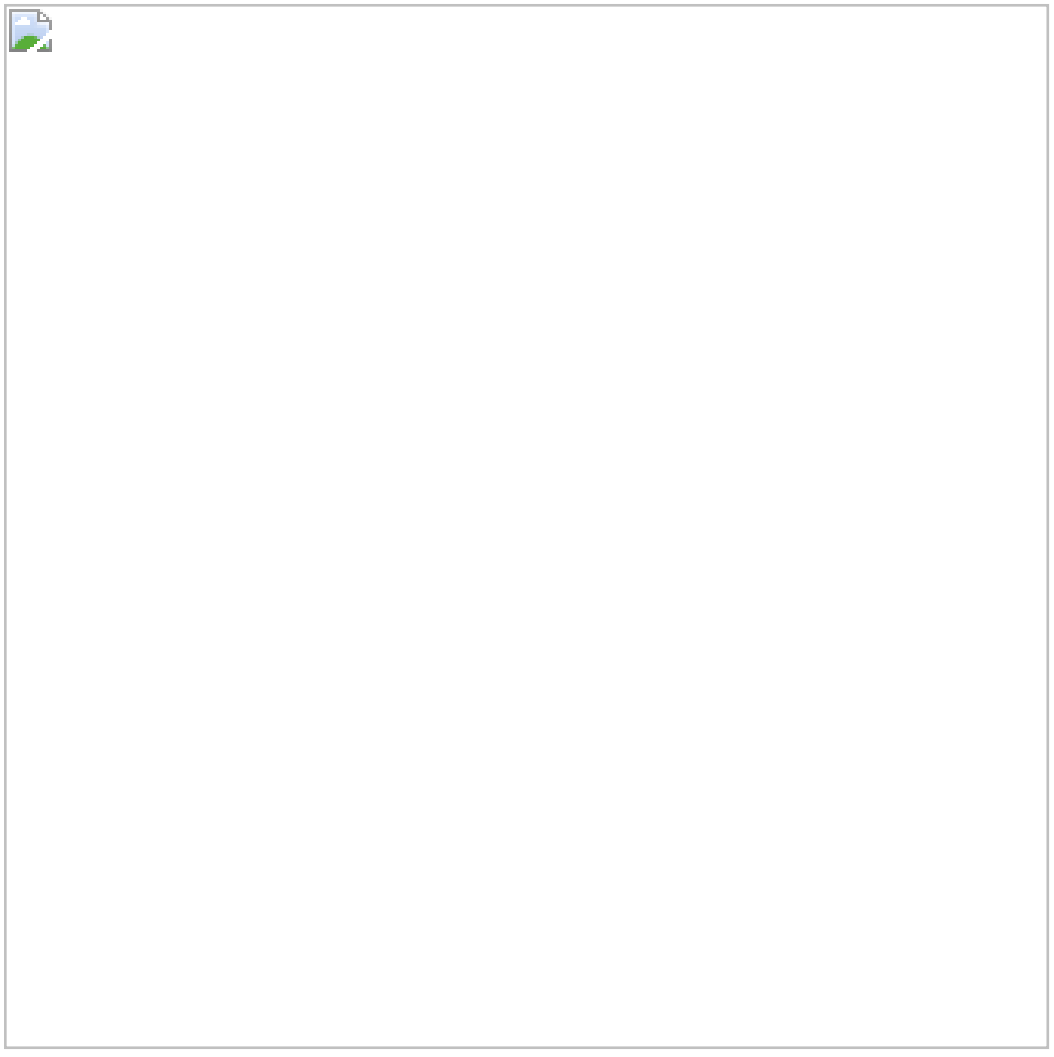

Electrical Properties of the Cerebral Prothoracicotropic Hormone Cells in Diapausing and Non-Diapausing Pupae of the Tobacco Hornworm, Manduca sexta

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Electrical Properties of the Cerebral Prothoracicotropic Hormone Cells in Diapausing and Non-Diapausing Pupae of the Tobacco Hornworm, *Manduca sexta*

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ABSTRACT—Prothoracicotropic hormone (PTTH) is an insect brain neuropeptide that is a primary factor regulating an insect development. Curtailment of its release is thought to be responsible for the pupal diapause of tobacco hornworm, *Manduca sexta*. The cell synthesizing and secreting the PTTH has been identified as a pair of neurosecretory cells in the pars lateralis on each brain hemisphere. Using intracellular recording techniques, we have demonstrated electrical properties of the PTTH cells in different physiological status, i.e., diapausing and developing pupae. In diapausing pupae, they showed threshold value increasing and input resistance decreasing with the progress of diapausing state, indicating that they were getting unexcitable. Spontaneous action potentials and excitatory postsynaptic potentials (EPSPs) were rarely observed in deeply diapausing state. Non-diapausing PTTH cells were almost silent except day-2, showing rather constant values of electrical properties. On day-2, a significant proportion of the cells had spontaneous action potentials, showing less negative membrane potential values than inactive cells. Exclusively inhibitory postsynaptic potentials (IPSPs) were observed in significant numbers of the cells during the period from day-2 to day-5. On the basis of the results obtained, we proposed a working hypothesis that electrical activities of the PTTH cell may be primarily regulated by its membrane properties which are further modulated by the synaptic mediation.

INTRODUCTION

Diapause is well understood in ecological and physiological stand points as the strategy by which insects integrate their life cycle with seasonal change of the environmental conditions [2]. By entering a state of diapause the insect circumvents climatic conditions; that would otherwise be detrimental to the continuation of its life cycle. The diapause state is initiated, maintained and terminated by specific environmental cues, the most common being photoperiod [12, 27].

The physiological process responsible for making a diapause state appears to involve the neuroendocrine system. In the case of pupal diapause in lepidopterous insects, the accepted concept is that environmental cues suppress the synthesis and/or release of the primary effector of insect post-embryonic development, the cerebral neuropeptide, prothoracicotropic hormone (PTTH) [12]. In the tobacco hornworm, *Manduca sexta*, photoperiod, the most powerful environmental cue for diapause initiation [25], is sensed by a brain centered photoperiodic clock which subsequently regulates the release of PTTH [8]. Pupal diapause in *Manduca* is well studied endocrinologically [7]. It is characterized by the absence of an increased ecdysteroid titer in the hemolymph during the first week of pupal life. This virtual absence of the steroid molting hormone is thought to be responsible for the diapause state and it is apparently a consequence of a failure of diapausing pupal prothoracic glands (PGs) to synthesize ecdysone at an increased rate [34]. This apparent failure of PG activation during diapause has been suggested to be in response to two endocrinological processes: the development of refractoriness of the gland to stimulation by PTTH [12], and the curtailment of PTTH release, which is evidenced by biochemical and electron microscopic studies [6, 13].

The biochemical nature of PTTH has been extensively studied by means of molecular biological techniques [15, 19]. In *Bombyx*, PTTH was revealed to be a homo-dimer and its constituent monomer comprises 109 amino acid residues [16]. Besides these molecular studies, morphological studies have been extensively progressed in relation to the secretory activity of the PTTH cell by means of immunocytochemistry [13, 32, 33]. However, the neurophysiological mechanism of PTTH release is still largely unknown, although some pharmacological studies have been made for larval PTTH release [29]. Shirai *et al.* [29] showed that acetylcholine is one of the neurotransmitters involved in the PTTH release and its action is mediated by a phosphoinositide hydrosis pathway.

By extracellular recordings, cerebral neurons of diapausing pupae have been shown to be less active than non-diapausing pupae in some insect species [28, 31]. Since

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Abbreviations: CA, corpora allata; CC, corpora cardiaca; EPSP, excitatory postsynaptic potential; IPSP, inhibitory postsynaptic potential; LD, light to dark cycle; NSC, neurosecretory cell; PG, prothoracic gland; PSP, postsynaptic potential; PTTH, prothoracicotropic hormone

important regulatory sites are likely restricted to PTTH cells, measurements of entire brains may mask important activity occurring within the cells. In *Manduca* the cells producing PTTH have been identified to be a pair of lateral neurosecretory cells by biochemical and immunocytochemical techniques [1, 22]. These cells are clearly visible in living brain by direct illumination [9], and hence easily accessible for intracellular recording with a microelectrode. In this study, we analyzed the electrical properties of the PTTH cells both in diapause and non-diapause *Manduca sexta* pupae. On the basis of the results, possible mechanisms for regulating the PTTH cell activity will be discussed.

MATERIALS AND METHODS

Experimental animals

Manduca sexta larvae were reared at 25°C on an artificial diet under either diapause inducing (8 hr light (L)/16 hr dark (D)) or diapause preventing photoperiod (16 hr L/8 hr D). About 90% of the animals reared under the 8L/16D became to be diapausing pupae. After pupation, animals were kept in constant darkness at 25°C. To see effects of chilling on the activity of diapausing PTTH cells, day-10 diapausing pupae were chilled at 5°C for about 30 days and then transferred to 25°C.

Electrophysiological preparations

A small square piece of the pupal cuticle was removed through from the head to neck region to expose the brain. The abdomen was cut off between the 4th and 5th abdominal segments, the gut and the fat bodies were removed, and the hemolymph was washed with a modified Weever's saline (in mM/l, NaCl, 140; $MgCl_2$, 1; $CaCl_2$, 5; dextrose, 20; KOH, 7; PIPES, 5; pH=6.4). The animal was then restrained dorsal side up in molded plasticine (Harbertt's Plasticine LTD, Bathamton, England) by inserting insect pins through the thoracic wing bases, and bathed in the saline. A metal platform was placed beneath the brain to get stable recordings. The sheath above the neurosecretory cells was partly removed by sharp forceps to have an access of the intracellular recording.

Intracellular recordings from the PTTH cell somata were made with glass microelectrodes filled with 4 M/l potassium acetate with resistance values of 30–60 MΩ Ag-AgCl reference electrode was placed in the saline in which the animal was bathed. Signals were amplified by a preamplifier (WPI, M-707A), monitored on a digital oscilloscope (Tektronix, 5116) and traced on a pen recorder (Gould, 220).

Each cell was stimulated with 500–1000 msec hyperpolarizing and depolarizing current steps of varying magnitudes, with an interpulse interval of 5 sec. Slope input resistance was estimated by calculating the linear regression from the linear portion of the current-voltage relation using small hyperpolarizing current steps. Threshold voltage was measured as the minimum depolarization from rest necessary to trigger the firing of an action potential.

In some cases, an anatomical feature of individual cells was revealed by intracellular iontophoresis of 1% Lucifer Yellow CH [30] (in 1 M/l LiCl), using a stimulus paradigm of 500 msec hyperpolarizing pulses of current (5 nA) passed at 1 Hz for 10–20 min. Brains with filled cells were subsequently dissected, fixed in a 4% solution of formaldehyde and processed for whole mount visualization with a Nikon epifluorescence microscope.

RESULTS

Morphological changes of the brain and identification of PTTH cells

In non-diapausing animals, pupation is immediately followed by adult development. Newly molted pupal brain is flattened dorsoventrally and has two major optic nerves on each side (Fig. 1A). Morphological change of the brain was characterized with aggregation of prohemocyte like-blood cells around the whole brain, increase in the number of the optic nerves, and swelling of the brain (Fig. 1B). The aggregation of the blood cells was already apparent on day-1. However, diapausing pupae did not show such a kind of blood cell aggregation and morphological change of the brain. The brain remained flattened and had only two optic nerves and a relatively hard sheath throughout the diapausing period (Fig. 1C).

Using a direct illumination on the living brain, four bilateral symmetrical groups of cerebral neurosecretory cell somata, two in the pars intercerebralis and two in the pars lateralis, were clearly visible and distinguishable according to their size, localization and color throughout the pupal stage as shown by Agui *et al.* [1]. A pair of PTTH cells were always clearly discriminated from the other cell types, because they were located most antero-laterally (Fig. 1). As a preliminary experiment, one of these cells was intracellularly stained by Lucifer Yellow in 10 animals, being demonstrated to be a PTTH cell with its axon running to the contralateral brain

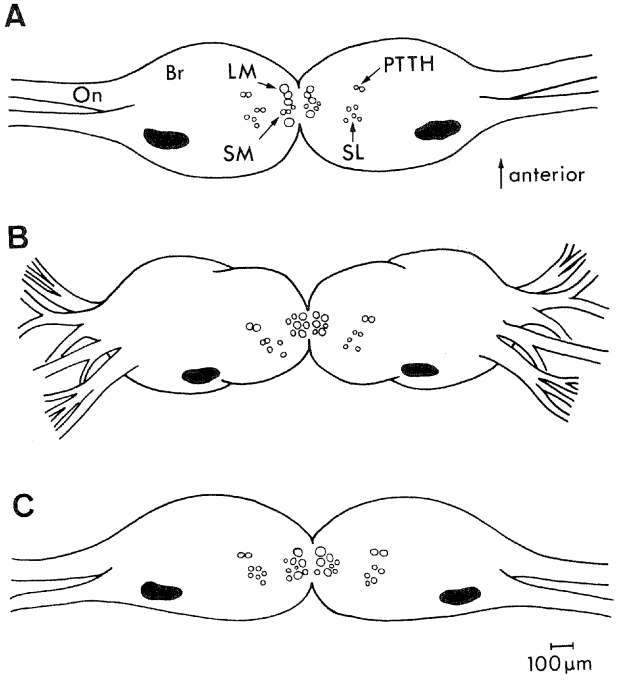

FIG. 1. Schematic drawings of *Manduca* pupal brain (Br) showing optic nerves (On) and the location of neurosecretory cell groups, i.e. large medial (LM), small medial (SM), small lateral (SL) and PTTH cells (PTTH). A,B and C show day-1 and -5 non-diapausing and day 30 diapausing pupal brain, respectively.

TABLE 1. Electrical characteristics of PTTH cells in diapausing pupa

	Days after pupation				Days after 30 days of chilling at 5°C[a]	
	1	5	10	30	0	2
N	17	10	11	10	12	25
Resting membrane potential(mV)	−36.2±4.8	−34.1±8.2	−34.5±4.3	−34.5±4.8	−37.3±6.9	−37.0±5.5
Spike height (mV)	44.4±9.4	57.8±11.2	55.8±10.7	57.7±9.7	50.6±6.8	54.6±9.7
Spike duration (msec)	11.9±5.1	6.1±1.5	8.0±1.4	9.0±2.5	10.9±3.2	14.9±3.3
Threshold current (nA)	0.19±0.14	0.23±0.11	0.37±0.35	0.41±0.25	0.23±0.12	0.28±0.15
Input resistence (MΩ)	104±38	63±21	54±30	48±15	64±15	65±15
% cells with spontaneous activity	0	0	0	10	16	20
% cells with PSPs	0	0	0	10[b]	0	24[b]

[a] Pupae were transferred to 5°C 10 days after pupation. After 30 days of chilling they were placed at 25°C.
[b] Observed PSPs were exclusively EPSPs.
Values are expressed as mean±SD.

hemisphere, and coming down to corpora allata as its terminal. The morphological characteristics thus revealed were consistent with those clarified by immunohistochemical staining of the cells using a monoclonal antibody against PTTH [22, 32, 33]. The size of the PTTH cell somata was larger in diapausing pupae than in non-diapausing developing pupae.

Electrophysiological properties of PTTH cells in diapausing pupae

Since PTTH release is a normal prerequisite for initiation of adult development, inactivation of the PTTH cells is thought to be one of the major reason for diapause development [12]. However, there is a lack of information about the mechanisms controlling the activation and inactivation of the cells. We intracellularly recorded electrical activities of individual PTTH cells at various stages of diapause to answer this issue.

Table 1 shows a summary of an electrophysiological analysis of individual PTTH cells. During 30 days of diapause, none of the cells, except one cell of day-30, showed spontaneous action potentials nor was there any evidence for ongoing inhibitory or excitatory synaptic inputs in the form of post-synaptic potentials (example Fig.2). Only one cell at day-30 showed spontaneous action potentials as well as depolarizing postsynaptic potentials (PSPs). These PSPs were excitatory (EPSPs), since their estimated reversal potentials were positive to threshold. The action potential had long duration of about 15 msec at half amplitude and a prolonged after hyperpolarization (Fig. 3). When individual PTTH cells were examined in day-40 animals which had been chilled for 30 days at 5°C since day-10, many cells displayed spontaneous action potentials as well as EPSPs (Fig. 4). In one animal spontaneous EPSPs occurred rhythmically to oscillate the membrane potential, and action potentials thus occurred rhythmically at the depolarizing phase (Fig. 5). The firing rate was low, ranging 0.05 to 1.0 Hz, but was modified by intrasomatic current injection; it increased when the depolarizing current was injected and was reduced by the

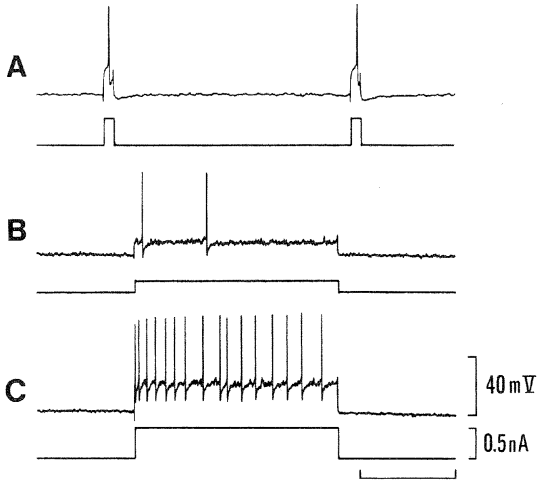

FIG. 2. Intracellular recordings from the cell body of a PTTH cell of a day-10 *Manduca* diapausing pupa. A: Responses to short depolarizing current pulses. B-C: Response to a long depolarizing pulse, showing fluctuation of membrane potential. Resting membrane potential was −34 mV. Time scale: A, 1 sec; B-C, 5 sec.

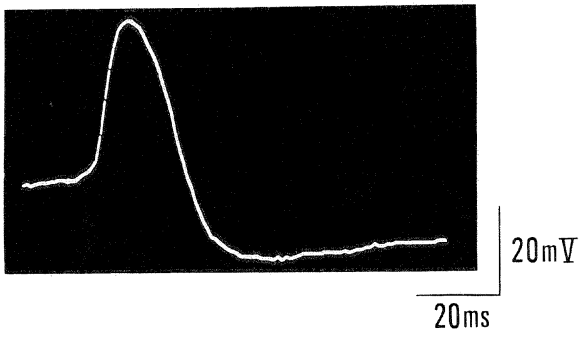

FIG. 3. Trace of a single action potential of PTTH cell body from a day-30 diapausing pupa showing long spike duration and prolonged after hyperpolarization. Resting membrane potential was −31 mV.

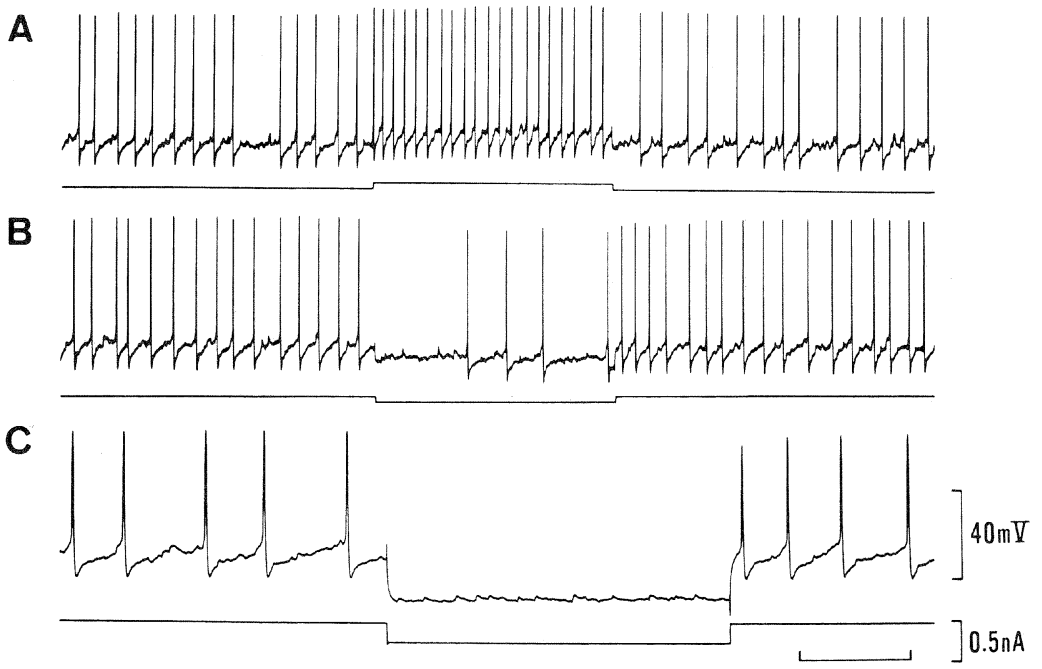

FIG. 4. Intracellular recording from a cell body of a spontaneously active PTTH cell of a diapausing pupa which was day-40 and had been transferred to 25°C after 30 days of chilling at 5°C. Resting membrane potential was −36 mV. Frequency of spontaneous activity was increased by depolarizing current pulse (A) and reduced by a hyperpolarizing current pulse (B). EPSPs could be visible when membrane potential was deeply hyperpolarized (C). Time scale: A-B, 5 sec; C, 1 sec.

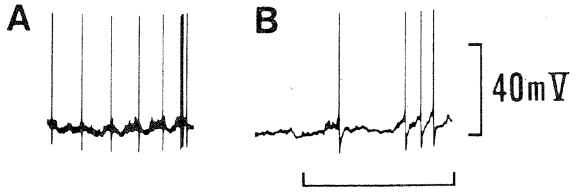

FIG. 5. Spontaneous oscillation in membrane potential of a PTTH cell body of a day-40 diapausing pupa which had been transferred to 25°C after 30 days chilling at 5°C. A: Action potential occurring at the depolarizing phase. B: Expanded record showing EPSPs causing depolarization of the membrane potential. Resting membrane potential was −38 mV. Time scale: A, 50 sec; B, 5 sec.

hyperpolarizing current injection (Fig. 4).

Resting membrane potential values on various days of diapause ranged from −30 to −50 mV, with no significant difference among the cells of different diapause stages. Injecting brief depolarizing current over threshold value into the cells elicited action potentials similar to spontaneous action potentials, with peak amplitude of 35–60 mV (overshoot of 5–20 mV) and typical duration of 5–15 msec measured at half-amplitude. Like the spontaneously generated action potentials recorded in active PTTH cells (Fig. 4), evoked potentials were followed by prolonged after-hyperpolarization (200–500 msec, Fig. 2). It is notable that depolarization of the membrane potential by intrasomatic current injection elicited a fluctuation of the membrane potential (amplitude, 2–5 mV), which sometimes generated the action potential (Fig. 2B, C). The current-voltage relationship of PTTH cells was linear in the range of −30 to −90 mV, but nonlinear, showing a rectification for the depolarizing current (example, Fig. 6). The rectification suggests that the voltage dependent potassium and voltage dependent calcium channels may be involved in this voltage dependent fluctuation of the membrane potential.

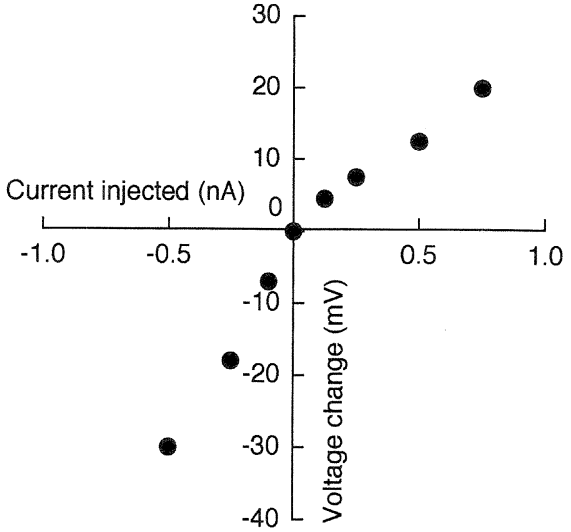

FIG. 6. Current-voltage relationships of a single PTTH cell from a day −30 diapausing pupa. A rectification is apparent when positive current was injected. Input resistance was estimated by calculating the linear regression through the linear portion of the current-voltage relation, i.e. for hyperpolarizing current steps. The estimated input resistance value for this particular cell was 56 MΩ.

In contrast to these relatively consistent valuables, input resistance and threshold level showed a change with days passing. During the course of diapause development, the input resistance calculated for the linear portion of the current-voltage relationship (cf. Fig. 6) became smaller from the largest value of 104±38 (mean±S.D.) MΩ on day-1 to the smallest value of 48±15 (mean±S.D.) MΩ on day-30. Conversely, the amount of current required to induce action potentials became larger and larger, while the threshold of firing somewhat increased. However, after chilling for 30 days these values became similar to those of the early diapausing stage. These results suggest that the PTTH cells become to be less excitable during a course of diapause development.

Electrophysiological properties of PTTH cells in developing pupae

Morphological change of the brain in developing pupae starts around 3 days after pupation. At the same day, the ecdysone titer in the hemolymph increases sharply [4]. According to these facts, it is probable that PTTH release occurs between day-2 and -3. We thus examined the electrophysiological feature of the PTTH cells in early developing pupal stages, i.e., day-1, 2, 3 and 5, to see the mechanism regulating the PTTH release in developing pupae. Intra cellular recordings were made at least 10 cells in each stage. Results are summarized in Table 2. In day-1 animals, PTTH cells showed neither spontaneous action potentials nor PSPs. The membrane potential was quite stable. When individual PTTH cells were examined at various times on day-2, which is the expected day for PTTH release, a significant proportion of the cells (10 out of 40) displayed ongoing train of action potentials and frequently showed evidence of inhibitory synaptic input (Fig. 7). The firing rates ranged from 0.2 to 2.7 Hz, which were higher than those of active cells in diapausing pupae, and were rather steady, but modified by intrasomatic current injection as were the cells in diapausing animals. Bursting patterns rarely occurred with slight oscillation of the resting level of membrane potential (Fig. 7C); depolarization of the membrane potential induced grouped discharge of action potentials. The inhibitory postsynaptic potentials (IPSPs) were often observed even in the inactive cells in this stage, while EPSPs were observed only in one inactive cell (Fig. 7D). After this stage, only one cell was found to be spontaneously active on day-3. However, spontaneous IPSPs were observed in significant number of the cells as late as day-5.

To determine whether specific changes in intrinsic properties of the PTTH cells accompany their activation, the electrical characteristics of individual cells were examined. Inactive PTTH cells from different groups showed similarities in all electrical properties measured (Table 2). Intrasomatic injection of depolarizing current induced the action potential in all inactive cells (Fig. 7A). Subthreshold depolarizing current injection caused a fluctuation of membrane potential as in the diapausing pupal cells. On the other hand, the active PTTH cells on day-2 were revealed to have significant differences from inactive cells in some properties (Table 2). Average resting membrane potential value measured at the pause of spontaneous action potentials was −36.7 mV and was significantly less negative than other cell groups ($P<0.05$, ANOVA followed by LSD), except day 5 cells, showing about −40 mV. Spike height of the active cells, was thus significantly reduced. Input resistance was significantly increased ($P<0.01$, ANOVA followed by LSD); mean input resistance values were 60–70 MΩ for the inactive cells, while the active cells averaged 91 MΩ. Conversely, the voltage threshold and the amount of current required to induce action potential in the active cells were significantly reduced ($P<0.01$, ANOVA followed by LSD).

TABLE 2. Electrical characteristics of PTTH cells in developing pupa

	Days after pupation				
	1	2		3	5
		inactive	active		
N	10	30	10	13	10
Resting membrane potential (mV)	−42.7±5.3	−41.1±6.2	−36.7±2.8[a]	−38.9±3.9	−38.5±6.3
Spike height (mV)	51.0±10.4	45.3±8.6	39.3±4.8	45.1±6.4	45.5±8.2
Spike duration (msec)	10.0±2.0	11.8±2.7	12.9±3.0	15.3±9.4	13.2±2.2
Threshold current (nA)	0.28±0.23	0.28±0.22	0.05±0.02[b]	0.20±0.11	0.33±0.2
Threshold voltage (mV)	−26.5±6.1	−26.0±7.2	−31.2±3.5[c]	−24.8±4.0	−22.9±6.8
Input resistence (MΩ)	61±17	66±28	91±33[d]	71±22	58±15
% cells with spontaneous activity	0	25		7.6	0
% cells with PSPs	0	27	60	30.7	20

[a)] Significantly different from other groups except day 5 ($P<0.05$, ANOVA followed by LSD).
[b,c,d)] Significantly different from other cell groups ($P<0.01$, ANOVA followed by LSD).
IPSPs occurred exclusively, except one cell of day-2 inactive cell group which showed both EPSPs and IPSPs. Values are expressed as mean±SD.

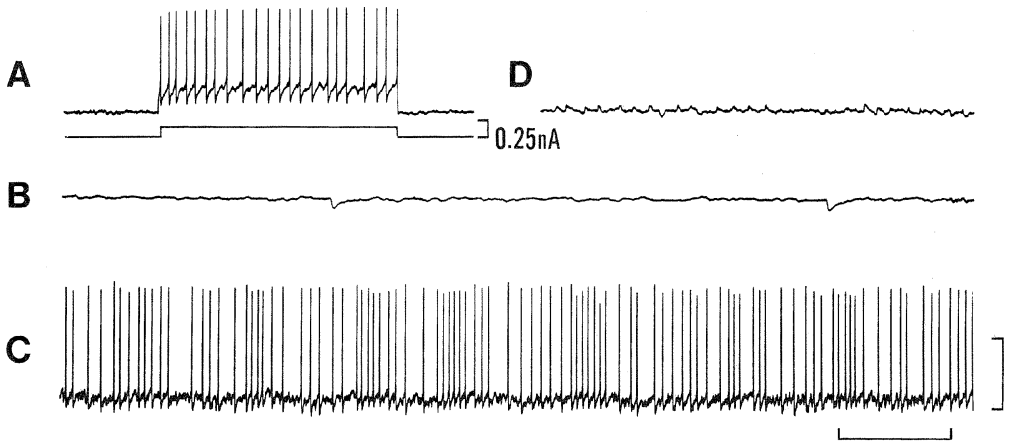

FIG. 7. Intracellular recordings from cell bodies of PTTH cells of day-2 non-diapausing pupae. A and B are from the same cell. A: Spike activity induced by a depolarizing current pulse. B: Spontaneous IPSPs. C: Spontaneous action potentials of which frequency was modulated with spontaneous slight oscillation of the membrane potential. D: Spontaneous EPSP and IPSPs. Resting membrane potentials: A-B, −26 mV; C, −34 mV; D, −40 mV. Vertical scale: A and D, 40 mV; B and C, 20 mV. Time scale: A and C, 5 sec; B and D, 1 sec.

DISCUSSION

Many efforts have been made to study the physiology of the PTTH cell during the diapause, employing the techniques such as ligation, brain extirpation, brain implantation, bioassay of PTTH titer as well as brain incubation [7, 20]. Some important pieces of information have thus been yielded about the quantity of PTTH in the brain or neurohemal organ and the critical period of PTTH release at pupal stage. Some recordings of electrical activity in brain were compared between diapausing and non-diapausing pupae in several lepidopterous insects, showing that the neuronal activity in the brain in diapausing pupae seems rather common phenomenon [28]. The techniques used in these researches, however, could not reveal any cellular mechanism regulating the release of PTTH such as the synaptic mediation. In this study, we analyzed the electrical activity of the PTTH cell intracellularly and obtained for the first time the basic information about the physiology of the cell in diapausing pupae as well as non-diapausing, developing pupae.

Electrical properties of the PTTH cells

Electrophysiological characteristics of the PTTH cells are similar to those described for invertebrate NSCs, which typically exhibit resting membrane potential between −30 and −60 mV and produce action potentials with duration of 10–50 msec and amplitude of 40–70 mV, and which are often followed by a prolonged after-hyperpolarization [9–11, 14, 21, 23, 24]. From these characteristics, the somatic action potential of the PTTH cell seems to be dependent on Ca^{2+} like that of the large medial cells (NSC Ia) [9]. In isolated, desheathed brain, the NSCs, including PTTH cells, reportedly often tend to be spontaneously active [9, 21]. In our preparation, however, in which the brain was left nearly intact and bathed in a physiological saline, the PTTH cells were revealed to be almost inactive except some particular stages. Intracellular current injection that exceeded the threshold value elicited spike activities in silent PTTH cells as does in the other *Manduca* NSC types [9, 11]. Importantly, current injection of subthreshold value often caused a fluctuation of the membrane potential ("noise") of 2–5 mV, which sometimes generates an action potential. Although the ionic mechanisms involved in this voltage dependent fluctuation have not been investigated in this study, the rectification in the current voltage relationship at the depolarizing range suggests that there may be involvement of voltage-dependent Ca^{2+} channels and voltage-dependent or Ca^{2+}-activated K^+ channels.

Diapausing vs non-diapausing pupal PTTH cells

Examination of individual PTTH cells during the temporal course of diapause and non-diapause pupal stage revealed several notable differences in electrical properties between diapause and non-diapause pupae. In diapausing pupae the voltage threshold value gradually increased with the progress of diapause development, being consistently higher than non-diapausing pupae ($P<0.01$, t-test). Conversely, the input resistance decreased from nearly 100 MΩ to about 50 MΩ. These changes in electrical properties indicate that the cell becomes less excitable with the progress of diapause development, and at the deep diapausing state (day 10–30) the cell is silent and least excitable. These features of unexcitability of the cell are consistent with the finding by Bowen *et al.* [6] showing that the relative titer of PTTH in the brain and CC-CA complex of diapausing pupae increases at the first 10 days of diapause development. However, these features of unexcitability is not reflected in ultrastructural changes in early diapause PTTH cell somata [13]. It is thus likely that there are different functional and structural levels at which diapause is expressed by the PTTH cells.

The cell in non-diapausing, developing pupae has relatively constant threshold values, input resistance and resting membrane potential except day-2: On that day, the cell showed high input resistance about 90 MΩ, lowest threshold value and least negative membrane potentials (Table 2). Since these differences between diapausing and non-diapausing pupae are apparent as early as one day after pupation (Table 1 and 2), a photoperiodically regulated hormonal balance between ecdysone and juvenile hormone during the larval stage (for review see [12]) may be responsible for the physiological change of the PTTH cell. This would also be convinced by the findings by Bowen *et al.* [7] that an important difference in the relative PTTH titer in the retrocerebral complex between diapausing and non-diapausing pupae is already detectable on day-1. Spontaneously active cells were most frequently encountered on day-2. This stage well corresponds to that for initial increase of ecdysone titer in the hemolymph [4, 7].

Possible mechanisms for PTTH cell activation

Although it is well documented for a number of cases that a photoperiodic clock regulates a PTTH release [12], there is very little information on how such mechanism is involved in activation of the cell. On the basis of the results obtained in this study, two separate mechanisms seem to be responsible for activation of the PTTH cell. One is the membrane property that regulates the membrane potential, the input resistance and the threshold value, and the other is synaptic drive.

As revealed by the current injection experiment, the depolarization of the membrane potential causes a fluctuation or a noise of membrane potential, which often generates the action potential without any synaptic mediation. In active cells the threshold value was as low as −31.2 mV, which is 5–8 mV lower than inactive cells, and in conversely, the input resistance was about 30 MΩ higher than inactive cells. As reported for the group Ia cells in the *Manduca* brain [11], these membrane properties would make the cells more excitable. In active cells of day-2, however, the increase in excitability cannot be interpreted by an increase of input resistance alone, since the current threshold was about 1/5 of that of the inactive cell while the input resistance was less than 1.5 times. In this context, the recent work by Hewes and Truman [14] is especially important. They revealed that the increase in excitability of the ventromedial cells of the *Manduca* brain, which are responsible for the release of eclosion hormone, occurs without alteration of input resistance and resting potential. It is suggested to be regulated by a transcription-dependent process including the synthesis of new RNA and protein. It may be possible that similar events may be involved in the regulation of PTTH cell excitability.

Although the mechanism controlling these membrane properties is unknown, one could argue some possible explanations for it. First, they could be controlled by a developmental program that may be further regulated by an inter-endocrine feedback, like in the ventromedial cells, in which the change in excitability is triggered by a declining ecdysteroid titer in the hemolymph [14]. Ionic composition of the hemolymph and other humoral factors could also be candidates for the regulator as in the case of mammalian vasopressin neurons [18, 26]. Further critical study is deserved to examine these possibilities.

Besides the intrinsic membrane properties, the PSPs may mediate the cellar activity. Although PSPs have been observed in many kinds of insect NSCs [9, 11, 14, 21], their role in activation of the cells has not been clarified yet. In *Manduca* pharate adults, EPSPs have been supposed to induce the spike activity in the group Ia cells [11]. In the PTTH cells of non-diapausing pupae, IPSPs were observed mainly on day-2, which is supposed to be a critical period for pharate adult development ([4], Bollenbacher et al., unpublished data). IPSPs occurred in inactive cells as well. They might prohibit the depolarization of the membrane potential to suppress the spike activity and the subsequent PTTH secretion until an appropriate time for the secretion. However, since the frequency of IPSPs sometimes quite low, we should carefully examine this hypothesis in the future study.

EPSPs seldom occurred in both active and inactive cells of non-diapausing pupae. Since the measurement of the electrical activity was done between 10:00–24:00, there still remains a possibility that excitatory synaptic input is restricted to occur at the remaining hours of a day and is involved in the activation of the cell, like group Ia cells, in which EPSPs occur at a particular time of day under a control of circadian clock [11]. However, hyperpolarization of the cell by injecting the current could reveal no EPSP even in the spontaneously active cell, while some procedure could demonstrate EPSPs in active cells of diapausing pupae. Thus no excitatory synaptic input seems to activate the cell in non-diapausing pupae. Since spike activity without synaptic drive has been reported in other neurosecretory cells such as ventromedial cells [14] and the cells in cockroach pars intercerebralis [17], it might be rather general in insect neurosecretory cells.

In diapausing pupae which underwent 30 days of chilling, EPSPs as well as spontaneous action potentials occurred in a significant portion of the cells (Table 2). It seems unlikely that these spontaneous activities as well as EPSPs are the direct cause of diapause termination, since only 2 (8%) out of 25 control animals which had received 30 days of chilling since day-10 and then were transferred to 25°C initiated adult development (Tomioka, Agui and Bollenbacher, unpublished observation). It is unclear whether these EPSPs occurred as an effect of chilling. We should be careful to discuss about the role of EPSPs in the diapausing pupae. However, one possible role of them may be that they are involved in the maintenance of the basic activity of the cell or in the release of slightest amount of the PTTH to keep the basic synthesis of ecdysone by PG which is detected in diapausing pupae [7]. This view is consistent not only with

the endocrinological evidence that the amount of PTTH in the brain and CC-CA complex gradually decreases 20 days after pupation in diapausing animals [7] but also with the electron microscopical observation demonstrating that the lateral cells of diapausing *Manduca* of the same age show structure relating to secretory activity [5].

The neurotransmitters responsible for the PSPs should be addressed in the future study. In *Bombyx* larvae, acetylcholine has been suggested to be a possible neurotransmitter for PTTH release [29]. Elucidating the involved transmitters is also required to understand the mechanism regulating the activity of the PTTH cells.

In *Manduca*, the photoperiodic clock regulating the PTTH release, hence the diapause or non-diapause development, has been not only localized in the brain but also revealed to work *in vitro* cultured conditions [8]: The brain retrocerebral complex from a short day animal which had been programmed to be diapause can be reprogrammed to be nondiapause *in vitro* by culturing under long-day conditions. A possibility has been suggested that the photoperiodic clock mechanism may reside in the PTTH cells themselves [3]. This is based on the following two findings in *Antheraea*. First, tetrodotoxin which blocks voltage dependent Na^+ channels hence the neuronal action potentials, failed to affect either the maintenance of diapause in LD12:12 or the termination of diapause in LD 17:7, indicating that nerve conduction probably does not play a role in the photoperiodic mechanism affecting PTTH release. Second, by surgical excision the area of the brain sensitive to photoperiod was demonstrated to be the region of lateral NSCs [34]. In the present study, no evidence has been yielded supporting the idea that the PTTH cell has the photoreceptor and the photoperiodic clock. Conclusion about this issue will be provided by the experiment during the sensitive stage for the photoperiod, i.e., the last larval stage in *Manduca* [8].

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