Synaptic Modulation by a Neuropeptide Depends on Temperature and Extracellular Calcium

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Dunn, Tyler W. and A. Joffre Mercier. Synaptic modulation by a neuropeptide depends on temperature and extracellular calcium. J Neurophysiol 89: 1807–1814, 2003; 10.1152/jn.00710.2002. The crayfish neuropeptide DRNFLRFamide increases transmitter release from synaptic terminals onto muscle cells. As temperature decreases from 20 to 8°C, the size of excitatory junctional potentials (EJPs) decreases, and the peptide becomes more effective at increasing EJP amplitude. The goal of the present study was to determine whether the enhanced effectiveness of the peptide is strictly a temperature-related effect, or whether it is related to the fact that the EJPs are smaller at low temperature, allowing a greater range for EJP amplitude to increase. Decreasing temperature reduced the number of quanta of transmitter released per nerve impulse (assessed by recording synaptic currents) and increased input resistance in muscle fibers. As in earlier work, the ability of the peptide to increase EJP amplitude was enhanced by decreasing temperature. However, the peptide was also more effective at increasing EJP amplitude when transmitter output was lowered by reducing the ratio of calcium to magnesium ions in the bath. Thus the effectiveness of the peptide may be related to the level of output from the synaptic terminals.

INTRODUCTION

Neuropeptides play important roles in mediating and modulating transmission at chemical synapses. One major “family” of neuropeptides consists of those structurally related to FMRFamide (Phe-Met-Arg-Phe-NH₂), a tetrapeptide originally isolated from a mollusk (Price and Greenberg 1977). FMRFamide-like peptides are present in every animal phylum (Greenberg and Price 1992) and have a variety of physiological effects, including modulating chemical synapses (e.g., Baux et al. 1992; Cottrell et al. 1992; Jorge-Rivera et al. 1998; Man-Son-Hing et al. 1989).

The FMRFamide-like peptide, DF₂ (DRNFLRFamide), is found in the pericardial organs of crayfish and is thought to be released into the circulation as a neurohormone (Mercier et al. 1993). This peptide increases the amplitude of excitatory junctional potentials (EJPs) in crayfish muscles (Mercier et al. 1993; Skerrett et al. 1995). This effect has been studied most extensively in deep extensor muscles (DEMs) of the crayfish abdomen. The neuropeptide increases the number of quanta of transmitter released per nerve impulse but does not alter muscle fiber input resistance or responsiveness to iontophoretic application of glutamate (Mercier et al. 2001; Skerrett et al. 1995). Thus the enhancement of EJP amplitude appears to involve presynaptic rather than postsynaptic mechanisms. Pharmacological studies suggest that the effect requires the activity of calcium/calmodulin-dependent protein kinase (Noronha and Mercier 1995), protein kinase C (Friedrich et al. 1998), and cyclic nucleotide-dependent protein kinases (Mercier et al. 2001).

One interesting aspect of DF₂’s ability to modulate transmitter release is its apparent temperature-dependence. In the crayfish DEMs, DF₂ is more effective at enhancing EJP amplitude at lower temperatures (Friedrich et al. 1994). The functional significance of this temperature dependence and the mechanisms that underlie it are not known. As temperature drops, EJP amplitude decreases in the DEMs (Friedrich et al. 1994) and in the deep abdominal flexor muscles (Czternasty and Bruner 1980). The enhanced capacity for DF₂ to increase EJP amplitude might help to compensate for reduced synaptic transmission at low temperatures.

The present work represents our first attempt to investigate mechanisms underlying the temperature-dependence of DF₂’s effect on synaptic transmission. Because lowering the temperature reduces EJP amplitude, it seems likely that the number of quanta of transmitter released also decreases under these conditions. Such a reduction in transmitter output may simply increase the range through which the peptide can increase transmitter release. The present study confirms that quantal content, a direct measure of transmitter release, decreases with temperature. It also shows that reducing the Ca²⁺/Mg²⁺ ratio, which would decrease calcium influx, enhances DF₂’s ability to modulate synaptic transmission.

METHODS

Crayfish (Procambarus clarkii), approximately 5–8 cm long, were obtained from Atchafalaya Biological Supply (Raceland, LA) and were kept in large holding tanks at 14–16°C. The freshwater in the tanks was aerated, circulated, and filtered. The crayfish were fed Tender Vittles dry cat food. Immediately before dissection, the crayfish were cooled in ice and killed by destruction of the cerebral and thoracic ganglia. The dorsal part of the abdomen, containing the DEMs (Parnas and Atwood 1966), was dissected away and secured in a 1.0-ml recording chamber that was perfused continuously with crayfish saline. Temperature was regulated by cooling the saline and monitored with a digital thermometer (±0.05°C). The crayfish saline was modified from that of Van Harreveld (1936). Most experiments were performed in a standard “low calcium, high magnesium” saline to prevent muscle twitching (Mercier and Atwood 1989). This saline had the following constituents (in mM): 200.7 NaCl, 5.36 KCl, 6.5

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CaCl₂, 12.3 MgCl₂, 5.0 HEPES; pH 7.4. In other experiments, the concentration of CaCl₂ was decreased to 4.2 mM, and the MgCl₂ concentration was raised to 14.9 mM to reduce transmitter output more drastically.

EJPs were elicited in muscle L1 of the fourth abdominal segment by stimulating excitatory axon 3 in the third abdominal segment, using methods described elsewhere (Mercier and Atwood 1989; Skerrett et al. 1995). Stimuli were applied at 0.2 Hz using a Grass S88 stimulator and a Grass SIU stimulus isolation unit. Postsynaptic potentials were recorded with glass microelectrodes filled with 3 M KCl. EJPs were monitored on a digital storage oscilloscope and were acquired on an IBM-compatible computer using a computerized data acquisition system designed and constructed by the Electronics Division at Brock University. EJPs were corrected for nonlinear summation (Martin 1955) as in previous experiments (e.g., Mercier and Atwood 1989; Skerrett et al. 1995).

Quantal synaptic currents were recorded using glass macropatch electrodes approximately 10 μm in diameter, as described elsewhere (Dudel 1982; Mercier and Atwood 1989). Electrodes were placed on the surface of the muscle fibers at locations that optimized the clarity and size of a single quantal current. Because it was impossible to count individual quanta at higher temperatures, quantal content was determined using the “failures method” described by Del Castillo and Katz (1954). According to this method, quantal content (m) can be estimated as

\[ m = \ln (N/N_0) \]

where \( N \) is the number of stimuli, and \( N_0 \) is the number of failures. The assumption underlying this method is that the frequency of quantal releases follows a Poisson distribution. This assumption is valid only when transmitter output is low. When quantal content is higher (>1.0), quantal release is fitted better by a binomial distribution, and the failures method underestimates quantal content (e.g., Johnson and Wernig 1971). For these reasons, recording sites were selected based on failure rate. In experiments where temperature was raised, sites were selected if the quantal failure rate was >70% at the low starting temperature; in experiments where temperature was lowered, sites were chosen if failure rate was <30% at the high starting temperature. Thus the temperature sensitivity of quantal content was assessed from temperatures where \( m < 1 \). Simultaneous intracellular recording in the muscle fiber allowed verification of postsynaptic response to stimulus pulses, so that a failed quantal current represents failed release at the monitored bouton and not sub-threshold stimulation of the axon. The failures method was used in preference to methods that involve comparing averaged responses to spontaneous quantas because spontaneous releases are infrequent in the DEMs and in at least some cases appear to indicate damage to the nerve terminals in this preparation. To insure that estimates of quantal content were accurate, the results from ≥50 stimuli were used.

Johnson and Wernig (1971) showed that at crayfish neuromuscular junctions, the Poisson calculation overestimates \( m \) by 4–39% when estimated values of \( p \), the release probability, are between 0.1 and 0.5. Although we sought conditions where \( p < 0.1 \), we could not estimate \( p \) reliably in the present experiments. One can approximate \( p \) from the relation

\[ m = np \]

where \( m \) is the number of quanta released per nerve impulse, and \( n \) is the number of active zones. Morphological studies indicate that crustacean synapses can have 20–100 active zones in the vicinity of a macropatch electrode of the size we used (Atwood and Tse 1988; Mghina et al. 1998). Using these estimates, a quantal content of 0.36 [which corresponds to \( \ln(N/N_0) \) with a 70% failure rate], would yield \( p \) values in the range of 0.004 to 0.02. A 70% failure rate, therefore, was considered sufficient to satisfy the criterion that \( p \) is sufficiently low to estimate \( m \) from the Poisson relation. Lower failure rates (as occurred at 17.5°C in the present study) overestimate \( m \) (see discussion).

Another way to address the question of whether the synaptic changes occur presynaptically or postsynaptically involves calculating the coefficient of variation (CV) associated with EJP fluctuations. This method does not assume that the data fit a Poisson distribution. CV is calculated from the relation

\[ CV = r_{\text{EJP}}/\text{mean EJP} \]

where \( r_{\text{EJP}} \) is the SD associated with EJP fluctuations. As \( m \) increases, CV decreases (Martin 1966). Factors that increase EJPs through presynaptic mechanisms decrease CV. Presynaptic and postsynaptic effects of an experimental treatment can be distinguished by comparing two ratios

\[ r = CV_{\text{before}}/CV_{\text{after}} \]

and

\[ \pi = M_{\text{after}}/M_{\text{before}} \]

where \( CV_{\text{before}} \) and \( CV_{\text{after}} \) are, respectively, the squares of the coefficients of variation before and after treatment, and \( M_{\text{after}} \) and \( M_{\text{before}} \) are, respectively, the mean EJP amplitudes before and after treatment (Faber and Korn 1991). A plot of \( r \) versus \( \pi \) will yield a positive slope if the treatment increases EJPs through presynaptic mechanisms. The same plot, however, will produce a horizontal line where \( r = 1 \) if EJPs increase only through postsynaptic changes (Faber and Korn 1991).

Input resistance in the muscle fibers was measured using two microelectrodes inserted approximately 50 μm apart into the same fiber. One electrode was used to inject electrical current, while the other was used to record voltage.

The temperature sensitivity of EJP amplitude, quantal content, and muscle fiber input resistance was assessed by calculating a \( Q_{10} \) value for each of these variables. \( Q_{10} \), the temperature coefficient, was calculated using the van’t Hoff equation

\[ Q_{10} = (kT_1/kT_2) = 10^{[(T_2 - T_1)/10]} \]

where \( k1 \) and \( k2 \) are mean values for a given variable at temperatures \( T1 \) and \( T2 \). Thus a \( Q_{10} \) of 1 represents no temperature sensitivity in variable \( X \), and a \( Q_{10} \) of 3 indicates a threefold increase in \( k \) with an increase in 10°C.

All values represent means ± SE, and statistical comparisons were made using a Mann-Whitney U test unless otherwise noted.

**Results**

EJP amplitude and quantal content (measured at a stimulus frequency of 0.2 Hz) were both affected by changes in the bath temperature between 8 and 20°C. Figure 1A displays a representative trial in which the temperature was reduced from 20 to 8.5°C while EJP amplitude and the quantal synaptic currents were monitored simultaneously. Quantal content (determined from the percentage of failures) and EJP amplitude both decreased as temperature decreased. The effect on quantal content was more pronounced than the effect on EJP amplitude in this example. This experiment was performed with a total of six preparations. Temperature was lowered in half the preparations and raised in the other half to ensure that the reduction in quantal content with decreased temperature was not the result of changes in muscle tonus that might move the macropatch from the original recording site. Mean EJP amplitudes,
the percentage of failures, and mean values for quantal content for all six preparations are shown in Fig. 2, A–C, respectively. As temperature decreased from 17.5 to 10°C, mean EJP amplitude decreased significantly ($P < 0.05$), the mean percentage of failures decreased significantly ($P < 0.005$), and mean quantal content decreased significantly ($P < 0.005$). The mean $Q_{10}$ value associated with changes in EJP amplitude (averaged from individual trials) was $3.46 \pm 0.52$, and the mean $Q_{10}$ for changes in quantal content was $7.62 \pm 1.49$.

The decrease in estimated quantal content suggests that the effects of temperature on EJP amplitude are mainly presynaptic, attributable to changes in transmitter output from the synaptic terminals. To obtain corroborative evidence for a presynaptic effect, the CV associated with EJP amplitudes was calculated for each of the six trials in which temperature was altered (Table 1). In each trial, CV was lower at 17.5°C than at 10°C, suggesting enhancement of transmitter output at the higher temperature. We also plotted $r$ (the ratio of CV$^2$ values) versus $\pi$ (the ratio of mean EJP amplitudes) for each of the temperature trials (see METHODS). The data from our six temperature trials (Fig. 2D) fell within an area where the slope is $>1$ (i.e., $r > \pi$). This result is predicted when EJP amplitude is enhanced by presynaptic mechanisms and not by postsynaptic mechanisms (Faber and Korn 1991).

In another set of trials (6 preparations), muscle fibers were

![FIG. 1. Reducing temperature decreased excitatory junctional potential (EJP) amplitude and quantal content, while increasing muscle fiber input resistance. A: in this representative trial, EJP amplitude (corrected for nonlinear summation) and quantal content (calculated using the failures method) increased as temperature increased. The large jump in quantal content above 17°C is a result of the overestimation of the failures method when $m > 1$ (for explanation of this, see Johnson and Wernig 1971). The inset box shows representative traces of single EJPs at 2 temperatures, 20°C (top trace) and 10°C (bottom trace). As observed in earlier work, reducing temperature increased the latency and slowed the time course of the EJP (Friedrich et al. 1994). Bars: 20 mV, 5 ms. B: muscle fiber input resistance decreased as temperature was increased. This representative trial shows the gradual decrease in input resistance to a 100-nA hyperpolarizing current pulse as temperature is increased by 12°C.

![FIG. 2. Evidence that changes in temperature alter transmitter release presynaptically. A: EJP amplitude was significantly decreased ($P < 0.05$) by a reduction in temperature from 17.5 to 10°C. The average EJP amplitude at 17.5 and 10°C was measured in 6 preparations. B: average percentage of failures at 2 temperatures in the 6 preparations used to determine quantal content. The percentage of failures was significantly lower ($P < 0.005$) at 10°C than at 17.5°C. C: quantal content, estimated from 50 stimuli using the failures method, was significantly reduced ($P < 0.005$) between 17.5 and 10°C ($n = 6$ preparations). These 2 temperatures were chosen because quantal currents were measured at these 2 temperatures in all preparations and $m < 1$. D: relationship of the ratio of the coefficients of variation squared for EJP amplitude at 2 different temperatures is plotted against the modification factor, which is the ratio of the mean EJP amplitudes at the 2 temperatures. Notice the points follow a positive slope and in all cases $r > P$.](http://jn.physiology.org/issue/issue/89/4/1809/SYNAPTIC-MODULATION-DEPENDS-ON-TEMPERATURE-AND-EXTRACELLULAR-CALCIUM.htm)
penetrated with two microelectrodes, and input resistance was measured as temperature was changed. Data from a representative trial are illustrated in Fig. 1B. As with EJP amplitude and quantal content measurements, temperature was changed in both directions, from 20 to 8°C and from 8 to 20°C. The Q10 value associated with changes in input resistance was $-1.82 \pm 0.25$; the negative value indicates that input resistance increased with decreasing temperature. Czernasty and Bruner (1980) also reported that input resistance of crayfish muscle fibers increased when temperature was lowered from 19 to 9°C, and they obtained a Q10 value equivalent to $-2.1$. The increase in input resistance with decreasing temperature probably helps to compensate somewhat for the reduction in quantal content and explains why the Q10 value for the effect on EJP amplitude is lower than that for quantal content.

As reported in Friedich et al. (1994), DF2 is more effective at increasing EJP amplitude at lower temperatures. Figure 3A shows the effect of 200 nM DF2 on deep abdominal extensor muscle L1 at two different temperatures, 12 and 20°C. (In this experiment, the Ca2+ and Mg2+ concentrations of the saline were 4.2 and 14.9 mM, respectively.) EJP amplitude increased by approximately 80% at 12°C and by approximately 30% at 20°C. Actual EJP amplitudes increased from 2.6 ± 0.5 to 4.2 ± 0.7 mV at 12°C and from 9.6 ± 3.5 to 15.5 ± 6.3 mV at 20°C. The EJP amplitudes observed in the presence of DF2 at 12°C were significantly lower ($P < 0.05$) than the EJP amplitudes prior to peptide application at 20°C. The initial rate of increase in EJP amplitude appeared to be similar at both temperatures, which is surprising because decreasing temperature generally slows the rate of biochemical and physiological processes. However, the maximal increase in EJP amplitude occurred slightly earlier at 20°C (7.5 min) than at 12°C (9.5 min), and the effect of the peptide appeared to decline slightly at 20°C but not at 12°C. A decline in the effect of DF2 on EJPs has been observed previously at 12°C, but only after approximately 15 min (Noronha and Mercier 1995). The mechanisms underlying this decline are not known, but the data suggest that raising the temperature from 12 to 20°C speeds up some of the subcellular processes activated by DF2.

To further examine the possibility that the enhanced effect of the peptide at low temperatures is directly related to reduced transmitter output, EJPs were raised slightly by increasing the ratio of Ca2+ to Mg2+ ions in the saline. A saline containing 6.5 mM Ca2+ and 12.3 mM Mg2+ was used in these experiments (which were performed at 12°C). Raising the Ca2+/Mg2+ ratio in this manner increased the initial EJP amplitude and reduced the effectiveness of the peptide (expressed as the percentage change in EJP amplitude). The mean EJP amplitude during the 8-min period immediately prior to peptide application was 6.3 ± 1.5 mV in 6.5 mM Ca2+/12.3 mM Mg2+. This was significantly larger than the mean EJP amplitude over the same time period in 4.2 mM Ca2+/14.9 mM Mg2+ (2.6 ± 0.5 mV; $P < 0.05$). Following application of DF2 in the higher Ca2+ saline, EJPs increased by approximately 30% (Fig. 3B) to an average value of 8.1 ± 2.0 mV. A comparison of data from the two saline solutions indicates that increasing the extracellular Ca2+/Mg2+ ratio increases the initial EJP amplitude and reduces the response to the peptide.

The effects of DF2 at the two different temperatures and Ca2+/Mg2+ ratios are summarized in Fig. 4A. The percentage increase in EJP amplitude was calculated by comparing EJPs 5–8 min after applying DF2 with EJP amplitudes averaged over the 3-min period immediately before peptide application. Reducing temperature and reducing the Ca2+/Mg2+ ratio have similar effects on the ability of DF2 to enhance transmitter output. Decreasing temperature and decreasing the Ca2+/Mg2+ ratio both significantly ($P < 0.05$) enhance the percentage increase in EJP amplitude elicited by DF2. DF2 elicited the largest percentage increase in EJP amplitude in the lower calcium saline at the lower temperature, when the two factors were combined.

The similarity between the effects of changing temperature and Ca2+/Mg2+ levels suggests that the effectiveness of DF2 is inversely related to initial EJP amplitude. To test this idea, data from both experiments were combined and plotted in Fig. 4B. There was a significant, negative correlation between the percentage increase in EJP amplitude elicited by DF2 and the

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TABLE 1. The coefficient of variation for EJP amplitude is shown for each trial at the two temperatures

<table>
<thead>
<tr>
<th>Trial</th>
<th>17.5°C</th>
<th>10°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.677</td>
<td>20.929</td>
</tr>
<tr>
<td>2</td>
<td>6.703</td>
<td>10.084</td>
</tr>
<tr>
<td>3</td>
<td>6.819</td>
<td>18.332</td>
</tr>
<tr>
<td>4</td>
<td>3.777</td>
<td>5.808</td>
</tr>
<tr>
<td>5</td>
<td>2.803</td>
<td>7.606</td>
</tr>
<tr>
<td>6</td>
<td>17.933</td>
<td>44.202</td>
</tr>
</tbody>
</table>

In all cases the CV is larger at the lower temperature. EJP, excitatory junctional potential.
The data suggest that the effectiveness of the peptide is inversely related to the log of the initial EJP amplitude.

An alternative method for reducing the initial EJP amplitude was explored. In some crustacean muscles, stimulating the axon repeatedly at low frequencies (e.g., 0.2 Hz or lower) causes a reduction in EJP amplitude, referred to as low-frequency depression (Bruner and Kennedy 1970; Bryan and Atwood 1981; Zucker and Bruner 1977). Such low-frequency depression is thought to be presynaptic in origin, resulting from a gradual reduction in transmitter output. Two sets of trials were conducted at room temperature to determine whether this method of reducing transmitter release would also enhance the ability of DF2 to increase transmitter output. In one set of preparations, the nerve was stimulated for approximately 30 min before DF2 was applied. This decreased EJP amplitude from 11.1 ± 1.7 to 5.9 ± 0.9 mV before peptide application. In a separate set of preparations, the nerve was stimulated for only 8 min before applying the peptide, causing very little low-frequency depression. (In this case, the EJPs decreased from 9.8 ± 2.0 to 9.1 ± 0.4 mV.) The initial EJP amplitudes at the beginning of each trial were not significantly different between the two sets of trials (P > 0.05). Representative examples of both types of trial are shown in Fig. 5, A and B. Figure 5A shows actual changes in EJP amplitude (in mV), and Fig. 5B shows the effectiveness of DF2, expressed as the percentage change in EJP compared with the average amplitude recorded 3 min before peptide application. In the example shown in Fig. 5A, the peptide increased EJP amplitude from approximately 17 mV to approximately 24 mV when there was little or no low-frequency depression and from approximately 8 mV to approximately 12 mV after substantial low-frequency depression. The percentage increase in EJP amplitude between the two trials was comparable (Fig. 5B). Data from six trials in each group were combined (Fig. 5C). The percentage increase

![Figure 4](http://jn.physiology.org/)

**Fig. 4.** Reducing temperature and extracellular calcium levels both lead to greater modulation by DF2. A: mean values of the increase in EJP amplitude measured between 5 and 8 min after peptide application. Amplitudes in this period are expressed as the percentage change from the average EJP amplitude during the prepeptide period. The trials at low temperature and in the low calcium saline are significantly different from all the other trials. (Error bars depict SE; n = 8, 7, 6, 7 from left to right.) B: initial EJP amplitude is inversely related to the effectiveness of DF2 to increase transmitter release. The initial EJP amplitude of each trial was plotted with the percentage change (increase) in EJP amplitude to 200 nM DF2 as compared with the prepeptide period. This trend is significant (r = −0.417; n = 32; P < 0.05), and the data are fit best by the complex function, y = −0.1393 ln (x) + 0.5984.

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![Figure 5](http://jn.physiology.org/)

**Fig. 5.** Tonic stimulation gradually reduces EJP amplitude but does not alter the percentage increase in EJP amplitude caused by DF2. A: in this figure the y-axis intersects at the point of peptide application in both trials; this is done to compare the effect of the peptide between the 2 trials. Both of these representative trials begin at similar EJP amplitudes and both are clearly modulated by DF2. One trial exhibits a 9-mV reduction in EJP amplitude prior to DF2 application. B: when the same trials as in Fig. 5A are compared by expressing EJP amplitude as a percentage change of the average EJP amplitude between 0 and 3 min before peptide application, there is no apparent difference in the peptide’s effects. C: 2 groups have no significant difference (P > 0.05) in the percentage increase in EJP amplitude 5–8 min after DF2 application as compared with the 3-min control period (n = 6 for each group and the bath temperature was 22°C for all trials).


in EJP amplitude 5–8 min after peptide application was not significantly different between the two groups (P > 0.05).

DISCUSSION

These experiments confirm earlier observations that DF2 increases EJP amplitude more effectively (when expressed as percentage change) at low temperature (Friedrich et al. 1994). These experiments also confirm that decreasing temperature reduces EJP amplitude, and they provide direct evidence that this is caused by a presynaptic reduction in transmitter release. An increase in input resistance compensates somewhat for reduced quantal content at low temperatures, but EJP amplitude remains highly temperature dependent (Fig. 1). This suggests that one of the physiological roles of the neuropeptide DF2 may be to compensate for reduced transmitter output when temperature is reduced. This idea is supported by observations that the pericardial organs of spiny lobsters, which contain and release peptides homologous to DF2, fire impulses when temperature is reduced from 20 to 14°C (Kuramoto and Tani 1994).

The reduction in quantal content with temperature is probably the result of reduced calcium influx through voltage-gated calcium channels in the synaptic terminals. The temperature coefficient quantifies the sensitivity of an event to a change in temperature of 10°C. Biochemical events involving changes in covalent bonding have Q10 values generally between 2 and 3, as do many physiological processes (Purves et al. 1995). The method used for estimating m may contribute somewhat to the high mean value (Q10 > 7.0) and large variance in the Q10 for quantal content. The failures method tends to overestimate quantal content when release levels are high and the data do not fit a Poisson distribution. This would tend to make estimated values of m excessively high only at the higher temperatures (e.g., at temperatures above 17°C in Fig. 1A). Such an effect would exaggerate the changes caused by temperature and probably contributes to the high Q10 values for m and for the high variance associated with them. However, even at 17.5°C, values of m approached 1.0 but did not exceed it. Using data from studies where m was estimated by directly counting quanta (Johnson and Wernig 1971; Mercier and Atwood 1989; Zucker 1993), ln (N/N0) overestimates m by 5–25% when ln (N/N0) is between 0.7 and 1.0. Even a 25% overestimate of m cannot account for the 200% increase in m that we observe when temperature increases from 10 to 17.5°C (Fig. 2C). Thus it is unlikely that the high Q10 values are wholly the result of overestimating m. In fact, since input resistance increased with a drop in temperature, the effect of temperature on m should be more pronounced than for EJP amplitude.

Calcium passage through the pore-forming region of an open calcium channel has little temperature sensitivity (Q10 of roughly 1.5; Klockner et al. 1990), as would be expected for diffusion through an aqueous medium (Hille 1991). However, Charlton and Arwood (1979) reported a large reduction in excitatory postsynaptic potential (EPSP) amplitude at the squid giant synapse that corresponded to a reduction in the presynaptic calcium current. The temperature-sensitivity of the calcium currents appears to be due to temperature-dependent changes in the calcium channels. The major temperature-sensitive components are not yet fully understood, but probably involve interaction between channel subunits, interaction between calcium channels and SNARE proteins, and phosphorylation state of the calcium channels (Allen 1996; Allen and Mikala 1998; Bunemann et al. 1999; Wiser et al. 1996, 1997). Some of the kinetic parameters of calcium channels have large Q10s, well above 3 (Allen 1996; McAllister-Williams and Kelly 1995; McNaughton et al. 1998), suggesting that the high temperature-sensitivity of calcium channels results from the coupling of multiple metabolic events (Morris and Clarke 1981). Thus the high Q10 values for quantal content in the present study probably reflect high temperature-sensitivity of the calcium channels to some extent.

Quantal content (m) is determined by two variables (Del Castillo and Katz 1954): n, the average number of synaptic vesicles ready for release; and p, the average probability of a single quantum being released (m = np). Thus DF2 could potentially increase transmitter release either by increasing p or by increasing n. An increase in p would be brought about by increasing calcium influx into the synaptic terminals, by releasing calcium from intracellular stores, or by increasing the sensitivity of the secretory apparatus to intracellular calcium. An increase in n would be brought about by increasing the number of vesicles that are docked at active zones.

It is well established that decreasing the extracellular Ca2+ level reduces quantal content and EJP amplitude by reducing calcium influx (e.g., Augustine and Charlton 1986; Dodge and Rahamimoff 1967; Dudel 1981). The similarities between the effects of reducing temperature and reducing extracellular calcium suggest that these two treatments enhance the ability of DF2 to increase transmitter release via a common mechanism. Since both these treatments are likely to reduce quantal content by lowering calcium influx, it seems likely that the enhanced effectiveness of the peptide is related to a reduction in calcium influx and, thus, to a reduction in binomial variable p. Reducing the extracellular Ca2+ level has been shown to reduce p without affecting n at crayfish neuromuscular junctions (Dudel 1981). The ability of DF2 to enhance transmitter output from the fast closer excitator axon of crabs requires N-type calcium channels (Rathmayer et al. 2002). This suggests that DF2 acts by increasing calcium influx, which would increase binomial variable p. The present data suggest that if p is reduced at low-temperature or low-extracellular Ca2+ levels, the scope for modulation by the neuropeptide may be enhanced. Experiments involving calcium imaging in nerve terminals would help to determine whether or not DF2 acts on calcium influx.

In this and a previous report (Friedrich et al. 1994), the effectiveness of DF2 in modulating chemical synapses has been expressed as the percentage change in EJP values, rather than in the actual change in millivolts. This was done primarily to minimize the effect of variation in EJP amplitudes between different preparations. However, it is worth noting that actual EJP amplitudes after peptide application at 12°C were still lower than those recorded at 20°C before peptide application. Thus although the percentage change in EJP amplitude induced by the peptide is greater at the lower temperature, the actual EJP amplitudes do not even approach those at the higher temperature with no peptide. Thus it seems unlikely that reducing temperature simply leaves a greater supply of transmitter quanta and that the peptide is less effective at the higher temperature because there are smaller reserves of quanta available for release.

To further investigate the effect of lowering transmitter
release on effectiveness of the peptide, the EJP amplitude was lowered through low-frequency depression. Tonic low-frequency stimulation (0.2 Hz) of crustacean phasic excitors often leads to a reduction in EJP amplitude over time (Bruner and Kennedy 1970; Lnenicka and Atwood 1985; Palahipil et al. 1986; Zucker and Bruner 1977). Although initial EJP amplitude was significantly reduced by low frequency depression, the magnitude of the modulation by DF2 was unaffected. Thus the mechanisms through which low-frequency depression reduces transmitter output may differ from those underlying the effects of reducing temperature and reducing extracellular calcium. The mechanisms underlying low-frequency depression are not well understood, but appear to involve the generation of nitric oxide (Aonuma et al. 2000). Treatments that reduce nitric oxide are not well understood, but appear to involve the generation of nitric oxide (Aonuma et al. 2000). Treatments that reduce calcium in the bath (Bryan and Atwood 1981), drastically reduce transmitter release without affecting low-frequency depression. Such observations suggest that this form of depression does not result from a reduction in calcium influx.

In summary, reducing extracellular calcium level and reducing the temperature both enhance the ability of neuropeptide DF2 to increase transmitter release. The similarity of the effects of these two experimental treatments suggests common underlying mechanisms related to changes in calcium influx. The results also suggest that the mechanisms through which low-frequency depression reduces transmitter output differ from those underlying reductions in temperature or extracellular calcium. Taken together, the present data and those of Rathmayer et al. (2002) suggest that presynaptic calcium channels are involved in the modulatory effect of DF2. The functional significance of the sensitivity and calcium sensitivity of the peptide’s effects are still to be determined.

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