11, and 13 for -III. To establish the positions of cysteine residues, 4K PTTH-II was treated with dithiothreitol and then with iodoacetamide to generate the *S*-carboxamide methyl (CAM) derivative, which was subjected to Edman degradation. The PTH derivative of CAM-Cys was identified at cycles 6, 7, and 11, indicating the presence of Cys at positions 6, 7, and 11 of 4K PTTH-II. This suggests that 4K PTTH-I and -III also have Cys at the equivalent positions (Fig. 1A).

Almost half of the amino acids in the NH_2-terminal sequences are common to the three peptides. Amino acid substitution apparently occurs in such a way that the hydrophilic or hydrophobic nature of the amino acid residues is retained at their respective positions; Val is replaced by Ile, Phe by Leu, Arg by Gln, Thr by Ser, Leu by Val, and Leu by Ala. All the substitutions described above, except for that of Leu by Ala at position 17, would be possible by single nucleotide changes. Because all three hormones have approximately the same level of activity, we conclude that 4K PTTH's can tolerate substitutions at these positions and maintain biological activity. Replacement of Arg by Gln at position 8 in 4K PTTH-III may account for its being more acidic than 4K PTTH-I and -II. It is yet unknown whether a single silkworm has all three molecular species.

Molecules have been purified from the tobacco hornworm, *Manduca sexta* (*9*), and the blow fly *Calliphora vomitoria* (*10*), which are homologous in amino acid composition to vertebrate insulin, although their primary structures have not been proposed. It is of great interest that 4K PTTH's contain regions homologous with insulin A chain (*11*), insulin-like growth factors (IGF) (*12*), and a polypeptide with multiplication-stimulating activity (MSA) (*13*). Figure 1B shows the sequences of IGF-I (from positions 42 to 60), 4K PTTH-II (from positions 1 to 19), and human insulin A chain (from positions 1 to 19). Approximately half of the 4K PTTH-II sequence is identical to the IGF-I and human insulin A chain sequences. Cod and toadfish insulin A chain, not shown here, are even more homologous to 4K-PTTH-II (58 percent) (*11*). The differences in insulin A chain that occur among vertebrates (including mammals, fish, and birds) are located at positions 4, 8 to 10, 12 to 15, 17, and 18 (*11*). The 4K PTTH-II differs from human insulin A chain in its sequence at all of these positions except for position 12. This is accompanied by an additional substitution at position 5. Porcine insulin (Sigma) failed to show PTTH activity at a dose of 1 μg, which is about 10^4 times the minimal active dose of 4K PTTH's. The 4K PTTH's failed to bind to guinea pig antibody to porcine insulin (Miles) as measured by radioimmunoassay (*14*). These data suggest the existence of a common ancestral peptide molecule that evolved into peptides with different functions in insects and vertebrates.

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5. Isolation of 4K PTTH species. *Bombyx* adult heads, homogenized successively in acetone and 80 percent aqueous ethanol, were extracted three times with 2 percent aqueous sodium chloride. The extract was heated (100°C) and centrifuged to remove the precipitate. This was followed by ammonium sulfate precipitation and two extractions with acetone. The precipitate was then solubilized in water, mixed with nine volumes of a saturated aqueous solution of picric acid, and the precipitate appearing after centrifugation was dissolved in 0.1*M* tris-HCl (*p*H 7.8). After a final extraction with acetone, the pale brown precipitate, designated as "crude 4K PTTH," was dissolved in 0.1*M* tris-HCl (*p*H 7.8), subjected to gel filtration on Sephadex G-50 (fine; 6 by 67 cm), and eluted with 0.2*M* ammonium acetate (250 ml/hour). The pooled active fractions were then applied to a DEAE-Sepharose CL-6B column (2.6 by 40 cm) that had been equilibrated with 0.2*M* ammonium acetate. The column was washed with 0.4*M* ammonium acetate and eluted with 1*M* acetic acid (20 ml/hour). The eluate was then charged onto an SP-Sephadex C-25 column (1.4 by 28 cm) that had been pretreated with 0.1*M* ammonium acetate buffer (*p*H 4.2). The pooled active fractions were lyophilized, dissolved in 0.1*M* tris HCl (*p*H 7.8), and applied to a Sephadex G-50 (superfine) column (1.8 by 170 cm), which was eluted with 0.2*M* ammonium acetate (8 ml/hour). The active fractions were combined (designated as "highly purified 4K PTTH") and applied to a DEAE-Sepharose CL-6B column (1 by 54 cm) equilibrated with 0.05*M* tris-HCl containing 0.1*M* sodium chloride. The column was washed with the same solution and then eluted with a gradient of 0.1 to 0.5*M* sodium chloride in 0.05*M* tris-HCl (*p*H 7.8) (8 ml/hour), while fractions (3.5 ml) were collected. The isolates 4K PTTH-I, -II, and -III were recovered from tubes 71 to 73, 74 to 76, and 83 to 89, respectively.
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Voltage-Dependent Calcium Channels in Glial Cells

Abstract. *The electrophysiological properties of glial cells were examined in primary culture in the presence of tetraethylammonium and Ba^{2+}, a treatment that reduces K^+ permeability of the membrane and enhances currents through voltage-dependent Ca^{2+} channels. Under these conditions, glial cells showed both spontaneous action potentials and action potentials evoked by the injections of current. These responses appear to represent entry of Ba^{2+} through Ca^{2+} channels because they were resistant to tetrodotoxin but were blocked by Mn^{2+} or Cd^{2+}.*

Glial cells are considered the ubiquitous yet silent partners of neurons. They appear to play a complex supportive role in nervous tissue (*1–3*). The syncytium formed by glial cells is believed to be important in controlling the extracellular milieu surrounding nerve cells by buffering potassium (*4*), by uptake of neurotransmitters (*2*) and transport of nutrients from the blood (*5*), and by providing mechanical support for the neuronal matrix. Glial membranes are also thought to be exclusively permeable to K^+ (*3*), and active voltage-dependent responses have not been reported. However, in their original work on the properties of glial cells, Kuffler and Potter (*6*) noted that the "steady-state resistance in some glial cells dropped when depolarizations were larger than 20–30 mV." Other workers have reported that Ca^{2+} influx is increased with high extracellular K^+ (*7*). The pronounced K^+ permeability of glial membranes could shunt and mask any voltage-dependent responses. Moreover, in most studies in which the permeability of glial membranes to ions other than K^+ was examined (*8*), the stable membrane potential was measured in response to changes in extracellular K^+.

Because of the time necessary to change extracellular K^+ (several minutes), any voltage-dependent conductances would be inactivated during the change and hence would not be observed after K^+ concentrations reached a new level. Therefore, I examined the electrophysiological properties of glial cells when K^+ currents were reduced. This report describes the observation in glial cells of voltage-dependent responses due to Ca^{2+} channels.

Glial cells were prepared in primary cultures by the method of Booher and Sensenbrenner (*9*) and Kimelberg *et al.* (*10, 11*). Recordings were obtained from cultures 2 to 8 weeks old. In growth medium containing 10 percent fetal calf serum, the cells had a polygonal shape and formed a flat layer, as reported earlier (*10*). However, after exposure to 1 m*M* dibutyryl adenosine 3′,5′-monophosphate (cyclic AMP) for 2 hours the cell bodies became rounded, with fine branching processes, and were much easier to impale. All recordings were obtained from cells after 2 to 8 hours of exposure to dibutyryl cyclic AMP. Cover slips with cultured cells were transferred to a temperature-controlled recording chamber (33°C) mounted on an inverted microscope. Cells were impaled with two microelectrodes. One microelectrode was for current injection, and sometimes used for intracellular staining, and contained either KCl (0.5*M*) or Lucifer yellow (5 percent); the other was for voltage recording and contained KCl (0.5*M*). The cells were continually superfused with physiological solution (*12*) with 5 m*M* K^+ or solution in which Ba^{2+} replaced Ca^{2+} and phosphates and sulfates were omitted.

Primary cultures prepared in this manner are 70 to 80 percent glial cells (*13*). The major contaminants are fibroblasts, but oligodendrocytes are occasionally seen on top of the astrocyte monolayer. However, after exposure to dibutyryl cyclic AMP only astroglia undergo the morphological change in which the cell body becomes rounded, with radially oriented processes (*13*). The fibroblasts are flat and spindle-shaped and are never impaled (*14*). Therefore, on the basis of electrophysiological properties in control solution (see below) and the morphological change in dibutyryl cyclic AMP, these cells were identified as glial. Further verification that the cells were glial was obtained when recorded cells were immunohistochemically stained for glial fibrillary acidic protein (GFAP), a specific glial marker (*13*). In six experiments in which the cells were visualized during recording and subsequently stained for GFAP, the impaled cell showed GFAP staining (Fig. 1).

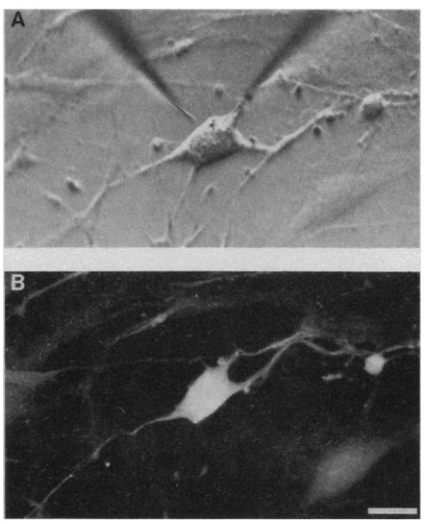

Fig. 1. Glial cells from which recordings are made in culture contain glial fibrillary acidic protein (GFAP). (A) Micrograph of a glial cell during recording. In the presence of dibutyryl cyclic AMP, glial cells had a distinct morphology, with a rounded cell body and radial processes. Only glial cells undergo such a morphological transformation in culture (*13*). The major contaminant in cultures are fibroblasts, which are flat and spindle-shaped and were not impaled. Glial cells were impaled with two microelectrodes, one for current injection and the other for voltage recording. (B) The cells were immunohistochemically stained for GFAP to ensure that the cells used in recording were indeed glial cells (*10, 13*). After the recording, the cultures were fixed for 30 minutes in 1 percent formaldehyde in phosphate-buffered saline (PBS). The cells were then washed three times with PBS and incubated with monoclonal antibody to GFAP (Lab Systems). This was followed by incubation with biotinylated antibody to mouse immunoglobulin G. The antigen was visualized with avidin-conjugated rhodamine. Cultured cells showed the typical GFAP staining, as reported by others (*10, 13*). Cells that had been previously used for recording were identified by the immunohistochemical staining as containing GFAP. With control procedures in which only incubation with the antibody to GFAP was omitted there was no staining of the intracellular fibrils in the glial cells. Scale bar, 20 μm.

Intracellular recordings were obtained from 40 glial cells in culture. In control solution (5 m*M* K^+), membrane potentials were 77.8 ± 4.8 mV and input resistance was 4.1 ± 2.1 megohms (mean ± standard deviation). These values are similar to those in other reports of glial properties in culture, in other in vitro preparations, and in vivo for the same K^+ concentrations (*3*). In no cells were action potentials recorded in control solution. Extensive dye coupling between glial cells was observed (*15*), so that when one cell was injected with Lucifer yellow, cells immediately surrounding it were stained, and the intensity of staining diminished with distance from the injected cell.

In testing for voltage-dependent calcium channels, recordings were obtained when 5 m*M* tetraethylammonium (TEA), 5 to 10 m*M* Ba^{2+}, and 10^{-6}*M* tetrodotoxin (TTX) were superfused. Tetraethylammonium blocks Ca^{2+}-activated K^+ channels in glial cells (*16*). In four cells in which impalements were maintained during the change from control solution

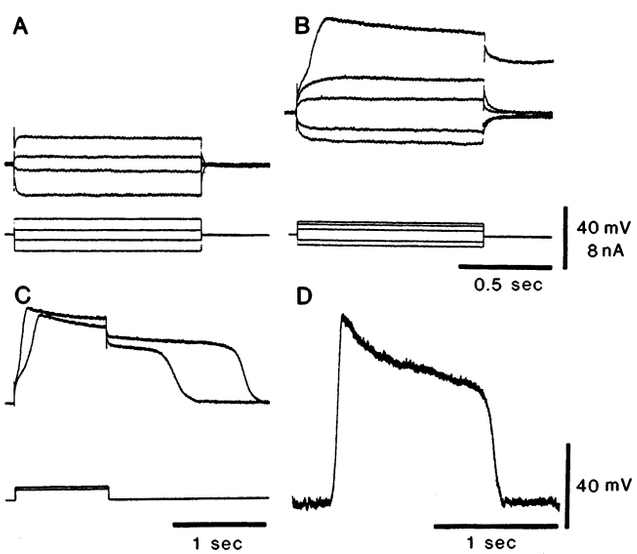

Fig. 2. Glial action potentials evoked in TEA-Ba^{2+}-TTX. (A) In control solution, glial resting potential was −75 mV and the input resistance was 7 megohms. No active responses were evoked by intracellular current injection. (B) After perfusion of TEA (5 m*M*), Ba^{2+} (10 m*M*), and TTX (10^{-6}*M*), the membrane potential depolarized by 33 mV and the input resistance increased by 130 percent to 16 megohms. At this time, depolarizing current pulses evoked prolonged action potentials. (C) The plateau of the action potentials outlasted the current injection, and the duration was variable. (D) Spontaneous action potential in TEA-Ba^{2+} solution. The illustrated Ba^{2+} spike was recorded in a glial cell that showed spontaneous rhythmic action potentials.

to TEA-Ba^{2+}-TTX solution, membrane potentials depolarized by approximately 25 to 30 mV, and the input resistance increased 76 percent (range, 30 to 130 percent). In control solution before superfusion with test solution, these cells had the characteristics of typical glial cells. However, after the extracellular solution changed to one containing TEA-Ba^{2+}-TTX, prolonged regenerative action potentials (Ba^{2+} spikes) were evoked by depolarizing current pulses (Fig. 2).

Ba^{2+} spikes were also studied in 20 other cells in which impalements were obtained initially in the TEA-Ba^{2+}-TTX solution. Glial action potentials were relatively slow, with durations up to several seconds, and were overshooting. The slow spikes were observed in the presence (or absence) of TTX and therefore were not due to sodium channels. Spontaneous Ba^{2+} spikes were observed in some cells (Fig. 2d). These had variable plateau durations but could occur with a regular interspike interval. The frequency of spontaneous Ba^{2+} spikes was dependent on membrane potential and could be blocked by hyperpolarization. Ba^{2+} spikes were blocked in the presence of Mn^{2+} (5 m*M*) or Cd^{2+} (1 m*M*) (Fig. 3). These experiments indicate that glial cells have voltage-dependent Ca^{2+} channels because the Ba^{2+}-dependent spikes are not blocked by TTX but are blocked by Cd^{2+} or Mn^{2+}. Ba^{2+} passes through the Ca^{2+} channel more easily than Ca^{2+} does (*17*) and decreases the resting K^+ permeability of membranes (*18*). Therefore, responses due to Ca^{2+} channels are enhanced in the presence of Ba^{2+}. Cd^{2+} and Mn^{2+} are effective blockers of Ca^{2+} channels in many systems (*19*).

Voltage-dependent responses have not been previously observed in glial cells (*8, 20*). However, examination of glial properties under conditions that maximize the observation of Ca^{2+}-dependent responses is critical. For example, Ca^{2+} spikes in presynaptic terminals of the squid giant axon were observed only in solutions containing TEA and increased Ca^{2+} (*21*). The high resting K^+ permeability and low input resistance of glial cells combined with the extensive electrotonic coupling between these cells (*15*) may make it difficult to observe voltage-dependent responses. Voltage-dependent changes in conductance to Ca^{2+} would not normally be observed electrophysiologically unless the Ca^{2+}-activated K^+ channel that is present in glial cells is blocked (*16*). Further work will be necessary to determine if glial cells in vivo also have Ca^{2+} channels and what the functional role of Ca^{2+} channels may be. Present hypotheses concerning the role of glial cells in neuronal tissue could be altered. For example, voltage-dependent influx of Ca^{2+} could activate Ca^{2+}-dependent K^+ channels (*16*) and displace intracellular K^+ from glia, thereby allowing glial cells to play a role in controlling extracellular K^+, not just passively redistributing K^+ (*22*). Hypothetically, this could occur when glial cells are depolarized by extracellular K^+ released during activity in adjacent neurons. Another potential role of Ca^{2+} influx into glial cells may be to control motility, such as retraction of glial processes during activation of hypothalamic neuroendocrine cells (*23*), by acting on contractile proteins (*24*).

Schmitt *et al.* (*25*) discussed how relatively small but widespread depolarization (for example, from raised extracellular K^+ levels) could affect integration in a neuronal network. Activation of Ca^{2+} channels in the glial syncytium could alter the kinetics and pattern of control of extracellular ions such as K^+. Ca^{2+} influx into glial cells could increase extracellular K^+. Therefore, the glial Ca^{2+} channel may be important in the regulation of excitability of central nervous system structures. Abnormalities may result in hyperexcitability disorders such as seizures.

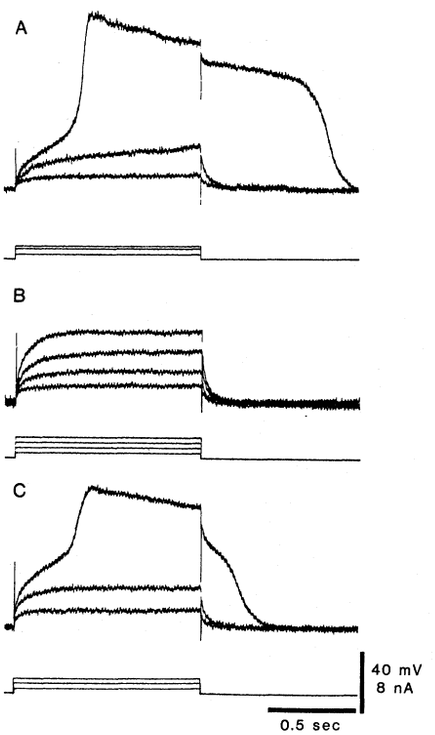

Fig. 3. Glial action potentials are blocked by Cd^{2+}. (A) In TEA (5 m*M*), Ba^{2+} (10 m*M*), and TTX (10^{-6} *M*), action potentials were evoked by depolarizing current injection. This cell also displayed spontaneous action potentials. (B) After perfusion of 1 m*M* Cd^{2+}, current-evoked action potentials were blocked, and no spontaneous action potentials were observed. The evoked (and also spontaneous) action potentials recovered when Cd^{2+} was washed out.

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