (E_a) of $41{\cdot}15 \pm 2{\cdot}17$ kJ mol^{-1}. For the

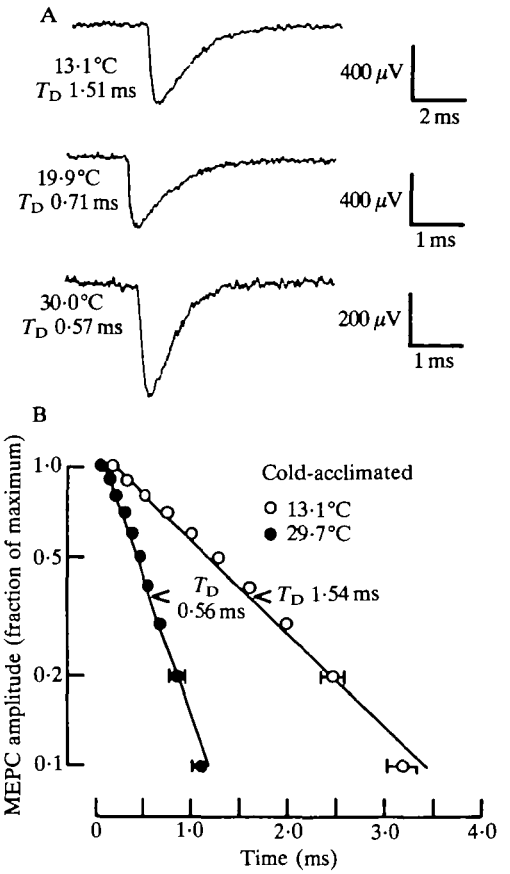

Fig. 1. (A) Examples of MEPCs recorded extracellularly from cold-acclimated fish muscle. (B) Analysis of T_D at the upper and lower test temperatures shown. The decay-phase was analysed by a log–linear regression analysis of current amplitude from its peak to 0·1 of this value. Each point represents the mean of eight individual currents. The error bars represent the S.E. of T_D at 0·1 and 0·2 of maximum amplitude. The error bars at 0·5 are contained within the symbols. The mean time constant T_D (the time taken for the current to decrease to 0·37 of maximum) was used in subsequent analyses.

cold group, Q_{10} was 1·79 and E_a was 41·43 ± 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

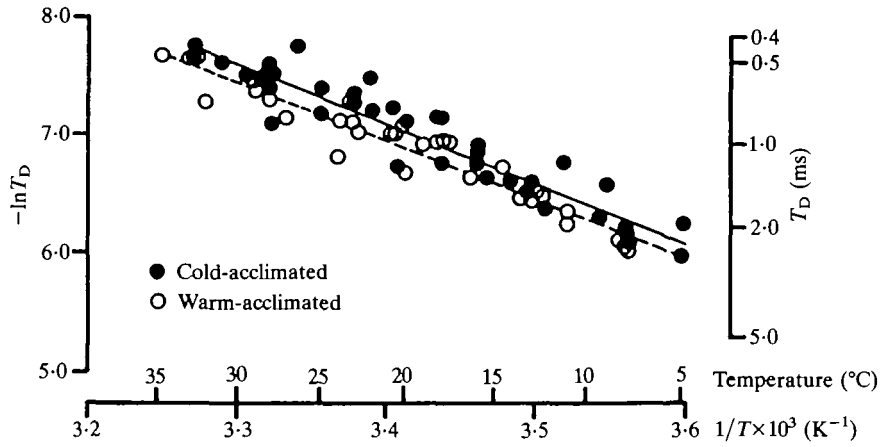

Fig. 2. Comparison of MEPC decay time constants T_D for muscles from warm-acclimated and cold-acclimated fish ($N = 6$ in each group). The data are fitted by linear regression lines: cold group $\ln T_D = 24{\cdot}037 - 41{\cdot}43/RT$ ($r = 0{\cdot}94$), warm group $\ln T_D = 23{\cdot}770 - 41{\cdot}15/RT$ ($r = 0{\cdot}96$), where R is the gas constant (8·314 J mol), and T is absolute temperature (K). All data points are the mean of measurements from eight individual MEPCs.

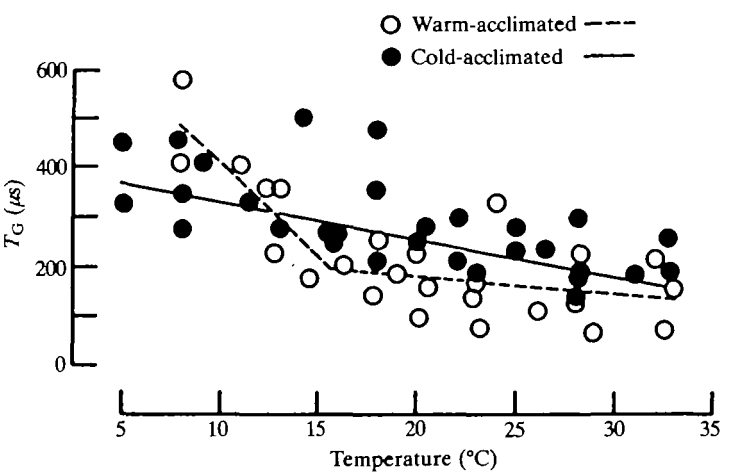

Fig. 3. The effect of temperature on the growth time T_G of MEPCs recorded from muscle from cold-acclimated and warm-acclimated fish. Each data point represents the mean of eight individual currents. The results for the cold group were fitted by a linear regression of T_G and temperature, $E_a = 20{\cdot}94 \pm 4{\cdot}75$ 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 ($P < 0{\cdot}01$), the E_a values at temperature above and below the break being $14{\cdot}15 \pm 12{\cdot}24$ and $82{\cdot}2 \pm 15{\cdot}47$ kJ mol^{-1}, respectively.

($r = 0{\cdot}59$) with a Q_{10} 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 ($P < 0{\cdot}01$). The Q_{10} 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 82·20 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 acetycholinesterase 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 ti[illegible] of these channels (Magelby & Stevens, 1972).

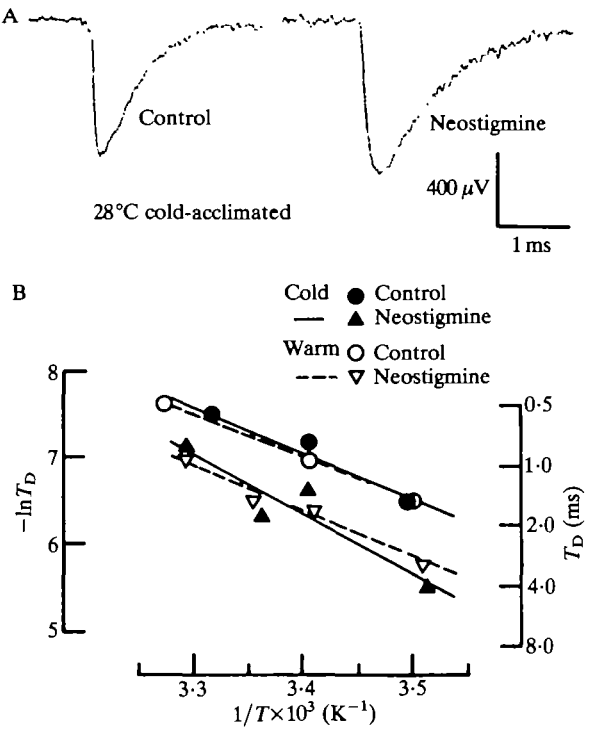

Fig. 4. (A) The effect of 5 μmol l^{-1} neostigmine on currents recorded at 28°C from a cold-acclimated fish. (B) Action of neostigmine on T_D of MEPCs recorded from muscles from cold-acclimated and warm-acclimated fish, one in each case. Neostigmine increased T_D approximately twofold across the temperature 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

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.

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