REVIEW ARTICLE

Application of the theory of homeoviscous adaptation to excitable membranes: pre-synaptic processes

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INTRODUCTION

For the purposes of this review, the term excitable membranes refers to membranes which generate action potentials or other electrical potentials and currents which initiate subsequent reactions. Typically they are part of the neuro-effector system of animals, but they also occur in single cell organisms. Excitable membranes also have many functions in common with inexcitable membranes.

The adaptation of an integrated neuro-effector system to an environmental factor such as temperature is presumably largely, but not totally, dependent on the adaptation of its component parts, of which excitable membranes are a distinctive element. This review considers the adaptation of excitable membranes in terms of the theory of homeoviscous adaptation. The theory is derived from the properties of lipid bilayers, which, it must be admitted, do not figure prominently in excitable membrane research. To that extent the *prima facie* case for applying the theory to excitable membranes is not particularly strong, but from a biochemical viewpoint it has some appeal.

The first section of this review comprises two parts. It begins with an outline of the homeoviscous theory of membrane adaptation, which sets out the criteria by which it may be tested. In the second part, environmental factors are considered. Some examples of the adaptation or survival of animals in extreme conditions are described, to set limits to the adaptation required of various 'excitable' mechanisms.

The second, major, section of this paper deals with the role of the synapse in adaptation. The effects of environmental factors, mainly temperature, on pre-synaptic processes are described, and the role of the lipid bilayer as a possible site of action, and of adaptation, is discussed. Post-synaptic phenomena will be reviewed elsewhere.

THE THEORY AND THE ENVIRONMENT

Of the physical factors which make up any organism's environment; temperature, pressure, radiation, chemical composition, and so on, two are particularly relevant to the type of membrane adaptation being considered here. They are temperature and hydrostatic pressure. Both are thermodynamic intensity factors whose occurrence in Nature and kinetic significance make them outstandingly important in adaptation studies, and in homeoviscous theory in particular.

Homeoviscous theory

The homeoviscous theory of membrane adaptation states that the 'viscosity' of the lipid bilayer is adjusted to offset changes imposed by environmental factors. Less explicit is exactly how bilayer 'viscosity' may affect the performance of the cell. Three distinct roles are generally attributed to the lipid bilayer; it provides a permeability barrier, an amphipathic matrix supporting enzymes, receptors and channels, and it also provides specific metabolic components in second messenger cascades. It is reasonable to invoke all three roles in homeoviscous theory, but the second is generally accepted as the main way by which 'viscous drag' affects functional components such as enzymes. Sinensky (1974) introduced the term homeoviscous to describe the change in the viscosity of lipids extracted from *Escherichia coli* grown at different temperatures. However, others, notably Chapman *et al.* (1966), had already appreciated the significance of bilayer fluidity and its metabolic regulation. The concept of 'micro-viscosity' applied to the bilayer has now given way to a more precise treatment, in which molecular structure and molecular motion are distinguished (Van der Meer, 1984). Structure is quantified as order by a variety of spectroscopic methods which have been devised for the purpose. Order parameters indicate the range of molecular movement which takes place in a bilayer, but give no information on the rate at which the molecules move. However spectroscopy also provides dynamic measurements of molecular motion. Often a disordered bilayer is one in which molecular motion is also rapid, and the convenient term 'fluidity' conveys such a condition, and is often used in a relative sense. In homeoviscous theory we are usually concerned with a comparison of properties in otherwise fairly similar bilayers, and so the ambiguities in the term 'fluidity' are minimized. It is still a matter for investigation as to whether the molecular constraints imposed by the bilayer lipids on particular functional membrane proteins are mainly structural or dynamic.

In many studies of membrane adaptation relatively simple techniques have been used, and these introduce their own empirical meaning. The molecular packing and types of motion which exist in bilayers have been widely discussed (for example, Van der Meer, 1984). The way they are affected by our two prime environmental factors, temperature and pressure, has been mostly studied by fluorescence spectroscopy, and because of its convenience, by the steady state fluorescence polarization (anisotropy) of diphenylhexatriene (DPH). This probe partitions into the lipid bilayer where it is largely

Abbreviations used: DPH, diphenylhexatriene; m.s.p., miniature synaptic potential(s); (m).e.p.p., (miniature) end plate potential(s); e.p.s.p., excitatory post-synaptic potential(s).

intercalated, but a proportion probably occupies the middle of the bilayer 'sandwich' (Lentz *et al.*, 1976; Parasassi *et al.*, 1984; Ameloot *et al.*, 1984; Genz & Holzwarth, 1986; Deinum *et al.*, 1988). The values of steady state anisotropy which are obtained from bilayers so labelled with DPH are therefore an average, weighted in favour of the hydrophobic region of the acyl chains. Theoretical arguments show that the steady state anisotropy of DPH is a fairly good measure of bilayer order, provided the system is not too fluid (Jahnig, 1979; Van Blitterswijk *et al.*, 1981). An objection to those measurements of bilayer order which are made on isolated, natural membranes, especially plasma membranes, is that changes occur during the isolating procedures which affect the bilayer (Kuhry *et al.*, 1985). Assessing these is not easy, but two points which counter the general objection are worth noting. First, relative fluidity is usually the main concern and such changes as arise from the isolation processes are likely to apply equally to both experimental and control membranes. Second, the order parameter of some membranes is remarkably unaffected by successive freezing and thawing, suggesting that membrane bilayers may not be all that susceptible to alteration during preparation.

Using the steady state fluorescence polarization of DPH the effect on bilayers of (a) lowering the temperature, and (b) increasing the hydrostatic pressure is seen to be an increase in anisotropy, i.e. in order. In pure phospholipid liposomes the temperature equivalent of hydrostatic pressure (i.e. the increase in temperature required to offset an increase in pressure) is $0.17\text{--}0.29\text{ }^{\circ}\text{C}\cdot\text{MPa}^{-1}$. In the case of natural bilayers it is $0.13\text{--}0.21\text{ }^{\circ}\text{C}\cdot\text{MPa}^{-1}$ (reviewed in Macdonald, 1987). Spin labels and other fluorescent methods give a similar result, but Raman spectroscopy yields $0.5\text{ }^{\circ}\text{C}\cdot\text{MPa}^{-1}$ in dipalmitoylphosphatidylcholine liposomes.

Homeoviscous adaptation is manifest as a change in the chemical composition of a lipid bilayer, with an ensuing compensatory change in bilayer order or motion. It is therefore of interest to equate chemical modulators of bilayer fluidity with temperature or pressure. Cholesterol tends to order a bilayer in the fluid (physiological) state because its relatively inflexible ring structure intercalates the phospholipid (Franks, 1976; Huang & Mason, 1982). The ordering is greatest in the outer part of the bilayer (Kutchai *et al.*, 1983). In dioleoylphosphatidylcholine bilayers, increasing the cholesterol:phospholipid ratio by 0.35–0.50 mole ratio causes the same ordering as subjecting the bilayer to 100 MPa, at 23–25 °C (Chong & Cossins, 1984). In dilauroylphosphatidylcholine bilayers an increase in 0.38 mole fraction is equivalent to 100 MPa at 37 °C (Macdonald *et al.*, 1988). Other, 'disordering', effects of compositional changes in bilayers have been published. The disordering effect of the *cis* double bond in fatty acids forming phospholipid bilayers has been shown to be very considerable (Stubbs *et al.*, 1981). Natural membranes contain complex mixtures of fatty acids incorporated into a variety of phospholipids, as well as cholesterol and proteins. Expressing the degree of saturation of the fatty acids present in the phospholipids as a saturation ratio (saturated:unsaturated), there seems to be a good correlation between saturation ratio and DPH fluorescence polarization (Cossins & Lee, 1985). Conversely membranes enriched with cholesterol show a concentration-dependent increase in order (e.g. Garda & Brenner,

1985). These correlations are based on data 'averaged' both spectroscopically and from a population of membranes. The inference is that the fluidity of the lipids which influence functional groups in the membrane, and their chemical composition, are at least as well correlated.

An interesting parallel to the natural chemical modulation of bilayer fluidity is the artificially 'fluidized' bilayer, which mimics high temperature. Small chemically inert molecules such as anaesthetics demonstrate this, at doses well above the clinical level. Another example is the incorporation of exogenous unsaturated fatty acids (Saum *et al.*, 1981). Conversely, a cholesterol-enriched 'ordered' bilayer mimics the effects of low temperature or high pressure, and may elicit similar physiological effects. However, the point to stress is that low temperature, high pressure and chemical modulators such as cholesterol affect bilayers in similar ways, as judged by the steady state anisotropy of DPH. In its simple version homeoviscous theory does not discriminate between these variables, and it implies they are interchangeable. Therefore a reaction which is rate-limited by the order of the lipid bilayer in which it is proceeding should be similarly affected by 'equi-ordering' experimental treatments. Due allowance has to be made for secondary or unrelated effects of the treatment, but this prediction should apply to the short time scale, as in a typical electrophysiological experiment in which, say, temperature is varied. If bilayer fluidity rate-determines the measured electrophysiological parameter then the effect of increased temperature should be mimicked by an appropriate fluidizing anaesthetic. The same principle might apply on the longer time scale of adaptation in the environment. This means the homeoviscous adaptation of membranes to low temperature and to high pressure, for example, should be similar. We shall see that there is some evidence consistent with this prediction.

Proof that the theory applies to the excitable properties of membranes, and thus to the adaptation of a nervous system, requires the detailed demonstration that the composition and order of boundary lipids are regulated to sustain the appropriate kinetics in such processes as the gating of ion channels and neurotransmitter release. Such evidence will be very difficult to obtain for many excitable processes; hence the 'equi-ordering' strategy is experimentally attractive, although simplistic. The purpose of this review is to consider existing knowledge of excitable processes in the light of the theory, and in particular the value of 'equi-ordering' experiments. A progression of questions should be kept in mind. What is a plausible molecular site of the rate-determining step in a given process? Is it likely to be a lipid bilayer and if so might fluidity rate-determine the process? Is there any evidence that the process undergoes adaptation to either temperature or pressure? And if so, is the lipid bilayer involved?

Several features of the theory have to be born in mind, particularly when applying it to excitable membranes. One already mentioned is the relationship between 'averaged' spectroscopic measurements from membranes and the condition of a localized population of lipids which may rate-determine a reaction mediated by a specific membrane protein. Spectroscopic analysis of the order and motion of such annular or associated lipids has been carried out in a few cases and much of the validity of the theory rests on these. One example is adenylate cyclase in the rat liver (Houslay, 1985), whose activity increases as

the order of a spin label in the membrane decreases. An example of a membrane-bound enzyme whose activity seems not to be influenced by the fluidity of its surrounding lipids is the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase in rabbit muscle sarcoplasmic reticulum (for chemical modulators, see Lee, 1985; for temperature and pressure effects, see Macdonald, 1987). A third example, in which head group, acyl chain length and fluidity have been evaluated in model membranes, is the sugar transporter from human erythrocytes (Carruthers & Melchior, 1986). Are the channel proteins, which open and close in response to voltage or to chemical ligands, affected by their bilayer lipids? Is neurotransmitter release under the influence of the fluidity of a bilayer? And if the bilayer lipids are that influential, has this physiological significance in both the short term and in the longer time scale of adaptation?

Another feature of the theory is its homeostatic nature. Generally membranes have a heterogeneous but characteristic lipid composition, which is subject to metabolic, short-term, control. Homeoviscous theory incorporates this, and adaptations to environmental stresses are accounted for by directing the metabolic regulation appropriately. Are excitable processes affected by changes in lipid metabolism? Whether a feedback loop (implying a bilayer 'fluidity' sensor) is involved or some 'steady state', open, form of regulation occurs, is a matter for future research.

Finally, the mechanistic appeal of the theory and its apparently universal application, from bacteria to brains, should not overshadow the relatively fragmentary and indirect evidence which supports it. Nevertheless the theory provides a rationale for investigating adaptation and its application to excitable phenomena has not yet received the attention it deserves.

The Environment

Temperature. What are the extremes of temperature to which excitable membranes are adapted? The fresh water fish *Barbus thermalis* (Johnson, 1954) and several species of Ostracod (small aquatic Crustacea) experience temperatures up to 50 °C (Wickstrom & Castenholz, 1973). The hot spring crustacean *Thermobathynella adami* appears to hold the record at 55 °C (Capart, 1951). Interestingly, animals in hot deserts do not have body temperatures higher than 50 °C, for example the desert snail *Sphincterochila* (Schmidt-Nielsen *et al.*, 1972) and the lizard *Dipsosaurus dorsalis*. The latter commonly has a body temperature at 45 °C and one exceptional and apparently healthy individual was recorded with a cloacal temperature at 46.4 °C (Norris, 1953). In view of the widespread existence of more primitive organisms at much higher temperatures it is interesting to speculate that the upper temperature limit for animals is set by some property of their excitable membranes.

Low temperatures in aquatic environments are limited by the freezing point of the sea (−1.9 °C) at which temperature many animals live and are studied, notably in the Antarctic. Terrestrial animals (i.e. in air) remain active at a temperature below 0 °C (Heinrich, 1987). Many animals survive freezing in natural conditions when their excitable properties are temporarily suspended. The most spectacular are frogs which overwinter on the ground (*Hyla versicolor*, *Rana sylvatica*, *Hyla crucifer*, and *Pseudocris triseriata*; Schmidt, 1982; Storey, 1985). About a third of their body water freezes solid and

Table 1. Low temperature blockade of conduction in the superficial peroneal nerve of *Gallus domesticus* and *Larus glaucescens*, after exposure to different environmental temperatures

From Chatfield *et al.* (1953) with permission.

Temperature of water (or air) surrounding the metatarsus (°C)	Range of blockade temperatures (°C)	
	Metatarsus	Tibia
<i>Larus</i> 37	24.7–33.5	32.8–38.3
26–40	20.6–24.4	36.7–39.4
(−1 to +6)	6.1–13.3	33.3–40.9
<i>Gallus</i> (26–40)	29.4–36.1	38.3–40.4

the animals can survive −60 °C for several days. Sea shore molluscs survive −30 °C for many months on polar beaches (Kanwisher, 1966; Aarset, 1982; Murphy, 1983). Cryopreservation techniques can also take mammals (e.g. hamsters) to below −3 °C for a period of minutes, partly frozen solid, and bring them back to an apparently normal physiological state (Smith *et al.*, 1954; Andjus, 1955). More extreme exposures (−196 °C for 10 years) seems to leave isolated rat hearts capable of only weak contractions when stimulated (Sumida *et al.*, 1984).

A good example of how excitable membranes can adapt to continue functioning at low temperatures is the case of the nerve in the leg of the arctic seagull *Larus glaucescens*. In the region of the metatarsus the nerve functions at close to 0 °C in winter whereas in the tibia, where it is insulated, it is closer to the normal body temperature. Table 1 [from Chatfield *et al.* (1953)] shows how the temperatures at which conduction is blocked is related to the environmental temperature. In the hen, *Gallus domesticus*, the metatarsus remains relatively warm and the nerve has a relatively high blockade temperature. Other examples of differentially 'tuned' excitable membranes include the heat and cool sensitive neurons in the hypothalamus of thermoregulators (mammals, birds) especially in those of hibernators which down-regulate and up-regulate their body temperature over a wide range (Aloica, 1980).

It is a striking feature of homeoviscous adaptation that some of the more persuasive data come from neuronal membranes. The hypothalamus of the lizard *Calotes versicolor* contains phospholipids with a fatty acid content and, in membrane dispersions, phase transitions, which are related to the temperature at which the animals were acclimated (Durairaj & Vijayakumar, 1984). The unmyelinated olfactory nerves of the garfish, one of the most pure excitable membranes available for biochemical analysis, undergo changes in phospholipid fatty acid composition, consistent with homeoviscous theory, and so too, do the myelinated trigeminal nerves (Friedman *et al.*, 1986). On an evolutionary scale, brain synaptic membranes from animals with different body temperatures show a similarity in their order, at their respective physiological temperatures (Cossins & Prosser, 1978). Are the excitable properties of these membranes affected by these compositional and physical changes? Or are only the 'pump and leak', maintenance functions involved?

Pressure. Most living organisms experience typical atmospheric pressure but in the deep oceans and oil well fluids, high hydrostatic pressures occur. In the deep oceans, the largest living space on planet Earth, the greatest depth of 10000 m generates a pressure of 100 MPa and invertebrates live at these extremes. Fish may inhabit such depths too, but have only been recorded at somewhat shallower trench depths, 7000 m (70 MPa), (Anon, 1970). All parts of the deep oceans have a temperature below 4 °C. As an example of high pressure adaptation, consider the fish *Coryphaenoides armatus*, which is probably typical of many which inhabit the oceans over the deep abyssal plain. Its depth distribution in the Atlantic ocean is 2200–4800 m; hence it normally experiences a pressure of 22–48 MPa and an exceedingly limited temperature range (> 1 °C) at approx. 2 °C. Axons in the vagus nerve isolated from *Coryphaenoides* freshly trawled from 4000 m depth may respond to stimulation at atmospheric pressure but if they do not, they recover after a period of time at high pressure (Harper *et al.*, 1987). In either case their excitable properties improve at high pressure and appear normal at 40 MPa, their pressure of origin. In comparison, axons from the shallow water cod, or indeed from the deeper *Mora moro*, trawled from 900 m depths (9 MPa), show compound action potentials with a depressed amplitude and a delayed time to peak at 40 MPa. There is little doubt that the excitable properties of the isolated axons from *C. armatus* are adapted to the animals' normal high pressure. As with the nerves in the seagulls' leg, we may ask, are the excitable channels alone responsible for the adaptation, or is the lipid bilayer involved as well, or does the adaptation take the form of an integrated, cellular change, with no simple 'target'?

Based on the temperature equivalent of pressure ($0.13\text{--}0.21\text{ }^{\circ}\text{C}\cdot\text{MPa}^{-1}$), it was predicted that the cell membranes in general from deep sea fish should show homeoviscous adaptation to pressure (Macdonald & Cossins, 1985). Evidence consistent with the prediction has been found in fluorescence anisotropy measurements of fish brain myelin fractions labelled with DPH and in the degree of saturation of the phospholipids in liver mitochondria (Cossins & Macdonald, 1984, 1986). The fact that a deep sea bacterium likewise exhibits appropriate changes in the degree of saturation of the fatty acids incorporated in its membrane phospholipids when grown at various pressures (DeLong & Yayanos, 1985) is also consistent with prediction. These examples show that adaptation is not merely a response to temperature but to bilayer order, and are important evidence for the homeoviscous theory.

EXCITABLE MEMBRANES: THE ADAPTATION OF SYNAPSES

Introduction

Despite the challenging examples of axons mentioned in the previous section, the emphasis given here is to the role of synapses. In the adaptation of any nervous system, synapses must play a major part because they are the sensitive integrating components of the system. Attempting to apply a kinetic theory of adaptation, such as the homeoviscous theory, to synapses is no easy task. The account which follows is necessarily highly incomplete as little research has been directed to the problem. However sufficient data have been gleaned

from the literature to present at least the outline of an argument, showing that synapses do undergo adaptation to temperature and pressure, and that the role of the lipid bilayer in a number of synaptic processes may be important. Because synapses are rather complicated creatures, only pre-synaptic processes will be considered here; post-synaptic events and axonal processes will be reviewed separately. This division unfortunately cuts across the physiological process, but it does enable the sites of interest to be dealt with systematically. The three aspects of pre-synaptic physiology which will be considered are, in order of increasing complexity: (1) the spontaneous release of neurotransmitter from the quiescent synapse, (2) the evoked release of transmitter, and (3) synaptic plasticity.

Pre-synaptic processes: effects of temperature on the spontaneous release of neurotransmitter

This account assumes that the vesicle mechanism of transmitter release is the physiologically important one (Cohen & Van Der Kloot, 1983). Three types of spontaneous potentials may be detected at the post-synaptic membrane of the vertebrate neuromuscular junction, corresponding to the release of neurotransmitter in distinctive ways. The best understood potentials are the spontaneous miniature synaptic potentials, m.s.p., either excitatory or inhibitory, which typically occur at a frequency of about $1\text{--}10\text{ s}^{-1}$ at room temperature with an amplitude of about 0.5–1 mV. The other two types of spontaneous potential, less understood, are the slow miniature end plate potentials (m.e.p.p.) (Thesleff & Molgo, 1983; Lupa *et al.*, 1986) and a longer depolarization described by Katz & Miledi (1977) and by Vyskocil & Illes (1977). In vertebrate neuromuscular junctions each m.s.p. arises from the release of a single vesicle containing 5000–10000 molecules of acetylcholine, and the simultaneous opening of about 1000 acetylcholine channels (Kandel & Schwartz, 1985). The frequency of m.s.p. is a function of the pre-synaptic cell, the nerve terminals. Synapses in various invertebrates manifest similar spontaneous miniature potentials, including animals as lowly as a jellyfish (Kerfoot *et al.*, 1985).

The spontaneous release of synaptic vesicles is a form of exocytosis. In the vertebrate, m.s.p. frequency is significantly affected by temperature, hydrostatic pressure and by other factors of which $[\text{Ca}^{2+}]_i$ may be a common constituent. M.s.p. may be regarded as background, quiescent phenomena, to be contrasted with evoked post-synaptic potentials (excitatory or inhibitory), which involve a highly synchronized release of vesicles and a post-synaptic voltage change much greater in amplitude. Spontaneous and evoked potentials in the vertebrate neuromuscular junction share certain similarities, in being enhanced by increased external osmotic pressure, cardiac glycosides, noradrenaline and the ionophore nystatin (Baker, 1972).

The m.s.p. frequency is a measure of the rate of exocytosis, and accordingly an Arrhenius plot is the appropriate way to express frequency as a function of variables such as temperature or pressure. Such plots are, at the very least, an empirical way of linearizing measurements of complex processes if the rate determining mechanism remains the same, and may, in favourable cases, yield the $\Delta H^\ddagger$ or $\Delta V^\ddagger$ of the rate-determining step. The post-synaptic membrane serves merely to indicate

an event, which is counted, and it is important that the synaptic terminals in question are electrically well separated from their neighbours. Thus m.s.p. are best detected focally on a post-synaptic membrane whose response is not significantly altered by a change in the cable properties of the post-synaptic cell. Many vertebrate neuromuscular junctions are safe in this respect, whereas invertebrate junctions are not (Washio & Inouye, 1980), which probably accounts for the paucity of m.s.p. frequency data in the latter.

When an isolated synapse, and especially a neuromuscular junction, is subjected to a change in temperature in order to vary m.s.p. frequency, co-variables may arise, such as a lack of dissolved oxygen, a change in the degree of stretch, a change in the resting potential of the pre-synaptic membrane or metabolic changes, perhaps involving cyclic AMP. Each of these may affect m.s.p. frequency and thereby obscure any sought-after correlation between, in this case, pre-synaptic bilayer fluidity and m.s.p. frequency. In the data in Fig. 1 co-variables are probably unimportant. First, hypoxia at high temperature is probably absent. Second, the stretching effect on neuromuscular m.s.p. frequency does not apply to the rat diaphragm (Turkianis, 1973) and was probably a constant in the other cases. Third, $[Ca^{2+}]_o$ is more critical in the m.s.p. frequency of mammalian neuromuscular junctions than it is in frog preparations and is given in the legend. Finally, the effect of temperature on the post-synaptic resting membrane potential is small, and if the pre-synaptic membrane is similar, then m.s.p. frequency is likely to be little affected by changes in resting membrane potential (Li, 1958; Li & Gouras, 1958; Takeuchi, 1958; Hoffmann *et al.*, 1966; Ward *et al.*, 1972; Jensen, 1972; Head, 1983). This last point is a weakness here, which has to be borne in mind. However, the data summarized in Fig. 1 are reasonably coherent and broadly interpretable.

Ectotherms: amphibian and fish neuromuscular junctions. The m.s.p. frequency in the inferior oblique extraocular muscle of the Antarctic fish *Pagothenia borchgrevinki* has been studied by J. A. Macdonald & Montgomery (1982) over the range 2–13 °C. The fish normally occupies water at between –1.9 and +4 °C, and succumbs in water temperatures higher than 6 °C. The temperature coefficient of the m.s.p. frequency is clearly similar to that of frog neuromuscular junctions (Fig. 1a). The muscle membrane was reported to hyperpolarize with the increase in temperature from 0–15 °C, and if the pre-synaptic membrane responded similarly, then the observed m.s.p. frequency is probably the net outcome of opposing factors, hyperpolarization and increased temperature. The range of temperature over which vesicle release occurs spontaneously is presumably related to the animal's normal temperature and is not, for example, a peculiarity of the ocular muscle.

M.s.p. frequencies in frog neuromuscular junctions show linear, or curvilinear, Arrhenius plots over a wide temperature range, and, unlike mammalian nerve terminals, show no sign of a negative temperature coefficient in the region 30–15 °C. Fig. 1(a) shows sets of data for nerve-muscle junctions from *Rana temporaria*, and for the same muscles in the subtropical *Rana pipiens*. The data in curve 6 are from animals kept at 5 °C, and significantly Duncan & Statham (1977) noted a displacement along the temperature axis in their data,

consistent with winter frogs being cold-adapted. The curves for *R. pipiens* are based on animals kept at 10 °C for 2 weeks (curve 2) and 1 week (curve 3), with the suggestion of the latter being rather different from the former, perhaps less cold-adapted. The data show that the temperature coefficients vary little between species but properly conducted temperature acclimation experiments could well reveal a characteristic m.s.p. frequency at the animals' adapted temperature. Differences between individual muscles should also be considered. M.s.p. frequency in the cutaneous pectoris muscle of *R. pipiens* is 6-fold that in the cutaneous dorsi muscle, because the pre-synaptic sites are bigger (Banner & Herreva, 1986).

Endotherms. Mammalian m.s.p. frequencies over the range 20–38 °C are shown in Fig. 1(b). The rat phrenic nerve-diaphragm junction has been the most popular preparation. The difference between the temperature coefficients measured by Hoffman *et al.* (1966) and Hubbard *et al.* (1971) (curves 3 and 6, Fig. 1) and by most others, has no obvious explanation. Other mammalian m.s.p. frequencies show temperature coefficients similar to the majority of diaphragm preparations, e.g. the nerve muscle junction in the tenuissimus muscle of the cat (curves 7 and 8), and the intercostal muscle of the mouse (curve 11). In the lumbar motoneurons of the young rat (curve 12, Fig. 1b) inhibitory m.s.p. occur at a frequency whose temperature coefficient is similar to that of excitatory m.s.p. frequencies. In viewing the mammalian m.s.p. data three points should be added. First the absolute frequency at a given temperature can vary a great deal. For example the two sets of data for the tenuissimus muscle of the cat show frequencies at 37 °C of 1.4 s⁻¹ and 35 s⁻¹. The former (Boyd & Martin, 1956) is a mean of at least 39 muscle fibres whilst the latter (Hubbard, 1970) is based on data from a single experiment. However, the temperature coefficients are similar, and indistinguishable from the majority of the data in Fig. 1(b). The second point to make from Fig. 1(b) is the relationship of the animals' normal body temperature (37 °C) to the absolute m.s.p. frequency. The data show that at 37 °C a frequency of more than 1 s⁻¹ is usual with 10 s⁻¹ being more representative. Despite all the variables which contribute to m.s.p. frequency, the impression is that the mammalian frequency is somewhat higher than that in ectotherms at their respective body temperatures. Perhaps this reflects the higher metabolic rate of mammals. Third, m.s.p. frequency in mammals takes on a somewhat erratic relationship with temperatures below about 25 °C, implying a marked susceptibility to temperatures normal for ectotherms.

A number of measurements of m.s.p. frequency in mammalian muscles at low temperatures have been published and to avoid confusion they are plotted separately (Fig. 1c). Non-linear Arrhenius plots, as in Fig. 1(c), suggest changes in the nature of the rate-determining reaction. The most likely explanation for an increase in m.s.p. frequency at low temperature is a rise in $[Ca^{2+}]_i$, due to a loss of buffering as Ca^{2+} fluxes between organelles and the extracellular fluid are disturbed.

The questions of prime interest to this review are, what is (are) the rate determining mechanism(s) in the Arrhenius plots of m.s.p. frequency and are the properties of lipid bilayers involved? $[Ca^{2+}]_i$ is clearly a focal point here. It is largely the net result of exchange processes

Fig. 1. Miniature end plate potential frequency as a function of temperature

The external concentration of calcium and magnesium is given in parentheses. (a) Ectotherms. 1, *Pagothenia borchgrevinki*, extraocular muscle (Macdonald & Montgomery, 1982). Data for 2–12 °C; frequency decreased above 13 °C (4.05 mM-Ca²⁺, 0.79 mM-Mg²⁺). 2, *Rana pipiens*, cutaneous pectoris muscle (Barrett *et al.*, 1978) (0.6 mM-Ca²⁺, 2 mM-Mg²⁺). Autumn and winter frogs kept for 2 weeks at 10 °C. 3, *Rana pipiens*, cutaneous pectoris muscle (White, 1976) (1.0 mM-Ca²⁺). Lines of best fit reproduced. Frogs kept 1 week at 10 °C. 4, *Rana pipiens*, sartorius muscle (Li & Gouras, 1958) (1 mM-Ca²⁺). Range of frequency shown at 20–25 °C. Frogs at 25 °C for 2 weeks. 5a, *Rana temporaria*, sartorius muscle (Bevan *et al.*, 1976) (1.8 mM-Ca²⁺, with neostigmine). 5b, *Rana temporaria*, Schwan cell, 12-day denervated end-plate (Bevan *et al.*, 1976) (1.8 mM-Ca²⁺; increasing Ca²⁺ reduced frequency). 6, *Rana temporaria*, cutaneous pectoris muscle (Duncan & Statham, 1977). Data normalized to 1 s⁻¹ at 22.5 °C (1.8 mM-Ca²⁺; curve for 0.5 mM-Ca²⁺ is similar). Animals kept at 5 °C. Winter frogs showed data which was shifted 'slightly to the right' of data for summer frogs. 7, *Rana temporaria*, sartorius muscle (Fatt & Katz, 1952) (1.8 mM-Ca²⁺). Frequency at 20 °C taken to be 4.52 s⁻¹, and temperature coefficient $Q_{10} = 3.8$. 8, *Rana nigromaculata*, sartorius muscle (Takeuchi, 1958) (2 mM-Ca²⁺). Frequency at 10 °C taken to be 0.1 s⁻¹ and line plotted for a temperature coefficient $Q_{10} = 4$. (b) Endotherms. 1, rat diaphragm (Ward *et al.*, 1972) (2 mM-Ca²⁺). 2, rat diaphragm (Hubbard *et al.*, 1970) (2 mM-Ca²⁺). 3, rat diaphragm (Hoffmann *et al.*, 1966) (1.5 mM-Ca²⁺; in Ca²⁺-free, slope unchanged but frequency reduced 40%). 4, rat diaphragm (Liley, 1956) (2 mM-Ca²⁺, 1 mM-Mg²⁺). 5, rat diaphragm (Head, 1983) (2.5 mM-Ca²⁺, 1.2 mM-Mg²⁺). 6, rat diaphragm (Hubbard *et al.*, 1971) (2 mM-Ca²⁺, 15 mM-Mg²⁺). 7, cat tenuissimus (Hubbard *et al.*, 1970) (2 mM-Ca²⁺, 1 mM-Mg²⁺). Data from a single preparation. 8, cat tenuissimus muscle (Boyd & Martin, 1956) (2.4 mM-Ca²⁺, 1.1 mM-Mg²⁺). Frequency raised in presence of increased Ca²⁺. 9, mouse diaphragm (Hubbard *et al.*, 1967) (2 mM-Ca²⁺). 10, mouse diaphragm (Nishimura, 1986) (2 mM-Ca²⁺, 1 mM-Mg²⁺; slope unaffected by Ca²⁺, frequency reduced in low Ca²⁺). 11, mouse intercostal muscle (2 mM-Ca²⁺, 1 mM-Mg²⁺) (Feigen *et al.*, 1963). 12, rat (5 day) lumbar motor neuron (Kojima & Takahashi, 1985) (2 mM-Ca²⁺, 1 mM-Mg²⁺). Frequency dependent on [Ca²⁺]_o. 13, chick (*Gallus domesticus*) ciliary ganglion (Martin & Pilar, 1964) (5 mM-Ca²⁺, 2 mM-Mg²⁺). (c) Endotherms, selected multiphasic plots. The numbers relate to the curves in (b) except for 14 (rat diaphragm; Turkani, 1973).

across the plasma membrane and between intracellular Ca^{2+} -sequestering compartments. There are two main transport pathways in the first category: (i) a Ca^{2+} -ATPase pump, (ii) a Na^{+} - Ca^{2+} exchange process, sustained in the longer term by a Na^{+} + K^{+} -ATPase pump. In the second category, sequestration of Ca^{2+} involves vesicular compartments (mitochondria, endoplasmic reticulum) and free proteins in the cytosol (Henkart, 1980). A steep non-linear (co-operative) relationship between $[\text{Ca}^{2+}]_i$ and m.s.p. frequency exists (Baker, 1972; Silinsky, 1985; Cohen & Van der Kloot, 1983). Thus temperature drives a Ca^{2+} -activated process, in which bilayer lipids may well modulate ion pump activity. However it is worth examining the process more closely, because in a compound process such as exocytosis there may well be a distinctive rate-determining reaction. For example, the temperature-jump experiments of Barrett *et al.* (1978), using the *Rana pipiens* neuromuscular junction, showed m.s.p. frequency to have the same temperature coefficient as in the much slower temperature changes (as in Fig. 1a). This means the steady state regulation of $[\text{Ca}^{2+}]_i$ may be of secondary importance, and the rate-determining step lies in the vicinity of the transmitter vesicle-docking equilibrium. This is consistent with the Schwann cell m.s.p. frequency (Fig. 1a, curve 5b) which is reduced by an increase in $[\text{Ca}^{2+}]_i$ (Ito & Miledi, 1977; Bevan *et al.*, 1976). Laterally diffusing protein components such as channel formers (as in mast cell secretion; Breckenridge & Almers, 1987), or bilayer fusion (Verkleij, 1984), are each potential rate-limiters. The importance of bilayer fluidity is not at all clear, but it is difficult to imagine exocytosis proceeding totally independent of it.

The 'translation' of the Arrhenius plots along the temperature axis (Figs 1a and 1b) is not, perhaps, surprising, but nevertheless is of considerable interest. M.s.p. frequency in antarctic fish, frog and mammalian neuromuscular junctions reflect their normal environmental temperature, that is, a 're-setting' of the exocytosis process to temperatures has taken place. Thus m.s.p. frequency is an adapted feature, and although the physiological function of spontaneous quantal release is not clear (Molenaar *et al.*, 1987; Tauc, 1982), it seems inconceivable that it does not serve some useful purpose, and is subject to adaptation, in this case to temperature.

Effects of pressure and chemical modulators of fluidity on m.s.p. frequency

M.s.p. frequency in the neuromuscular junction of the frogs *R. temporaria* and *R. pipiens* at 20 °C is reversibly reduced to nearly 20% of the control rate by 10 MPa (Ashford *et al.*, 1982). A similar reduction is achieved by cooling from 22.5 to 16.5 °C, i.e. by 6 °C, or 4 °C at slightly higher temperature range (Duncan & Statham, 1977) (Fig. 1a). The general temperature-equivalent of the bilayer-ordering effect of pressure is 0.13–0.21 °C · MPa⁻¹, so clearly 10 MPa pressure (equivalent to a reduction of 1.3–2.1 °C) inhibits m.s.p. frequency rather more than might be expected from the effects of cooling. Increasing $[\text{Ca}^{2+}]_o$ from 1 mM to 10 mM accelerated the control frequency, presumably by increasing $[\text{Ca}^{2+}]_i$, and it increased the onset pressure for inhibition by some 1 MPa. It did not, however, affect the subsequent sensitivity of m.s.p. frequency to pressure. In *R. pipiens* Barrett *et al.* (1978) noted that increasing $[\text{Ca}^{2+}]_i$ reduced the temperature coefficient of the m.s.p. frequency. It

therefore appears as if applying high pressure does not mimic cooling in its effects on m.s.p. frequency, and to that extent it appears unlikely to act through a mechanism involving bilayer fluidity. However it is conceivable that should exocytosis involve a mechanism of bilayer fusion in which a non-bilayer (hexagonal H_{II}) lipid phase plays a part (Verkleij, 1984), then pressure may block fusion by opposing the lamellar- H_{II} phase transition. The dT/dP for this transition in the case of egg phosphatidylethanolamine lipids is 0.47 °C · MPa⁻¹ (Chang, 1986). Indeed in one experiment using certain model vesicles which are thought to fuse by such a lamellar- H_{II} transition, a pressure of 10 MPa significantly blocked fusion at a temperature close to the phase transition (Chang, 1986).

A requirement of the homeoviscous theory is that bilayer-fluidizing agents should mimic increased temperature. The ways in which ethanol and other fluidizers accelerate m.s.p. frequency in the frog neuromuscular junction have been discussed by Wann *et al.* (1980). The reduction in m.s.p. frequency caused by benzyl alcohol, another fluidizer, shows the system's response is far from simple and consistent. The attractive experiment of equilibrating a neuromuscular junction with cholesterol and observing m.s.p. frequency does not appear to have been carried out.

Effects of temperature on the evoked release of transmitter

Consider the consequences of cooling the giant synapse of the squid *Loligo pealei*. This synapse provides technical advantages to the experimenter and, perhaps surprisingly, is similar in a number of its properties to the frog neuromuscular junction, the other major research synapse. Both usually transmit one post-synaptic action potential per pre-synaptic potential, which is probably not the case in many central synapses. The main effect of cooling the squid synapse is the disappearance of the evoked excitatory post-synaptic potential, e.p.s.p., which initiates an action potential 'downstream' from the synapse. This marks the cessation of conduction through the synapse, but interesting events are detectable 'upstream' (Charlton & Atwood, 1979; Llinas *et al.*, 1978; Weight & Erulkar, 1976). The effect of temperature on the resting membrane potential can be disregarded, because the voltage clamp technique maintains it constant. The e.p.s.p. fails to initiate an action potential because its amplitude is diminished, largely because of the reduced amount of transmitter released. Several factors contribute to this. One is the small amplitude of the pre-synaptic action potential and the associated delayed and diminished influx of Ca^{2+} ions (I_{Ca}). Alternatively, low temperature blockade might arise from the depressed metabolism of the synapse. Adams *et al.* (1985) have shown how various metabolic inhibitors, which tended to increase $[\text{Ca}^{2+}]_i$, blocked the e.p.s.p. in the giant synapse of the squid *Alloteuthis subulata* without affecting pre- or post-synaptic resting potentials, or the pre-synaptic action potential. Increased $[\text{Ca}^{2+}]_i$ might (i) inactivate the I_{Ca} channels and/or reduce I_{Ca} by decreasing the electrochemical gradient, (ii) cause the loss of transmitter by some non-evoked leakage or (iii) desensitize exocytosis. Thus, in general synaptic blockade can arise from a loss of $[\text{Ca}^{2+}]_i$ -buffering and the adaptation of at least certain synapses to low temperature might, to

Fig. 2. Synaptic delay as a function of temperature and pressure

The reciprocal of the delay, ms^{-1} , is plotted against the reciprocal of temperature and provides a measure of the rate of exocytosis; see the text. Pressure is shown on a linear scale (inset). Curves 1, 2, 3 and 4: *Loligo pealei*, giant synapse (respectively, Charlton & Atwood, 1979; Llinas *et al.*, 1978; Lester, 1970; Henderson *et al.*, 1979); curve 5: *Rana pipiens* and *R. temporaria*, sartorius muscle (Katz & Miledi, 1965b).

a first approximation at any rate, be regarded as the adaptation of $[\text{Ca}^{2+}]_i$ -buffering (see below.)

However, synaptic blockade is a relatively crude end point and in considering the graded effects of cooling a synapse, two other criteria are used, (a) synaptic delay and (b) quantal synchrony and content. Synaptic delay has been defined as the interval between the peak (Katz & Miledi, 1965a) or the mid-point (Lester, 1970) of the repolarizing phase of the presynaptic action potential and the onset of the e.p.s.p. The main part, 80% in the case of the squid giant synapse at 18 °C, is taken up with the influx of Ca^{2+} ions (I_{Ca}), i.e. the rise in $[\text{Ca}^{2+}]_i$ and the rest comprises the Ca^{2+} -activated exocytosis, transmitter release, diffusion and the response of the post-synaptic channel. I_{Ca} might therefore be expected to be an important site of action of low temperature, which prolongs synaptic delay. In the giant (stellate) ganglion of the squid (*Loligo pealei*), I_{Ca} normally starts midway through the repolarizing phase of the action potential. It rises in a voltage-dependent manner, relatively slowly following a sigmoid time course to a plateau with little evidence of short term inactivation (Llinas *et al.*, 1981a). Despite the marked increase in $[\text{Ca}^{2+}]_i$, transmitter release is phasic (Zucker & Stockbridge, 1983). Lowering the temperature some 10 °C delays the onset of I_{Ca} slightly, it slows the kinetics of activation but reduces the amplitude only a little. The increased synaptic delay is greater than can be accounted for by these effects and so presumably it arises from a slowing of the second part of the delay (i.e. exocytosis, transmitter diffusion and binding to the receptor-channel) (Llinas *et al.*, 1978; Charlton & Atwood, 1979).

Calculations show that diffusion of the transmitter

from its point of release to the post-synaptic membrane is a relatively rapid process, and observations show that the rate of channel opening is also very rapid (both in the μs range). It therefore appears that exocytosis and not I_{Ca} is the main temperature-sensitive site in the squid synapse, and perhaps in synapses in general. Significantly, the results of temperature jump experiments with the frog cutaneous pectoris muscle are consistent with this idea (Barrett *et al.*, 1978). Using a laser pulse to warm the preparation from 2 °C to 10 °C during the first third of synaptic delay had little effect on the total delay or indeed on quantal content. Fig. 2 shows Arrhenius plots of synaptic delay, in which the reciprocal of the delay provides a measure of the rate of evoked exocytosis, according to the above argument. Note that acetylcholine and, in the case of the squid synapse, probably glutamate, are the two transmitters involved. The data are very similar and unfortunately no information on species from different temperatures is available. Attempts to formulate a kinetic scheme for exocytosis readily accommodate a temperature term; the problem is knowing whether temperature acts via bilayer fluidity, surface charge or a protein 'barrier' (Bass & Moore, 1971; Llinas *et al.*, 1981b). The important factors appear to include $[\text{Ca}^{2+}]_i$, the availability of 'docked' vesicles and sub-membranous proteins which can act as a barrier to vesicle fusion. The pre-synaptic membrane bilayer might well impose a rate-determining step and could therefore be involved in the homeoviscous adaptation of evoked transmitter release (see below).

Temperature affects the synchrony and quantal content of transmitter release. Katz & Miledi (1965b) published a histogram of synaptic unit delays recorded at the frog

Fig. 3. Quantal content, m , as a function of temperature

Calcium and magnesium concentrations are in parentheses. Curve 1: *Pagothenia borchgrevinki* extraocular muscle (Pockett & Macdonald, 1982), 10 Hz for 200 e.p.p. (4.05 mM- Ca^{2+} , 0.79 mM- Mg^{2+}); curve 2: rat diaphragm (Hubbard *et al.*, 1971), (a) in presence of curare (normal Mg^{2+}), (b) 2 mM- Ca^{2+} , 15 mM- Mg^{2+} , last 20 of 40 stimuli at 100 Hz; curve 3: rat diaphragm (Hoffmann *et al.*, 1966), last 25 of 50 stimuli at 50 Hz (1.5 mM- Ca^{2+} , 10 mM- Mg^{2+}); curve 4: *Rana pipiens* cutaneous pectoris muscle (Barrett *et al.*, 1978) (0.45 mM- Ca^{2+} , 2 mM- Mg^{2+}); curve 5: cat tenuissimus muscle (Boyd & Martin, 1956) (0.6 mM- Ca^{2+} , 3.8 mM- Mg^{2+}); curve 6: guinea pig serratus muscle (Thies, 1965), last 110 of 200 stimuli in single fibre (1.8 mM- Ca^{2+} , 8 mM- Mg^{2+}).

neuromuscular junction and emphasized the extent to which quanta became temporally dispersed at low temperature. However the 'desynchronization' is no greater than would be anticipated from the Arrhenius plot of synaptic delay (Fig. 2).

Quantal content (m) per impulse varies little with temperature. Fig 3, curve 2a, shows that in the curare-blocked rat phrenic nerve-diaphragm preparation, a decrease in temperature from the normal 37 °C causes little effect, although the Mg^{2+} -poisoned neuromuscular junction shows mostly a negative temperature coefficient (curve 2b). Curves 3 and 5 also show a negative coefficient. Over the range 6–15 °C the amount of acetylcholine per quantum is reduced some 25% (Cohen & Van Der Kloot, 1983). Even in the Antarctic fish *Pagothenia*, warming the extraocular muscle from its normal -1.9 °C has little effect on quantal content. Over the 10–20 °C range the quantal content of the *Pagothenia* muscle declines whilst that of the cutaneous pectoris muscle in *Rana pipiens* increases (in high Mg^{2+}). So, although Fig. 3 shows no particular temperature coefficients of interest, it does suggest that the evoked release mechanisms are adapted to the environmental temperature.

Temperature acclimation experiments are relatively scarce in this area, despite the attractions offered by the sartorius muscle of *Rana temporaria* in which cooling can prolong synaptic delay at least 10-fold before conduction is blocked. However, there are acclimation data showing that the blockade temperature is related to the environmental temperature. Table 2 shows that the

synapse is more sensitive than the 'upstream' motor nerve or the 'downstream' muscle membrane, and that it is indeed adapted to temperature. From the previous discussion it may be predicted that, of the main stages in synaptic transmission (pre-synaptic stimulus, exocytosis, diffusion of the transmitter and the receptor-channel response), the first two are the main sites of adaptive change to low temperature. They are not the only focus of adaptation, however, as the activity of acetylcholinesterase from fresh water fish correlates well with acclimation temperature (Kusakina, 1963).

Effect of pressure and chemical modulators of fluidity on the evoked release of transmitter

Does pressure qualitatively mimic low temperature, and if so, does it quantitatively match the 'temperature-equivalent-of-pressure' range of 0.13–0.21 °C·MPa⁻¹?

Synaptic delay in the giant synapse of *Loligo pealei* is prolonged 15% at 15 °C by 20 MPa (Henderson *et al.*, 1977), but in the neuron R15 in *Aplysia* it is not prolonged (Parmentier *et al.*, 1981). Fig. 2 (curves 1–4) shows that quantitatively the gross delay is not consistent with pressure acting in the same way as low temperature.

Generally, high pressure impairs transmitter release, but we lack the detailed analysis to make the comparison with low temperature really interesting. For example, 20 MPa applied to the neuromuscular junction in the stretcher muscle of the lobster (*Homarus americanus*) reduces the excitatory junction potential, consistent with the release of transmitter being reduced (Campanot, 1975), but the data do not distinguish between effects on

Table 2. Adaptation of the vertebrate neuromuscular junction to temperature

Temperature at which transmission in the neuromuscular junction is blocked whilst excitability in the nerve and muscle membrane remains is shown.

Animal	Temperature of exposure	Neuromuscular junction	Synaptic blockade temperature (°C)	Reference
High temperature				
Fish (<i>Raja erinacea</i>)	Natural habitat (warmer than that of <i>R. radiata</i>)	Coracomandibular muscle and hypobranchial nerve	28.3	Battle (1926)
Fish (<i>Raja radiata</i>)			26.1	
Frog (<i>Rana pipiens</i>)	7 days at 10 °C	Cutaneous pectoris	36	White (1976)
Low temperature				
Frog (<i>Rana pipiens</i>)	8 days at 25 °C	Sartorius	4 ± 0.5	Jensen (1972)
	8 days at 10 °C	Sartorius	1 ± 0.1	
Frog (<i>Rana pipiens</i>)	14 days at 25 °C	Sartorius	5	Li & Gouras (1958)

exocytosis or prior events. Quantal content was shown to be reduced 27% in the same preparation by a mere 1 MPa for 10 min (Colton & Colton, 1982). In vertebrate muscles the evoked release of acetylcholine is reduced by pressure (Kendig *et al.*, 1975, 1976). In the rat phrenic nerve diaphragm, 14 MPa inhibits release by some 70% and in guinea pig cerebral cortex synaptosomes, the release of γ -aminobutyrate (GABA) induced by depolarization with elevated $[K^+]_o$ is delayed at 6.7 MPa (Gilman *et al.*, 1986). In neuron R15 in the abdominal ganglion of *Aplysia*, varying $[Ca^{2+}]_o$ had no effect on evoked potentials, whose amplitude was reduced by pressure, which from other evidence, was clearly acting pre-synaptically. It is tempting to speculate that exocytosis is a pressure-labile site in these excitable preparations, especially as exocytosis in bovine chromaffin cells and platelets is relatively sensitive to pressure (Heinemann *et al.*, 1987; Macdonald *et al.*, 1988). As to whether pressure and low temperature share a critical site of action, and of adaptation, the question is still an open one. In contrast to the spontaneous release of transmitter in the frog muscle, which is highly sensitive to pressure, evoked release is less sensitive, and therefore closer to the temperature-equivalent-of-pressure in the ordering of lipid bilayers.

The bilayer-fluidizing effect of general anaesthetics at high dose does not seem to correlate with a uniform effect on evoked transmitter release (Gage & Hamill, 1981). The subject has been reviewed recently with the conclusion that no single target site such as the bilayer is conspicuous in pre-synaptic processes (Wann & Macdonald, 1988). The bilayer-ordering effects of, for example, cholesterol enrichment, have not been applied to synaptic preparations, and so we cannot yet compare such chemical modulators with low temperature and high pressure.

Effects of temperature on synaptic plasticity

Synaptic transmission undergoes a variety of modifications, some of which provide a basis for learned behaviour in simple animals (Klein *et al.*, 1980; Kandel,

1981). These processes are susceptible to temperature and other environmental influences, and here two examples are considered, frequency facilitation and post tetanic potentiation.

Facilitation, in which paired stimuli are applied to a synapse to elicit an enhanced second e.p.s.p., is quantified as $F = (epp_2 - epp_1)/epp_1$. The units may be in mV or in quantal content, the latter ensuring that facilitation is pre-synaptic and free of a post-synaptic component, such as a changed sensitivity to the transmitter. In the sartorius muscle from the Australian frog *Hyla aurea* kept at 14 °C, Eccles *et al.* (1941) showed that facilitation was greater at low temperature. Furthermore, the rate at which the facilitation decayed was reduced at low temperature (Fig. 4). In the sartorius muscle from the subtropical toad *Bufo marinus*, variation in temperature had no effect on the degree of facilitation but markedly affected the rate at which the facilitation decayed (Balnave & Gage, 1970). In curare-treated muscles at 20 °C the time constant of facilitation decay was 40 ms, and at 13 °C it was 100 ms. Fig. 4 shows that the temperature coefficient of the rate at which facilitation decays in these two preparations is the same. It would be interesting to compare animals acclimated to different temperatures. Because ouabain and azide had no effect on the decay, ion transport utilizing ATP appears to play no part, leaving the possibility that the facilitation is due to a persistent Ca^{2+} conductance in the pre-synaptic membrane. In this respect this example of facilitation differs from post-synaptic potentiation, which is affected by inhibitors of ion pumps.

The differential effects of temperature on evoked transmitter release and the decay of facilitation are neatly shown by the temperature jump experiments of Barrett *et al.* (1978), which have been mentioned before. Using the cutaneous pectoris muscle of *Rana pipiens*, paired stimuli separated by a few hundred ms were interposed by a temperature jump of 2–10 °C. The abrupt increase in temperature increased facilitation in this case, but the earlier the temperature jump in the inter-stimulus gap, the less the facilitation was increased.

Fig. 4. Decay of facilitation and post-tetanic potentiation as a function of temperature

Rate as the reciprocal of the time constant of decay to normal. *Facilitation* (left-hand scale, ms^{-1}): curve 1, sartorius, *Hyla aurea* (14°C) (Eccles *et al.*, 1941) paired stimuli, 5–400 ms intervals. Curare present; curve 2, sartorius, *Bufo marinus* (Belnavé & Gage, 1970), paired stimuli at 5–40 ms intervals. Curare present; curve 3, giant synapse, *Loligo pealei* (Charlton & Bittner, 1978a), reciprocal of time constant for decay, paired stimuli at 5–15 ms intervals. *Post-tetanic potentiation* (right-hand scale, min^{-1}): curve 4, frog sartorius treated with curare (Rosenthal, 1969), stimulus 62.5 s^{-1} for 20 s, thereafter test e.p.p. with single stimulus. E.p.p. potentiation continued after 'tetanus', reached same maximum at 20.2°C and 11.5°C but more slowly in the latter case. The rate of decay of the post-tetanic potentiation is plotted; curve 5, frog (*R. pipiens*, *R. temporaria*, *R. catesbiana*) sartorius muscle, glycerol treated with $[\text{Ca}^{2+}]_0$ present, stimulated 50 Hz for 1 min (Kita *et al.*, 1980); curve 6, reciprocal of time to recover to normal, cell R_{15} , abdominal ganglion of *Aplysia californica* (Schlapfer *et al.*, 1975). Stimulate 2 s^{-1} for 100 stimuli, after which test stimulate at wide intervals to determine potentiation at 12°C . The decay of the potentiation was then measured at different temperatures. Two groups of animals were used, exposed for 2 days to (a) 11°C and (b) 20°C .

This was due to the accelerated decay of facilitation. Furthermore, if the temperature jump preceded the first stimulus an enhanced e.p.s.p. occurred (due to increased quantal release), but the second e.p.p. showed no facilitation, presumably again due to its enhanced decay.

In the squid (*Loligo pealei*) giant synapse the facilitation at 20°C and 15°C is indistinguishable, but the time constant of the early decay is prolonged at the lower temperature. This decay of facilitation is about half as sensitive to temperature as that in the sartorius muscle of *Bufo marinus* (Charlton & Bittner, 1978a) (Fig. 4). The transmitter is probably glutamic acid and the mechanism underlying facilitation may well involve a calcium current, but residual $[\text{Ca}^{2+}]_i$ cannot be ruled out (Charlton & Bittner, 1978b). Facilitation in this synapse is not diminished by prolonged depolarization nor is it enhanced by hyperpolarization.

In contrast, crayfish (*Procambarus clarkii*) neuromuscular junctions appear to have a balance of properties such that pre-synaptic depolarization, which favours transmitter release, outweighs its tendency to diminish the amplitude of action potentials by inactivating I_{Na} (Wojtowicz & Atwood, 1984). Long term facilitation (8 Hz, 20 min) in the same crayfish stretcher muscle appears to involve a pre-synaptic mechanism in its response to temperature. The effect of lowering the temperature is to enhance long-term facilitation, probably by a process in which pre-synaptic $[\text{Na}^+]_i$ increases as a result of repetitive stimulation. This in turn probably

increases $[\text{Ca}^{2+}]_i$ by way of $\text{Na}^+-\text{Ca}^{2+}$ exchange and perhaps other mechanisms, thereby enhancing transmitter release. Other Ca^{2+} -independent mechanisms may also be involved. Ouabain enhances long term facilitation (Atwood *et al.*, 1975).

In one study crayfish were acclimated at 7°C and 17°C . Muscles from both groups of animals were compared at the common temperature of 10°C . In the 7°C muscles the onset of long-term facilitation was slower than in the 17°C muscles. In other words, muscles from animals acclimated at 17°C and abruptly exposed to 10°C rapidly facilitated, which tended to compensate for other immediate effects of low temperature on muscular contraction. If the fluidity of the lipid bilayer in the pre-synaptic cell is appropriately 'adjusted' to 17°C then the abrupt exposure to 10°C will cause it to be highly ordered. This in turn could well reduce the outward pumping activity at the membrane Na^+ pump, leading to a significant increase in $[\text{Na}^+]_i$, with the consequences for $[\text{Ca}^{2+}]_i$ above. This idea has a certain appeal, but it is hardly an orthodox case of homeoviscous adaptation.

Post-tetanic potentiation (the enhanced synaptic potential following a train of stimuli) in the frog sartorius muscle was first studied as a function of temperature by Rosenthal (1969). She showed: (a) the amplitude of the potentiation was unaffected by temperature and (b) the decay of potentiation was reduced at low temperature (Fig. 4, curve 4). The rat diaphragm neuromuscular junction shows the same phenomena, and Nussinovitch

& Rahamimoff (1988) have shown that $[Ca^{2+}]_i$ is a major contributing factor, but $[Na^+]_i$ also plays a part in the $[Ca^{2+}]_o$ -independent mechanism. The post tetanic decay of $[Ca^{2+}]_i$ (presumed) was followed by Kita *et al.* (1980) who used m.e.p.p. frequency as an indicator of $[Ca^{2+}]_i$. As is common to such decays, at least two exponentials were analysed, consistent with rapid and slow transport processes, with, respectively high and low temperature coefficients. The data in Fig. 4, curve 5, show the overall rate of decay.

More direct evidence that $[Ca^{2+}]_i$ mediates post-tetanic potentiation is seen in the cell L_{10} in the abdominal ganglion of *Aplysia californica*. A similar time course for the decay of both $[Ca^{2+}]_i$, measured by the Arsenazo III method, and post-tetanic potentiation, was measured by Connor *et al.* (1986). During stimulation Ca^{2+} entered the cell mainly through the voltage-gated Ca^{2+} channels. Although temperature was not varied in these experiments, Schlapfer *et al.* (1975) have investigated the temperature dependency of the post-tetanic potentiation in cell R_{15} in the same ganglion. Fig. 4 shows the rate at which potentiation decays at various temperatures, and additionally the effect of exposing the animals for 2 days to 20 °C and 11 °C. At low temperature the rate is higher in the 11 °C-exposed preparation, which is evidence for some mechanism counteracting the effect of low temperature. Note how the 20 °C-exposed preparation shows a marked non-linearity which the authors attributed to a critical change in bilayer fluidity. They supported this idea with the statement that ethanol and butanol (4 % and 0.1 %, respectively) accelerated the rate of decay.

Effects of pressure on synaptic plasticity

In the cell R_{15} in the abdominal ganglion of *Aplysia* 100 atm pressure enhances both frequency facilitation and post-tetanic potentiation by 127 % (Parmentier *et al.*, 1981). Frequency facilitation is quantified as the ratio of the amplitude of the last synaptic potential in a train to the amplitude of the first. The post-tetanic potentiation refers to the amplitude of the synaptic potential 10 s after the train of stimuli has stopped. The same pressure had no post-synaptic effects. The enhanced facilitation was accompanied by a reduction in the first e.p.s.p. in the train of impulses, exactly like the effect of the increasing $[Mg^{2+}]_o$. Transmitter release rather than mobilization within the pre-synaptic cell would therefore appear to be a likely site of action. This effect of pressure is quite unlike that of lowering temperature over the range 20–8 °C, which caused no effect on the first e.p.s.p. in a train of impulses, and a slight reduction in frequency facilitation and no effect on the degree of post-tetanic potentiation (Schlapfer *et al.*, 1975). Unfortunately, as no measurements were made of the effect of pressure on the decay of post-synaptic potentiation, no comparison can be made with the temperature coefficients which Schlapfer *et al.* (1975) measured for this process.

Facilitation and the modulation of ion channels

A special form of pre-synaptic facilitation occurs in the abdominal ganglion of *Aplysia*, in which serotonin acts on excitatory interneurons involved in withdrawal reflexes. The resultant facilitation underlies the behavioural sensitization of the reflex (Kandel & Schwartz, 1982; Shuster *et al.*, 1985). The essentials of the facilitation are as follows. Serotonin produces a surge in cyclic

AMP via the activation of the membrane-bound adenylate cyclase. A protein kinase which is dependent on cyclic AMP then phosphorylates a type of K^+ channel, causing it to close. Pre-synaptic action potentials are now prolonged, because the normal function of the K^+ channel is to open and repolarize the membrane. The effect of a prolonged action potential is to enhance I_{Ca} and hence transmitter release. This mechanism is clearly dependent on the functioning of several membrane proteins, of which adenylate cyclase, in the mammalian liver at any rate, is much influenced by the order of its annular lipids (Houslay, 1985). Serotonin binding is also influenced by membrane fluidity (Wesemann *et al.*, 1986). We may predict that this kind of channel modulation, which is widespread (Dudai, 1987; Paupardin-Tritsch *et al.*, 1986; Nairn *et al.*, 1985), will be sensitive to temperature and pressure, and will require homeoviscous adaptation for its continued operation over the range of conditions met in Nature.

CONCLUSIONS

This review is based on the premise that synapses are particularly important in the adaptation of a nervous system and its scope has been confined to pre-synaptic mechanisms.

As implied in the section in 'The Environment' above, adaptation takes two forms: (a) the tolerance of extreme conditions, when normal functions may be suspended (as in the case of frozen frogs, for example) and (b) the continued functioning in other conditions, which may vary rapidly, or seasonally or on an evolutionary time scale.

The role of the bilayer in the suspended condition (a) is presumably confined to ensuring fully reversible changes in the organization of the bilayer, avoiding for example 'protein shedding' (Deckmann *et al.*, 1985). In this the excitable components of the membranes are probably no different to the inexcitable.

In applying the homeoviscous theory of membrane adaptation to condition (b) I have examined three tiers of synaptic function; spontaneous activity, evoked activity and synaptic plasticity. In each case some evidence from the literature showed that particular pre-synaptic functions were probably adapted to the temperature which the donor animal experienced. Furthermore, although bilayer fluidity is in no case clearly established as a rate limiter or an adapted feature, its significance is ever-present.

Four roles may be proposed for the bilayer lipids in the temperature adaptation of pre-synaptic mechanisms, involving (1) ion transport mechanisms, (2) second messengers, (3) exocytosis, and (4) gated ion channels. The first is an established role in excitable membranes, but in the present context it is particularly interesting to relate bilayer fluidity to $[Ca^{2+}]_i$. The second role is the most speculative, but from the information given above it appears to be potentially very important, bearing in mind the growing knowledge of how the properties of ion channels are under metabolic influence. The third role, exocytosis, is likely to be distinctive, and there are surely possibilities for varying bilayer fluidity to examine its impact on exocytosis. The fourth role, the influence of bilayer lipids on pre-synaptic gated channels, is presumably similar to that in post-synaptic and axonal

channels and will be discussed elsewhere. In pre-synaptic processes I_{Ca} is of prime importance.

Testing homeoviscous theory in synapses is technically demanding, and the strategy of comparing the effects of temperature with those of high pressure and chemical modulators of fluidity is a somewhat despairing attempt to devise a test which can be applied to a single cell, often in a complex ganglion. Not surprisingly, perhaps, no marked similarities between the effects of pressure and low temperature emerged. This is probably to do with the sensitivity of physiological mechanisms and protein complexes to pressure (Macdonald, 1987; Low & Somero, 1975). The outcome heightens the interest in comparing, say, the adaptation of a neuromuscular junction to high pressure (and low temperature) in a deep sea fish with that in polar fish. Selectively, the use of inert chemical modulators to fluidize a membrane has yielded some provocative results (e.g. Fig. 4, curves 6a and 6b), and should be pursued.

Microscopic methods are required to provide measurements of bilayer fluidity in localized patches of membrane in synapses. Methods for isolating important constituents of synaptic membranes, such as specific calcium channels, from 'difficult' excitable tissues will be of the utmost importance in future studies of the adaptation of synapses.

Finally, it is necessary to answer the question implied in the title of this paper: does homeoviscous theory apply to synapses, and in particular, to pre-synaptic functions? The answer must be 'not proven', but the electrophysiological evidence presented in this review adds weight and specificity to the 'bulk biochemical' evidence well known in the literature. The case can only be proved, or rejected, when a sufficiently detailed analysis of adapted mechanisms can be carried out.

I thank Drs. K. T. Wann and A. A. Harper for commenting on the manuscript.

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