Temperature Dependence of Action Potential Parameters in *Aplysia* Neurons

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**Introduction**

Temperature is one of the most important stimuli and produces numerous effects in most organisms. The effects of temperature change on the activities of neurons and axons have been studied in various species, including *Aplysia*, squids, snails, lobsters, crabs, crayfish, locusts, and frogs [1–8].

The large, well-studied neurons of *Aplysia* are suitable for studying the physiological effect of temperature because these neurons are long-lived in vitro compared to mammalian neural cells and so are apt to withstand heat stress for relatively long periods. Because of this, it is possible to obtain long-term intracellular recordings. The effects of heat stress on the neuronal activity of *Aplysia* have been observed as changes in the signal transduction pathways, synaptic physiology, muscle contractility, behavior, and neuronal firing patterns [9–13]. A regular and reproducible increase in the generation frequency of pacemaker potentials in *Aplysia* neurons between 5 and 25 °C was found to be caused by an increase in temperature [14]. A temperature change from 10 to 20 °C produced a noticeable hyperpolarization in the membrane of the right giant cell in the abdominal ganglion of *Aplysia californica* [15]. In other studies, the resistance increased with decreasing temperatures [1]; however, the burst duration (BD), spike height, and number of spikes per burst of the *Aplysia* R15
cell all decreased with an increase in temperature from 23 to 37°C [16]. Hakozaki et al. [17] examined the temperature-induced responses of the cells in an Aplysia ganglion, and their results suggested that a GTP-binding protein should be activated by raising the temperature to open the K+ channels; however, few recent studies have reported any effect of temperature on the electrical activity of the Aplysia neurons.

Experiments involving action potential (AP) parameters using Aplysia neurons are found in papers published more than a decade ago, when there were few personal computer systems capable of calculating and saving large data files. This meant that it was difficult to determine the temperature dependence of AP parameters because of the slow acquisition rate when collecting data on a computer at the resolution shown on an oscilloscope. In the present study, we performed such an experiment using Aplysia neural cells in vitro over a long time and sought to determine the function describing the temperature dependence of the AP parameters with a high acquisition rate.

Although it is known that some AP parameters are altered with temperature for neurons within the Aplysia abdominal ganglion, it has not yet been verified whether the temperature dependence of these AP parameters is reproducible. Therefore, our aim was to determine which AP parameters for Aplysia neurons have reproducible temperature dependences. Because this experiment allowed recording over a relatively long period, we could include changes in temperature while monitoring individual neurons. We analyzed the recorded data using a computer program to obtain the quantitative temperature dependences of 14 AP parameters, which are defined in the next section.

**Materials and Methods**

**Animals**

The animals (*A. juliana*) were collected locally in Jeju, South Korea, and, prior to use, they were maintained in a recirculating sea water bath at 17°C. *A. juliana* has an appearance similar to that of *A. californica* in its body color patterns. However, the abdominal ganglion of *A. juliana* deviates in its overall structure from the prototypic ganglia of *A. californica* and *A. kurodai* [18].

**Dissection**

Before dissection, isotonic MgCl₂ amounting to half of each *A. juliana* specimen’s weight was injected into it as an anesthetic. Abdominal ganglia were then removed from the specimens and from the prototypic ganglia of *juliana*.

**Parameters**

**Temperature Dependence of AP Parameters**

**Parameters for** *Aplysia* neurons have reproducible temperature dependences of 14 AP parameters, which are defined in the next section.

**Data Acquisition**

Each abdominal ganglion was immersed in an L-15:ASW (1:1, v/v) medium, and the Petri dish with the abdominal ganglion was placed on a small copper plate (50 × 100 mm), under which two thermostatic coolers were set up side-by-side (HMM 3940; Acetec Co., Korea), one with the hot side facing up and one with the cold side facing up. The bath temperature was increased or decreased using a temperature controller system connected to the thermoelectric module. A PT100 platinum resistance temperature sensor was connected to a digital thermometer (TRM-006; Toho, Japan) to monitor the temperature. Intracellular recordings were obtained to measure the membrane potential. A line was connected to an electrode, which was inserted into a cell in the abdominal ganglion of *A. juliana* to obtain the values of the membrane potential, and was linked to channel 2. The PT100 temperature probe was placed near the abdominal ganglion. The membrane potential was measured using a Neuro Probe Amplifier (Model 1600; A-M Systems, Carlsborg, Wash., USA) with a microelectrode filled with 3 M KCl. Data were acquired using an A/D board (NI PCI-6221; National Instruments). The electrical signals of the APs were identified using a digital oscilloscope (54622A; Agilent, Colorado Springs, Colo., USA). Because we found that the beating or bursting cells generated APs at a rate of 0–4 Hz, it was necessary to record the data onto a computer hard disk at a rate of 3 kHz to clearly reproduce the APs. Thus, 180,000 pairs of temperature and AP values were automatically saved on the hard disk per minute, and each minute of data was stored under an independent file name over 60 h of recording, which amounted to approximately 20 GB of data. During this long, continuous recording period (~60 h), we inevitably encountered electrode slips and other problems. However, after reintroducing the electrode, we only continued the recording of cell data when the resting potential returned to its value (within ±2 mV) before the recording interruption. Otherwise, we stopped recording and did not include the data for further analysis.

**Definitions of AP Parameters**

The graph in figure 1A represents the AP, V(t). As shown in figure 1A, the positive peak membrane potentials $V_{np,i-1}$ and $V_{pp,i}$ have approximately the same value of +30 mV at the two positive peak points $P_{pp,i-1}$ and $P_{pp,i}$, at the times $t_{pp,i-1}$ and $t_{pp,i}$, respectively. The negative peak membrane potentials $V_{np,i}$ and $V_{np,i+1}$ have nearly the same value of −45 mV at the times $t_{np,i}$ and $t_{np,i+1}$ at the negative peak points $P_{np,i}$ and $P_{np,i+1}$, respectively. The graph in figure 1B represents the waveforms of the differentiated signals (DSs), which represent the digital differentiation of the membrane potential shown in figure 1A with respect to time. The digital differentiation has a maximum AP slope at the end of the temperature rising phase, MSR, at the positive peak point $Q_{D_{np},i}$ at the times $t_{D_{np},i}$. The digital differentiation also has an absolute maximum AP slope during the temperature falling
and the half point of the potential difference, $t_{hwr}, i$, in the falling caudal quarter-ganglion are defined as follows.

The interspike interval, ISI (ms), is defined as the mean interval (ID), ISI, of the neural spike trains, from $t_{pp}, i$ to $t_{np}, i$ [fig. 1A, (2)] $\text{ISI} = \frac{\sum \text{ISI}_i}{N} = \frac{\sum |t_{pp}, i - t_{np}, i|}{N}$. The first half of the temperature rising phase, $\Delta t_{r1}$ (ms), is defined as the mean interval, $\Delta t_{r1}, i$, between $t_{np}, i$ and $t_{pp}, i$ [fig. 1A, (4)] $\text{ISI} = \frac{\sum \text{ISI}_i}{N} = \frac{\sum |t_{pp}, i - t_{np}, i|}{N}$. The last half of the rising phase, $\Delta t_{r2}, i$, between $t_{pp}, i$ and $t_{np}, i$ [fig. 1A, (4)]

Fig. 1. Definitions of AP components. A The membrane potential vs. time for a neuron firing spontaneously. $P_{pp}, i$ and $P_{pp}, i$ designate arbitrary peak points and $t_{pp}, i$ and $t_{pp}, i$ are the times corresponding to those points. A positive peak point occurs at the maximum point on the upper part of an AP. A negative peak point occurs at the minimum point on the lower part of an AP. The electrophysiological parameters measured include the potential difference $[A (1): A_{AP}, i]$; the duration between the two points $P_{pp}, i$ and $P_{pp}, i$ [A (2): ISI], the time interval between the two points of a negative peak, $t_{np}, i$ and the half point of potential difference, $t_{hwl}, i$ during the rising phases of temperature change $[A (3): \Delta t_{r1}, i]$; the time interval between the half point of potential difference, $t_{hwl}, i$ and the point of a positive peak, $t_{pp}, i$ [A (4) in inset: $\Delta t_{r2}, i$]; the time duration between the two points of a positive peak, $t_{pp}, i$ and the half point of the potential difference, $t_{hwr}, i$ in the falling phase $[A (5) in inset: \Delta t_{f1}, i]$; the time duration between the half point of potential difference, $t_{hwr}, i$ and the point of the negative peak, $t_{pp}, i+1$ [A (6): $\Delta t_{f2}, i$], and the time duration between the two points, $t_{hwl}, i$ and $t_{hwr}, i$ at the half amplitude membrane potential $[A (7) in inset: \Delta t_{AP}, i/2, i]$. The electrophysiological parameters calculated include the duration between the two points, $t_{DSwl, i}$ and $t_{DSwr, i}$ at the half amplitude DS $[B (1): \Delta t_{DS}, i/2, i]$, maximum slope of action potentials during the rising phases of temperature change at the positive peak point, $Q_{DSSpp, i}$ and peak-to-peak amplitudes of DS $[B (2): A_{DS}, i]$. C The membrane potential vs. time for a neuron repeatedly firing discrete bursts of spikes $[C (1): BD]$, followed by a quiescent period $[C (4): IB]$, before the next burst occurs. The electrophysiological parameters measured include the ISI in intraburst spikes $[C (2): BI]$, the last ID of the spike train during a burst $[C (3): BI_{last}, i]$, and the first ID of the spike train during a burst $[C (5): BI_{first}, i]$.
\( \Delta t_{12} = \sum \Delta t_{i,1}/N = \sum |t_{pp,1} - t_{bwl,1}|/N \). The first half of the temperature falling phase, \( \Delta t_{i1} (ms) \), is defined as the mean value of time interval, \( \Delta t_{i1} \), from \( t_{pp,1} \) to \( t_{bwl,1} \) [fig. 1A, (5)] \( \Delta t_{i1} = \sum \Delta t_{i1}/N = \sum |t_{bwl,1} - t_{pp,1}|/N \). The last half of the falling phase, \( \Delta t_{i2} (ms) \), is defined as the mean value of time interval \( \Delta t_{i2} \), between \( t_{bwl,1} \) and \( t_{pp,1} + 1 \) [fig. 1A, (6)] \( \Delta t_{i2} = \sum \Delta t_{i2}/N = \sum |t_{pp,1} + 1 - t_{bwl,1}|/N \). The duration of the temperature rising phase, \( \Delta t_{i} (ms) \), is defined as the mean time interval from \( t_{pp,1} \) to \( t_{pp,1} + 1 \) [fig. 1A, (1)] \( \Delta t_{i} = \sum |t_{pp,1} - t_{pp,1}|/N = \Delta t_{i1} + \Delta t_{i2} \). The duration of the temperature falling phase, \( \Delta t_{i} (ms) \), is defined as the mean value of time interval from \( t_{pp,1} \) to \( t_{pp,1} + 1 \) [fig. 1A, (1)] \( \Delta t_{i1} = \sum \Delta t_{i1}/N = \sum |t_{pp,1} - t_{pp,1}|/N \). The frequency, \( fi \), is defined as the mean minimum value of digital differentiation, \( \Delta V/\Delta t \), where \( \Delta V/\Delta t \) is the maximum slope of the AP during the temperature rising phase, \( \Delta t_{i} (ms) \), is defined as the mean maximum value of digital differentiation, \( \Delta V/\Delta t = \left( MSR^i \right) \). The minimum slope of the AP during the temperature rising phase, MSF \( (Vs^{-1}) \), is defined as the mean minimum value of digital differentiation, \( \Delta V/\Delta t = \left( MSF^i \right) \). The last half of the falling phase, \( \Delta t_{i2} (ms) \), is defined as the mean half-width duration, \( \Delta AP_{1/2,1} \), between \( t_{bwl,1} \) and \( t_{pp,1} + 1 \) [fig. 1A, (7)] \( \Delta AP_{1/2,1} = \sum \Delta AP_{1/2,1}/N = \sum |t_{pp,1} + 1 - t_{bwl,1}|/N \). The spontaneous firing frequency, simply referred to as Frequency \( (s^{-1}) \), is defined as the inverse of the ISI, i.e., Frequency \( = 1/\text{ISI} \), where \( \text{ISI} = \Delta t_{i1} = \Delta t_{i2} = \Delta t_{i1} + \Delta t_{i2} + \Delta t_{i1} + \Delta t_{i2} \).

The maximum slope of the AP during the temperature rising phase, MSR \( (Vs^{-1}) \), is defined as the mean maximum value of digital differentiation, \( \Delta V/\Delta t \), at the points of the positive peaks, \( Q_{DSP}^i \) (fig. 1B) MSR \( \equiv \sum MSR /N \). The minimum slope of the AP during the temperature falling phase, MSF \( (Vs^{-1}) \), is defined as the mean minimum value of digital differentiation, \( \Delta V/\Delta t \), at the points of negative peaks, \( Q_{DSP}^i \) (fig. 1B) MSF \( \equiv \sum MSF /N \). The DS half-width duration, \( \Delta DS_{1/2,1} (ms) \), is defined as the mean half-width duration, \( \Delta DS_{1/2,1} (ms) \), from \( t_{pp,1} \) to \( t_{pp,1} + 1 \) [fig. 1B, (2)] \( \Delta DS_{1/2,1} = \sum \Delta DS_{1/2,1}/N = \sum |t_{pp,1} - t_{pp,1}|/N \). The DS amplitude, \( AP_{1/2} (Vs^{-1}) \), is defined as the mean peak-to-peak amplitude of the DSs, \( A_{DS} \). [fig. 1B, (1), (2)] \( A_{DS} = \sum A_{DS}/N = \sum |MSR - MSF|/N \). The burst duration (BD) \( (ms) \) is the mean duration of each burst, BD [fig. 1C, (1)] \( BD = \sum BD /N \). Such bursts are followed by a quiescent period before the next burst occurs. The intraburst ISI, BI (ms), is defined as the mean ISI within bursts, \( BI \), between \( t_{i-2} \) and \( t_{i-1} \) [fig. 1C, (2)] \( BI = \sum BI /N = \sum |t_{i-1} - t_{i-2}|/N \). The last ID of the spike train during the burst, \( BI \), is defined as the mean last ID within bursts, \( BI \), between \( t_{i-1} \) and \( t_{i} \) [fig. 1C, (3)] \( BI = \sum BI_{i-1} /N = \sum |t_{i-1} - t_{i-1}|/N \). The first ID of the spike train during the burst, \( BI_{i-1} \), is defined as the mean first ID within bursts, \( BI_{i-1} \), between \( t_{i-1} \) and \( t_{i} \) [fig. 1C, (5)] \( BI_{i-1} = \sum BI_{i-1}/N = \sum |t_{i-1} - t_{i-1}|/N \). The interburst interval, IBI (ms), is defined as the mean time interval between two groups of spikes, IBI, that are at least twice as far apart in time as \( BI_{i-1} \) and \( BI_{i-1} \) [fig. 1C, (4)] \( IBI = \sum IBI /N = \sum |t_{i-1} - t_{i-1}|/N \), on the condition that \( IBI > 2 \cdot BI_{i-1} \) and \( IBI > 2 \cdot BI_{i-1} \).

**Computation Analysis of AP Components**

The relationship between the current, I, flowing across the patch of membrane, the capacitive current, \( I_C \), and the ionic current, \( I_I \), is simple: I is the sum of \( I_C \) and \( I_I \), i.e.

\[
I = I_C + I_I
\]

where I is the stimulus current that initiated the action potential by depolarizing the cell above the threshold. When we performed experiments using Aplysia neurons, no external current was applied to generate APs. Therefore, the stimulus current was zero during the AP. Therefore,

\[
I = I_C + I = C \frac{dV}{dt} + I = 0, \quad (2)
\]

where \( C \) is the capacitance of the system. Thus, we find

\[
I = -I_C = -C \frac{dV}{dt}, \quad (3)
\]

for nonpropagating APs [19]. The capacitance of a pair of plates of width \( A \) and length difference \( d \), with a dielectric between the plates, is given by

\[
C = \frac{\varepsilon A}{d}, \quad (4)
\]

where \( \varepsilon \) is the permittivity of the dielectric. If no biomembrane structural phase transition occurs in the cell membrane of a neuron in the Aplysia abdominal ganglion as its temperature changes from 8 to 34 \( ^\circ \)C, the membrane capacitance of this cell will stay constant [20]. If \( C \) in equation (3) is constant, then \( I_C = -dV/dt \), and thus \( -C \cdot dV/dt \) can be qualitatively treated as equivalent to the ionic current. Thus, \( C \cdot (MSR) \) and \( C \cdot (MSF) \) can be interpreted as the maximum inward and maximum outward currents during the APs, respectively.

During the experiments, we obtained a vast amount of data. Every minute, 180,000 pairs of temperature and AP values were saved onto the hard disk. These data were consecutively and automatically recorded using an independent file name for each minute of data from 1 to 1,200. We kept recording new data for the next 1,200 min and so on. However, the mean values of each of the 14 AP parameters could be easily calculated using the computer program we designed.

If the value of temperature, \( T_j \), and the value of membrane potential, \( V_j \), were simultaneously recorded onto the computer hard disk at time \( t_j \) at a rate of 3,000 samples/s, then the minimum time interval was \( \Delta t = t_{j+1} - t_{j-1} = 2/3,000 \) s. The first-order derivative of a digital function (in this case, voltage) by another variable (in this case, time), \( t \), is defined using centered differences, as follows:

\[
\frac{\Delta V}{\Delta t} = \frac{V_{j+1} - V_{j-1}}{t_{j+1} - t_{j-1}} = \frac{3}{2} \cdot \frac{V_{j+1} - V_{j-1}}{(Vs^{-1})}. \quad (5)
\]

Electrophysiological data were processed using the scientific data analysis tools, C++, Origin 6.0, and Mathematica 5.1. All the data are presented as mean ± standard deviation in the text and table except in the figures where the data are presented as mean ± standard error.

**Selection of Experimental Data for Further Analysis**

To obtain reproducible temperature-dependent AP parameters, the transmembrane potentials and temperature values were obtained simultaneously from seven neurons. This resulted in about 300 experiments on neurons from the dorsal surface of the left caudal quarter-ganglion. Table 1 displays the conditions under which the seven experiments (A–G) were performed on separate animals. The weights of animals were between 115 and 324 g. The seasons during which the experiments were performed varied from winter to early summer. We chose cells with cell bodies larger than 100 µm in diameter. The media used were L-15 and ASW mixed in a ratio of 1:1 (volume to volume) or ASW only. The temperature

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**Temperature Dependence of AP Parameters**

The table except in the figures where the data are presented as mean ± standard deviation in the text and
was varied between 8.1 and 33.9 °C and 4–16 temperature cycles were used. The total length of time over which the transmembrane potentials were recorded varied between 720 and 3,943 min. The total numbers of spikes in the transmembrane potential varied from 60,537 to 360,845. The average firing rates for the experiments were between 35.9 and 123.4 min−1. Both beating and bursting spontaneous firing patterns were observed in the acquired data.

**Results**

We calculated the maximum and minimum values for 10 of the AP parameters and the IBI using the experimental data shown in table 1 and analyzed the temperature dependence of these parameters. We found qualitative changes in these parameters as the temperature increased. These values and their temperature dependences are summarized in table 2. The ranges of the A AP, V pp, t AP, 1/2, tr1, t r2, tf1, t f2, Frequency, |−MSF|, |−MSR|, and IBI values are 47.3–89.5 mV, 0.3–44.7 mV, 2.9–21.1 ms, 235–4,170 ms, 1.2–6.9 ms, 1.7–15.4 ms, 13.2–136.7 ms, 0.2–3.3 s−1, 9.2–32.2 Vs−1, 4.1–20.4 Vs−1, and 4.5–22.3 s, respectively. The values of A AP, V pp, t AP, 1/2, t r1, t r2, t f1, t f2, and IBI decreased as the temperature increased; in some cells, t r1 and t f2 increased slightly after the minimum value was reached. However, the values of |−MSF|, |−MSR|, and Frequency increased with temperature; in some cells, they decreased after the maximum value was reached.

In order to obtain a more quantitative analysis of the temperature dependences of the AP parameters from the acquired data shown in tables 1 and 2, we focused on the two data sets recorded from two spontaneously beating neurons (experiments A and C). We found that the standard errors of the means of the AP parameters were much smaller in these two cells than in the other cells (experiments B and D–G), which allowed a quantitative analysis.

A comparison of the temperature dependences of the data (experiments A and C) is shown in figure 2. The characteristics of the temperature dependences of the 14 AP parameters of the two experiments are similar. Nine parameters, A AP, V pp, t AP, 1/2, t r2, ISI, t r1, t r2, and t f2, decreased, whereas the other 5 parameters, V np, A DS, |−MSR|, |−MSF|, and Frequency, increased as the temperature increased. Furthermore, two cells showed similar quantitative changes in most of the AP parameters in response to a temperature change. However, the values of V np were different in the cells because V np is related to the resting membrane potential (V r), which is known to differ greatly depending on the cell type. Taken together, these qualitative and quantitative analyses suggest that the temperature-dependent changes in most of the AP parameters could be general phenomena in Aplysia neurons.

Although the mean values of the AP parameters and the experimental conditions for experiments A and C were similar, we further analyzed the data from experi-

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**Table 1. Dissection conditions and electrophysiological properties of seven dissected Aplysia specimens**

| Weight of animal, g | Season of experiment | Media, ext. cell fluid | Temperature cycle range, °C | Total recording time, min | Total number of spikes | Average firing rate, min−1 | sel. dur. for anal., min (n of rep. tem. changes) | Spon. firing patterns |
|---------------------|----------------------|-----------------------|----------------------------|--------------------------|--------------------------|---------------------------|------------------------------------------------|-------------------|
| 235                 | winter               | L-15:ASW (1:1, v/v)   | 10.2–27.5                  | 3,943                    | 360,845                  | 91.5                      | 1,800 (16)                  | beating            |
| 251                 | summer               | ASW                   | 13.4–28.7                  | 720                      | 88,858                   | 123.4                     | 580 (4)                     | beating            |
| 252                 | winter               | L-15:ASW (1:1, v/v)   | 8.1–27.6                   | 3,496                    | 298,030                  | 85.2                      | 854 (8)                     | beating            |
| 115                 | spring               | L-15:ASW (1:1, v/v)   | 12.4–33.7                  | 1,174                    | 108,954                  | 92.8                      | 792 (7)                     | beating            |
| 275                 | spring               | L-15:ASW (1:1, v/v)   | 12.6–26.0                  | 1,800                    | 64,756                   | 35.9                      | 1,163 (8)                   | beating            |
| 152                 | spring               | L-15:ASW (1:1, v/v)   | 14.1–33.9                  | 2,165                    | 60,357                   | 27.8                      | 801 (7)                     | bursting           |
| 324                 | spring               | L-15:ASW (1:1, v/v)   | 12.2–27.8                  | 906                      | 84,431                   | 93.1                      | 727 (5)                     | bursting           |

These are shown as 7 separate experiments, named A–G. ext. cell fluid = External cell fluid; sel. dur. for anal. = selected section duration for analysis; n of rep. tem. changes = number of repetitive temperature changes; Spon. firing patterns = spontaneous firing patterns; v/v = volume/volume.
Table 2. Analysis results for ten AP parameters and IBI: maximum values, minimum values, and analyzed patterns of temperature dependence

| AP parameters | External values and patterns | External values and patterns of APs |
|---------------|------------------------------|-------------------------------------|
| $A_{AP}$, mV  | max. values 78.0 ± 1.9 | exp. A 1.680 ± 462  |
|               | min. values 55.3 ± 2.4  | exp. B 321 ± 51  |
|               | patterns of tem. dep.  | exp. C 235 ± 47  |
|               |                           | exp. D 250 ± 5   |
|               |                           | exp. E 419 ± 58  |
|               |                           | exp. F 706 ± 45  |
| $V_{pp}$, mV  | max. values 32.3 ± 2.6 | exp. G 670 ± 250  |
|               | min. values 1.0 ± 2.9   |                                    |
|               | patterns of tem. dep.   |                                    |
| $\Delta t_{AP/2}$, ms | max. values 17.8 ± 1.6 | exp. A 1.680 ± 462  |
|               | min. values 5.3 ± 0.4   | exp. B 321 ± 51  |
|               | patterns of tem. dep.   | exp. C 235 ± 47  |
| $\Delta t_{1}$, ms | max. values 1,680 ± 462 | exp. D 250 ± 5   |
|               | min. values 321 ± 51    | exp. E 419 ± 58  |
|               | patterns of tem. dep.   | exp. F 706 ± 45  |
|               |                           | exp. G 670 ± 250  |
| $\Delta t_{2}$, ms | max. values 6.1 ± 0.3 | exp. A 1.680 ± 462  |
|               | min. values 2.2 ± 0.2   | exp. B 321 ± 51  |
|               | patterns of tem. dep.   | exp. C 235 ± 47  |
| $\Delta t_{3}$, ms | max. values 11.6 ± 1.3 | exp. D 250 ± 5   |
|               | min. values 3.1 ± 0.1   | exp. E 419 ± 58  |
|               | patterns of tem. dep.   | exp. F 706 ± 45  |
| $\Delta t_{4}$, ms | max. values 118.4 ± 8.0 | exp. G 670 ± 250  |
|               | min. values 35.8 ± 13.0 | exp. A 1.680 ± 462  |
|               | patterns of tem. dep.   | exp. B 321 ± 51  |
| Frequency, s⁻¹ | max. values 2.9 ± 0.5 | exp. C 235 ± 47  |
|               | min. values 0.5 ± 0.1   | exp. D 250 ± 5   |
|               | patterns of tem. dep.   | exp. E 419 ± 58  |
| $|-\text{MSR}\|$, Vs⁻¹ | max. values 20.0 ± 1.8 | exp. F 706 ± 45  |
|               | min. values 11.0 ± 0.7  | exp. G 670 ± 250  |
|               | patterns of tem. dep.   | exp. A 1.680 ± 462  |
| $|-\text{MSF}\|$, Vs⁻¹ | max. values 10.9 ± 0.3 | exp. B 321 ± 51  |
|               | min. values 4.5 ± 0.4   | exp. C 235 ± 47  |
|               | patterns of tem. dep.   | exp. D 250 ± 5   |
| IBI, s        | max. values 22.3 ± 9.9  | exp. E 419 ± 58  |
|               | min. values 4.5 ± 1.0   | exp. F 706 ± 45  |
|               | patterns of tem. dep.   | exp. G 670 ± 250  |

These patterns show the overall qualitative changes as temperature increased: ↓ = decrease; ↑ = increase; ↑↓ = first decrease, then increase; ↑↑ = first increase, then decrease.

max. = Maximum; min. = minimum; patterns of tem. dep. = patterns of temperature dependence.

Temperature Dependence of AP Parameters
tions in detail by plotting $A_{AP}$ and temperature, $T$, as a function of time, as described below.

The upper trace in figure 3 is $A_{AP}$, and the lower trace is the temperature. The temperature was maintained at room temperature for the first 400 min. The temperature value and some AP parameters were calculated between 81 and 320 min. This is represented by (a) in the upper left corner in figure 3 ($T = 18.70 \pm 0.15^\circ C$, $\tau_{t1/2} = 8.58 \pm 0.38$ ms, Frequency = 2.01 $\pm$ 0.12 s$^{-1}$, IBI = 0, and $A_{AP} = 75.01 \pm 0.79$ mV). Because of the low standard errors, the spiking pattern could be considered as regular while the temperature was held constant. Over the period from 401 to 3,943 min, we varied the temperature between 9 and 28 $^\circ C$. Over this time, the mean temperature was 20.12 $\pm$ 5.77 $^\circ C$. Both heating and cooling occurred at an average rate of approximately 0.3 $^\circ C$ min$^{-1}$. No AP data were recorded between 394 and 442 min because the electrode slipped from the cell during the experiment for some unknown reason. It can also be seen from the upper trace of figure 3 that the value of $A_{AP}$ was unstable between 360 and 560 min. However, because the cell kept spiking in a regular and reasonable manner after 560 min, it was thought that this did not greatly affect the data to be analyzed. It was useful to analyze the data represented by (b), in the upper middle part of this graph and recorded between 601 and 2,400 min, under a periodically alternating temperature; the mean $A_{AP}$ value over this period was 67.80 $\pm$ 8.25 mV. The data after 2,400 min were not useful for analysis because the values of $A_{AP}$ dropped and silent types of firing patterns appeared after 3,000 min. Finally, it was necessary to determine whether the spiking pattern of this selected section was influenced by bursting activity.

Bursting is one of the three signaling patterns of neurons, and in bursting patterns, silent periods are followed by periods of spiking. All of the bursting activities could be calculated from the 30 consecutive hours of data that were saved between 601 and 2,400 min. Spontaneous bursting activities did not occur for the time period from 601 to 1,837 min; however, seven bursting patterns were found between 1,838 and 1,842 min, and these occurred at a temperature of approximately 10 $^\circ C$ (BI = 2.57 $\pm$ 0.40)

Fig. 2. Comparison of temperature dependences of 14 AP parameters between experiments A and C. The data in the 'selected duration for analysis' section in table 1 were averaged. The overall tendencies listed in table 2 are clearly shown in these figures.
0.148 s and IBI = 6.53 ± 0.819 s). They occurred 54 more times with their durations totaling 19 min between 1,843 and 2,320 min. Because the ratios of IBI to BI (IBI/BI) of these bursting activities at low temperatures were between 2.3 and 3.2, the spiking patterns of these bursting activities were not fundamentally different from beating activities. A typical bursting activity, in which the IBI to BI ratio became 19.5, occurred only once, at 2,321 min, at a temperature of 20.7°C. From this, we can conclude that the spiking analysis of the selected section was not influenced by the effects of bursting activity. Finally, we selected a good section of data from experiment A, specifically the data recorded between 601 and 2,400 min, to discuss the temperature dependences of the AP parameters. Before presenting these results, it is necessary to give a brief account of the statistical method used to treat the experimental data.

Figures could be plotted using the experimental data recorded between 601 and 2,400 min presented by (b) in figure 3. In this section, there were a total of 211,698 spikes; therefore, each of the 1,800 files contains an average of 117.6 APs. First, the mean values of the AP parameters at each corresponding temperature were calculated, one file at a time, using a C++ program, and the results were saved to text files. The saved files were then copied to the Origin program and sorted by temperature. The files were divided into ten groups, each corresponding to a 2°C temperature interval, and each group contained 180 values for each of the AP parameters. In the Origin program, the averages of the 180 values for each AP parameter were calculated; each filled dot in figure 2 is an average of 21,169 measurements of APs, averaged with error bars. Each data point in figures 4–6 is an average of 10,585 measurements; the temperature rising and falling phases and the first and second half-time periods are plotted separately in the numbered panels of the same subfigure, i.e. in figures 4A1–6C1 and figures 4A2–6C2, respectively. It is possible to draw multiple plots in figure 4 using these mean values for the AP parameters, and it is not difficult to find apparently reproducible temperature dependences for the AP parameters in these figures.

**AP Parameters That Decreased with Increasing Temperature**

As shown in figures 4A–C, the parameters $A_{AP}$, $V_{pp}$, $\Delta t_{AP, 1/2}$, and $\Delta t_{DS, 1/2}$ all decreased as the temperature was raised. The standard errors of these values were small. The values of $A_{AP}$ and $V_{pp}$ during the falling phases were almost identical to those during the rising phases of temperature change (fig. 4A). The values of $A_{AP}$ and $V_{pp}$ from 1 to 900 min were also very similar to those from 901 to 1,800 min (fig. 4A2). Figure 4B1 presents plots of $\Delta t_{AP, 1/2}$ as a function of temperature. The values of $\Delta t_{AP, 1/2}$ were very similar, irrespective of the rising or falling phases of the temperature changes. The values of $\Delta t_{AP, 1/2}$ from 1 to 900 min were a little larger than those from 901 to 1,800 min, and the difference between the two sets of values increased as the temperature was decreased (fig. 4B2). The DS half-width duration, $\Delta t_{DS, 1/2}$ was calculated as the width at half-maximum amplitude of $A_{DS}$. Figure 4C presents plots of $\Delta t_{DS, 1/2}$ as a function of temperature. The values of $\Delta t_{DS, 1/2}$ during the rising phases were slightly larger than those during the falling phases of temperature change at low temperatures, whereas at high temperatures, the values of $\Delta t_{DS, 1/2}$ during the falling phases were slightly larger than those during the rising phases of temperature change (fig. 4C1). The values of $\Delta t_{DS, 1/2}$ from 1 to 900 min were slightly higher than those from 901 to 1,800 min, but it was reversed at low temperatures (fig. 4C2).

Plots of ISI, $\Delta t_{14}$ and $\Delta t_{23}$, and $\Delta t_{14}$ and $\Delta t_{23}$ as functions of temperature are shown in figure 5A–C, respectively. The values of ISI and $\Delta t_{14}$ during the rising phases were slightly larger than those during the falling phases of temperature change (fig. 5A1). The values of ISI and $\Delta t_{14}$ from 901 to 1,800 min were slightly larger than those from 1 to 900 min, and the difference between these two

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values increased slightly at lower temperatures (fig. 5A2). The values of $\Delta t_{r2}$ during the rising phases were slightly larger than those during the falling phases of temperature change at low temperatures; however, at high temperatures, the values of $\Delta t_{r2}$ during the falling phases were slightly larger than those during the rising phases of temperature change. In contrast, the values of $\Delta t_{r1}$ during the temperature falling phases were slightly larger than those during the temperature rising phases (fig. 5B1). The values of $\Delta t_{r2}$ and $\Delta t_{r1}$ from 1 to 900 min were slightly larger than those from 901 to 1,800 min; however, in the latter case, the difference between the two values became larger as the temperature decreased (fig. 5B2). Figure 5C presents plots of $\Delta t_{r2}$ as a function of temperature. The values of $\Delta t_{r2}$ during the rising phases were slightly larger than those during the falling phases of temperature change (fig. 5C1). In addition, the values of $\Delta t_{r2}$ from 901 to 1,800 min were slightly larger than those from 1 to 900 min between 18 and 28°C, and the difference between these two values increased with temperature (fig. 5C2).

**AP Parameters That Increased with Temperature**

Plots of $V_{pp}$, $A_{DS}$, $-MSR$ and $-MSF$, and Frequency as functions of temperature are shown in figure 6, respectively. The absolute values of $V_{pp}$ during the temperature falling phases were slightly larger than those during the temperature rising phases at low temperatures, but it was reversed at high temperatures (fig. 6A1). The absolute values of $V_{pp}$ from 901 to 1,800 min were slightly larger than those from 1 to 900 min at low temperatures, but not at high temperatures (fig. 6A2). The parameters $A_{DS}$, $-MSF$, and $-MSR$ are plotted in figure 6B as functions of temperature. The $A_{DS}$ amplitude, $A_{DS}$, was defined as the absolute value of the difference between $-MSR$ and $-MSF$, i.e. $A_{DS} = |(-MSF) - (-MSR)|$. The parameters $A_{DS}$, $-MSF$, and $-MSR$ all increased with temperature. The values of $A_{DS}$, $-MSF$, and $-MSR$ during the temperature falling phases were almost identical to those during the temperature rising phases (fig. 6B1). The values of $A_{DS}$, $-MSF$, and $-MSR$ from 1 to 900 min were also almost identical to those from 901 to 1,800 min (fig. 6B2).

The spike frequency (Frequency) is plotted in figure 6C as a function of temperature. The Frequency values during the temperature falling phases were slightly larger than those during the temperature rising phases between 10 and 26°C. The difference between the Frequency values of the rising and falling phases increased as the temperature increased. However, at 28°C, the Frequency val-
ues during the temperature rising phases were slightly larger than those during the temperature falling phases (fig. 6C1). The Frequency values from 1 to 900 min were slightly larger than those from 901 to 1,800 min (fig. 6C2).

**Discussion**

All animals that are active over a range of temperatures require an internal temperature system constructed of individual temperature-sensitive reactions in order to maintain function throughout the entire temperature range. For example, circadian oscillators cannot allow the period of their clock to change in response to a change in temperature [11]. In this study, we showed that 14 AP parameters changed in response to a temperature change in *Aplysia* neurons. However, the functional implications or evolutionary explanations of these temperature-dependent changes in the AP parameters are still unclear. They may be involved in the process of temperature detection by changing the AP frequency and/or other parameters, or they could be part of the cell’s protective response to temperature shocks. One aim of this study was to understand the underlying mechanism of the physiological response of a neuron to temperature change in terms of ionic currents by using *Aplysia* neurons as an experimental model. We would like to eventually define this temperature-driven response of a neuron using a set of mathematical equations composed of ionic currents. In order to accomplish this, we need to acquire quantitative data such as the 14 AP parameters analyzed in this study.

Experiments involving temperature-dependent AP parameters such as $A_{AP}$, $\Delta t_{AP,1/2}$, frequency, and resting membrane potential ($V_r$) in *Aplysia* neurons have previously been performed [13, 14, 16]. The general conclusion from these past studies on *Aplysia* neuronal firing patterns is that a rising temperature increases the spike frequency [14, 21] and absolute value of the resting membrane potential [13, 14], and that there is a correlation with a decrease in the spike amplitude and $\Delta t_{AP,1/2}$ [16]. However, the analysis methods used in the past had limitations from a technical point of view because they dealt with the temperature dependences of AP parameters by depending on the data acquired from a pen writer or magnetic tapes over a relatively short time interval. It was possible for us to use a higher resolution in the functional relations that described the temperature dependences...
of 14 AP parameters than used in former experiments. Moreover, we used *Aplysia* neural cells, which remain alive for a very long time in vitro; in experiment A, data were acquired continuously for over 60 h. Thus, it was possible to obtain data for 14 AP parameters that were dependent on a physical variable such as the temperature in *Aplysia* cells with relatively small error bars. We can be assured of the reproducibility with living experimental targets by examining the variation in the parameters recorded. Accordingly, we found that the 14 defined parameters were influenced by temperature in a highly reproducible manner.

Temperature-driven changes in AP parameters could affect the function of a neuron by changing the conduction velocity in the afferent axon as well as the firing rate [22]. Considering the importance of AP parameters in neuronal function [23], a question arises: how does the temperature affect these AP parameters in a neuron? Several studies have examined the temperature dependence of the intrinsic membrane conductance influenced by ion channel conductance. For example, Thompson et al. [24] found an increase in Ca$^{2+}$-activated K$^+$ conductance at a low temperature in hippocampal neurons in vitro. In addition, it was reported that alterations in temperature have a critical role in the conformational changes seen in a voltage-gated potassium channel, leading to their activation and inactivation [25]. Although they remain elusive in *Aplysia* neurons, transient receptor potential channels, which are nonselectively permeable to cations, are thought to be a group of ion channels used in animals to sense hot or cold sensations by being activated by a change of temperature [26]. Indeed, Chen et al. [27] reported that transient receptor potential-like channels contribute to the firing pattern of APs in the modified Hodgkin-Huxley neuron model. It is unclear how a temperature alteration can affect the specific ionic conductance that is responsible for the changes in the 14 AP parameters in our *Aplysia* model.
In order to address this issue, we aim to eventually develop nonlinear differential equations based on our recorded data for the 14 AP parameters and their temperature-dependent changes. Although the Hodgkin-Huxley-type Huber-Brown cold receptor model can simulate the temperature dependences of cold receptors [28, 29], a computer simulation model that explains the temperature dependence of the firing patterns in *Aplysia* neurons has not yet been found. The temperature-dependent reproducible properties of the 14 AP parameters described in this study can be considered as the basis for setting up a set of nonlinear differential equations that approximate the temperature-dependent firing patterns of neurons in *Aplysia*. However, because it is not easy to set up a system of equations using these results, it is necessary to perform another experiment to obtain the quantitative temperature-dependent properties of the APs. When the temperature was increased from 11 to 32 °C, we found that seven firing patterns of the neurons in *Aplysia* changed sequentially from the silent state to beating, doublets, beating-chaos, bursting-chaos, square-wave bursting, and bursting-oscillation patterns [30]. In addition, as the temperature was decreased, the spiking patterns changed in reverse order. It would be beneficial to develop equations using scaling factors that depend on temperature and to trace whether alterations in temperature play a critical role in voltage-gated channels. Finally, it will be interesting to analyze the temperature-dependent properties of the 14 AP parameters using such equations.

(2) Two AP parameters were characterized by larger values during the falling phases than during the rising phases of temperature change: Frequency and Δt1. Three AP parameters were characterized by larger values during the rising phases than during the falling phases of temperature change: ISI, Δt1, and Δt2. However, if errors are considered, little difference is found between the rising and falling phases.

(3) Six AP parameters were characterized by larger values between 1 and 900 min than between 901 and 1,800 min: A_AP, V_pp, Frequency, Δt_AP, 1/2, Δt2, and Δt1. Two AP parameters were characterized by larger values between 901 and 1,800 min than between 1 and 900 min: ISI and Δt1. However, if errors are considered, little difference is found between the first and last 1 h.

**Concluding Remarks**

To determine functional relations that describe the temperature dependences of AP parameters, better experimental tools than those used in previous experiments were required. Using these more advanced tools, we performed experiments on long-lived in vitro *Aplysia* neural cells. In this paper, the AP parameters were analyzed and calculated using the data from an experiment with a cell from the abdominal ganglion of *A. juliana* to quantitatively understand the temperature dependences of the AP parameters. The basic experimental results relating to the temperature dependences of the 14 AP parameters displayed in figures 2 and 4–6 can be summarized as 3 findings:

(1) Nine AP parameters were found to decrease with increasing temperature: A_AP, V_pp, ISI, Δt1, Δt2, Δt1, Δt2, Δt_AP, 1/2, and Δt_DS, 1/2. Five AP parameters were found to increase with temperature: Frequency, | V_np |, A_DS, | − MSR |, and | − MSF |.

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