Passively propagated spikes from the soma of cells in pineal gland of guinea pigs

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PARKINGTON, H. C., H. A. COLEMAN, AND I. MCCANCE. *Passively propagated spikes from the soma of cells in pineal gland of guinea pigs.* Am. J. Physiol. 257 (Cell Physiol. 26): C802–C809, 1989.—The membrane properties of cells within the pineal gland of guinea pigs were studied using intracellular electrophysiological techniques. The electrotonic responses to intracellular current injection decayed with a single exponential in ~60% of cells but was preceded by a quicker component in the remainder. The membrane time constant was 2.8 ms. Depolarization beyond −29 mV activated an outward current that reversed at around the value of the resting potential. Hyperpolarization activated a slow inward current. Spikes occurred in response to activation of α-adrenoceptors. They were resistant to tetrodotoxin but were abolished by nifedipine and verapamil, suggesting that calcium carries the current during their upstroke. Spikes could not be evoked by depolarizing current pulses of 1-ms to 2-s duration. The responses to hyperpolarizing current steps or voltage-clamp steps applied during the peak of spikes evoked by nerve stimulation were indistinguishable from the responses to those applied between spikes. During nerve stimulation, fluctuations were observed in the current trace of cells under voltage clamp, indicating that the spikes could not be voltage clamped successfully. It is concluded that the spikes occurring in response to nerve stimulation are generated on the processes of the pinealocytes and are passive in the soma.

pinealocytes; voltage clamp; membrane properties; cell processes

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STUDIES IN THIS LABORATORY using intracellular microelectrodes have identified two types of cells in isolated pineal glands of guinea pigs. The responses of cells of type II to nerve stimulation resemble observations in some blood vessels (20). In the present study we describe the electrophysiological properties of cells of type I, as these are considered to be pinealocytes.

In a previous study it was demonstrated that cells of type I respond to sympathetic nerve stimulation with depolarization that is a consequence of activating β-adrenoceptors and spikes that result from α-adrenoceptor activation. Intracellular injection of Lucifer yellow showed that each cell consists of a soma that is 10–20 μm in diameter and two to five processes, at least one of which terminates in a bleb that is ~5 μm in diameter. Dye injected into any one cell does not spread to neighboring cells, indicating that the cells are not coupled to each other (14).

In rats, induction of serotonin N-acetyltransferase, essential for the synthesis of melatonin, is mediated by β-adrenoceptor stimulation and does not occur in the absence of external calcium (24). Activation of α-adrenoceptors also results in an increase in cytoplasmic calcium in pinealocytes in this species, and it has been shown that this is a consequence of calcium influx rather than release from internal stores (19). Because calcium influx is controlled by membrane potential in a variety of tissues (1, 2, 7, 10), the question remains as to the role, if any, of changes in membrane potential in the secretion of melatonin. A study of the membrane properties of pinealocytes using intracellular electrophysiological techniques, including single-electrode voltage clamp, would seem to be important for an understanding of the mechanism of transmitter action and thus for melatonin production and/or release.

METHODS

Male guinea pigs (200–400 g) were housed at 22°C under conditions of 12-h artificial light and 12-h dark and were fed fresh vegetables and pellets ad libitum. Each animal was killed by cervical dislocation. The pineal gland was removed from the brain; freed from the attached sheet of dura, habenulae, and striae medullaris; and transferred to a bath through which flowed physiological solution having the following composition (in mM): 120 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1 Na_2HPO_4, 25 $NaHCO_3$, and 11 glucose, gassed with 95% O_2-5% CO_2 and maintained at 37°C. The gland was attached to the silicone rubber base of the bath by pins through adhering connective tissue, and the habenular end was anchored when introduced into a small suction pipette (14). The depth of solution was kept low in order to keep electrode capacitance to a minimum.

The suction pipette was used for electrical stimulation of the nerves. Preparations were stimulated with trains of pulses from a Digitimer stimulator. The duration of each pulse within the train was 0.1 ms, the voltage intensity was 10–30 V (stimulator dial setting), the frequency of pulses was 1–10 Hz, and the number of pulses in the train was 2–10.

Membrane potentials were recorded using conventional glass microelectrodes pulled on a Brown-Flaming puller (Sutter Instruments), filled with 1 M KCl and having resistances of 50–100 MΩ. The microelectrodes were connected to a high-input impedance preamplifier (Axoclamp-2, Axon Instruments), and the electrode capacitance was optimally neutralized by monitoring the voltage decay in discontinuous current-passing mode. The electrode time constant was then negligible compared with the membrane time constant. In some experiments the Axoclamp was used in its single-electrode, voltage-clamp mode with a switching frequency of 2–7 kHz. With this arrangement it is possible to record membrane currents from voltage-clamped pineal cells within intact glands. However, this technique has a limited response time; in the present study it was not possible to resolve the currents within the first 1–2 ms of a step change in potential. The data were stored in analog form on magnetic tape (Tandberg tape recorder) with an overall frequency response of either 1.25 or 2.50 kHz and were subsequently analyzed on a Pericom computer using the software package pClamp (Axon Instruments).

Intracellular impalements were made all along the gland from its apex to within 1 mm of the stimulating electrode. Results used were only from those impalements that had been maintained for at least 30 min. In estimating the resting membrane potential and the characteristics of the spikes the results from four to seven cells were combined for each tissue.

The statistic quoted with each mean is its standard error, which was based on the number of animals rather than the number of cells, except when stated otherwise. A significance level of 0.05 was used in testing (Student's *t* test).

The drugs used in this study were tetrodotoxin (Boehringer Mannheim), phenylephrine HCl (Sigma Chemical), prazosin (Pfizer), tetraethylammonium chloride (TEA; Sigma Chemical), nifedipine (Bayer), and verapamil (Isoptin, Knoll).

RESULTS

Passive membrane properties. The resting membrane potential of type I cells was −51 ± 2 mV (44 animals). The membrane depolarized by 43 ± 2 mV (7 animals) per 10-fold increase in external potassium concentrations in the range of 15–100 mM. There was a smaller dependence of membrane potential on the concentration of external potassium when the latter was <15 mM, as might be expected from the Goldman-Hodgkin-Katz voltage equation.

Square steps of current were injected into the cells through the microelectrode in order to study the passive membrane properties. An average of 20–100 steps that hyperpolarized the cells by <5 mV was used. The rising phase of the electrotonic potential, and its decay after the end of the current step, were analyzed separately and, for any given cell, the time courses of the two were not different. In glands from 14 animals, the change in the membrane potential followed a single exponential in 13 of 22 cells (59%) (Fig. 1*A*). For the other nine cells a faster component preceded an exponential change in the membrane potential (Fig. 1*B*). A similar effect occurs in many neurons in which the initial rapid component is ascribed to the current flowing down electrically significant cell processes, while the final exponential component is considered to be a function of the membrane time constant (τ_m) (11, 15). Therefore τ_m was calculated from the final, exponential component for these pineal cells. τ_m was not significantly different in cells in which the response followed a single exponential compared with cells in which the final exponential component was preceded by a faster one and so the data from all 22 cells were combined. Overall, τ_m was found to be 2.8 ± 0.2 ms and the input resistance (R_{in}) was 128 ± 11 MΩ.

Changes in membrane conductance. When both hyperpolarizing and depolarizing current steps of larger amplitude were injected into the cells, conductances were activated such that the membrane potential relaxed back in the direction of the resting potential (Fig. 2).

These conductances were studied in greater detail in cells from 10 preparations using a switching single-electrode voltage clamp: an example is shown in Fig. 3*A*.

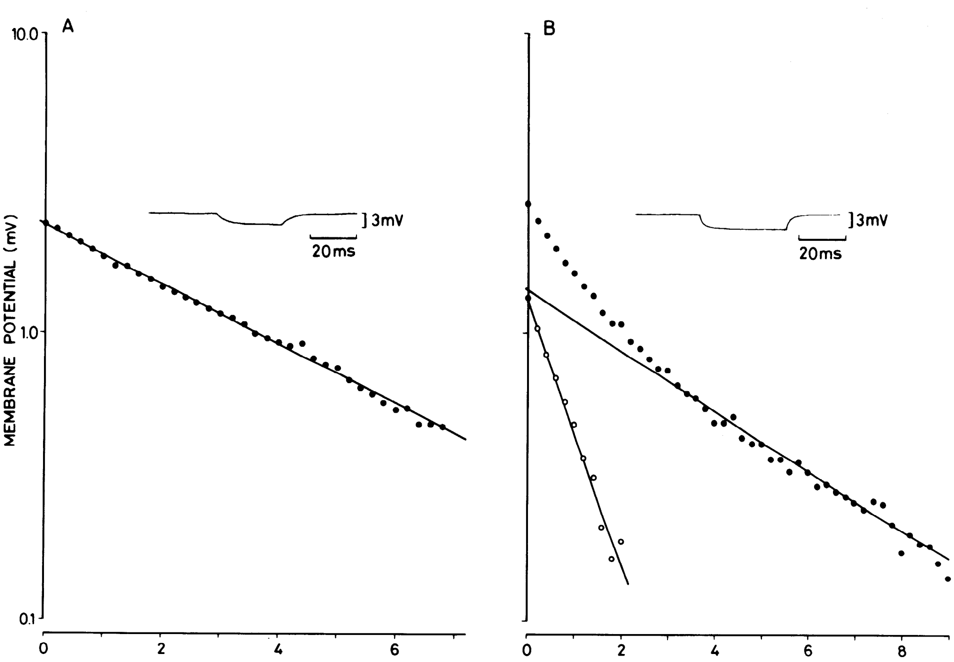

FIG. 1. *A*: small hyperpolarizing current pulses evoked an electrotonic potential with a single exponential in 59% of pinealocytes. Shown is an example of average of 100 steps in 1 such cell (with electrode time constant subtracted). *B*: an example in 1 cell of 41% in which final exponential decay (filled circles) was preceded by a faster component (open circles) (average of 80 steps).

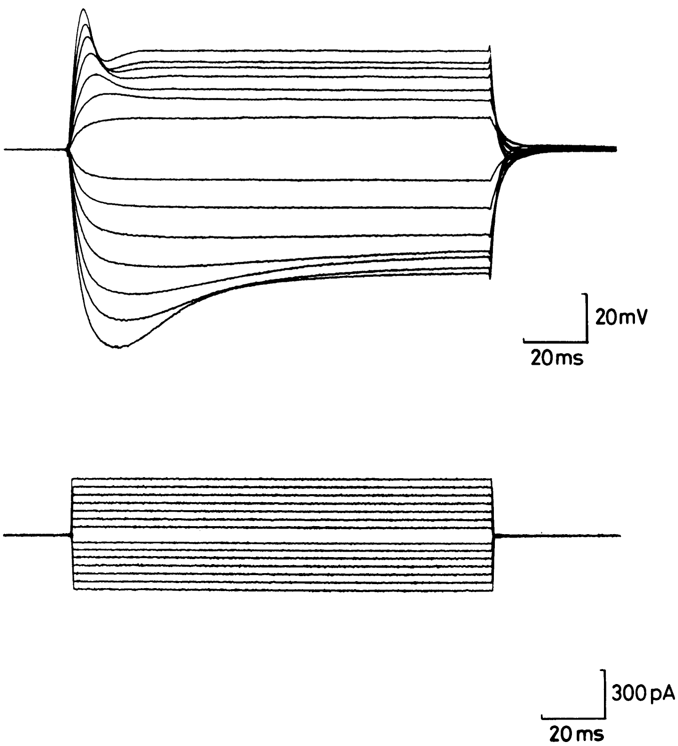

FIG. 2. Hyperpolarizing and depolarizing current steps evoked time-dependent changes in membrane potential.

The cells had a resting slope conductance of 7.9 ± 0.7 nS (n = 10). The resting conductance appeared to rectify slightly in the depolarizing direction. This is probably due in large part to the slowness of the clamp to settle and thus represents contamination with other currents, although some rectification might be expected according to the Goldman-Hodgkin-Katz current equation. During depolarization of the membrane with voltage-clamp steps, a time-dependent outward current became evident at −29 ± 3 mV, i.e., 22 ± 3 mV positive of the resting potential. It reached its maximum value within 50 ms at this potential. This current became larger and quicker in onset at more depolarized potentials, such that the slope conductance at 40–150 ms was increased 4.9 ± 0.9-fold to reach a maximal value of 40 ± 9 nS (n = 10). Partial inactivation of the outward current seemed to occur for the larger voltage steps in 5 of 10 cells, and the possibility cannot be ruled out that a slow inward current was developing. For these cells, the slope conductance for the peak current increased 4.8 ± 1.3-fold from rest to 42 ± 13 nS and sagged back to ~40 nS in <100 ms. An example of a current-voltage curve is illustrated in Fig. 3*B*, which shows the leakage current, taken as the current immediately after the initial capacitive current, and the steady-state current at the end of the voltage steps.

The outward current was studied in greater detail by applying a two-step voltage-clamp routine. The membrane was held at the level of the resting potential and was then stepped to a potential at which the channel was maximally activated (usually −10 to −15 mV) and held for a duration of 40–100 ms (which remained constant for all steps in a given cell but varied for different cells). The membrane was then stepped back to various potentials (Fig. 4*A*). The current at the beginning of the second step was plotted against the membrane potential for that step and gave the current-voltage curve for the maximally activated channels carrying the outward current (Fig. 4*B*). This current rectified strongly, such that the inward component was much smaller than most of the

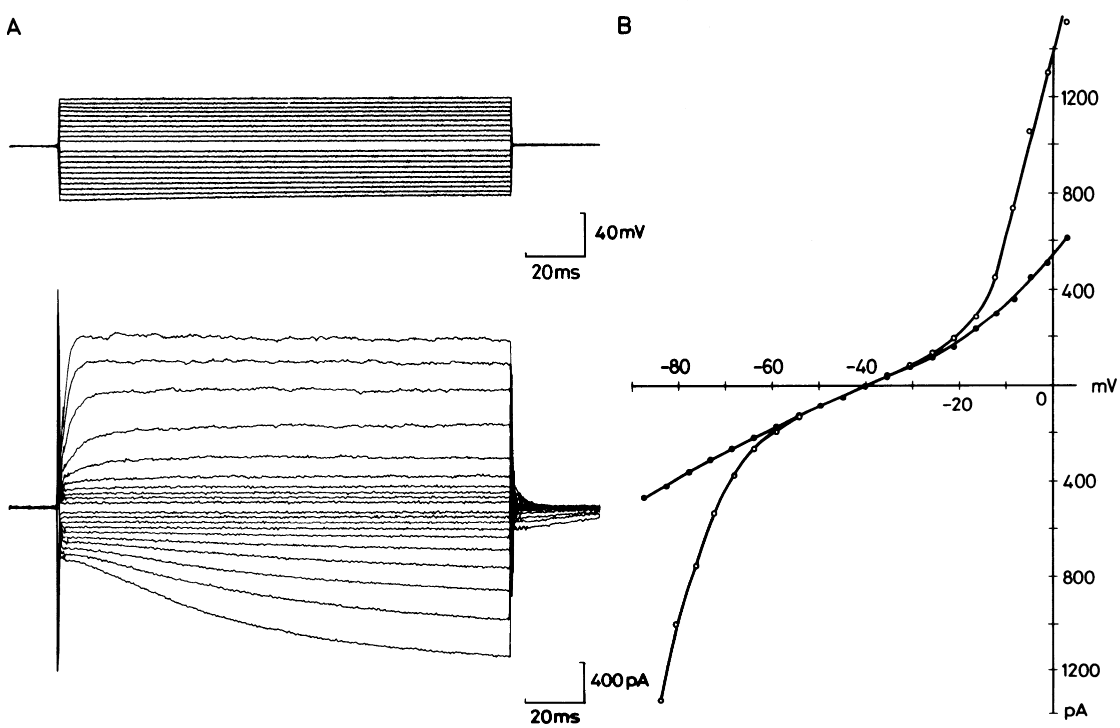

FIG. 3. *A*: membrane was depolarized and hyperpolarized under voltage clamp (*top*), and currents (*bottom*) were recorded. These recordings were from a different cell to that in Fig. 2. *B*: leakage (filled circles) and final (open circles) currents were plotted against membrane potential.

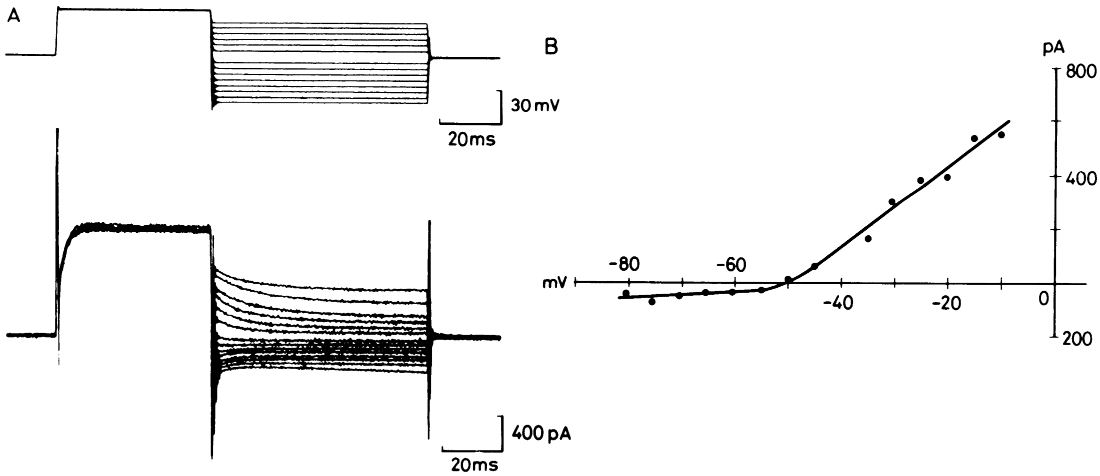

FIG. 4. *A*: membrane was depolarized by 45 mV for 50 ms and then stepped to various potentials (*top*), and current (*bottom*) was recorded. *B*: current at beginning of 2nd step was plotted against membrane potential for that step. (Leakage current was subtracted.)

outward current. Rectification is to be expected from the Goldman-Hodgkin-Katz current equation for a current that is carried mainly, if not solely, by potassium ions as a consequence of a much greater concentration of the charge carrier inside the cell compared with outside the cell. The apparent degree of rectification may be exaggerated by the limited response time of the single-electrode voltage clamp. This is because the inward currents have a faster time course than the outward currents, and therefore the amplitude of the "instantaneous" inward currents would tend to be underestimated compared with the outward currents. The maximum slope conductance of the curve, which occurred when the current was outward, was 22 ± 4 nS (n = 4). The reversal potential, taken as the intercept of this current-voltage curve and the current-voltage curve for the leakage current, was −5 ± 2 mV (n = 4) from the resting membrane potential.

Hyperpolarizing voltage-clamp steps activated a voltage- and time-dependent inward current that activated with a slower time course (Fig. 3*A*). For cells in which voltage-clamp steps of 120 ms or longer duration were applied, the membrane slope conductance increased 4.5 ± 0.4-fold from rest to a value of 34 ± 5 nS (n = 6) (Fig. 3*B*). When voltage steps of 1.5- to 1.7-s duration were applied, this current became evident when the membrane was hyperpolarized by <5 mV. The current did not appear to inactivate, even for larger steps.

TEA (10 mM), a blocker of some potassium channels, was applied to seven cells (in 7 preparations) under voltage clamp with voltage steps of 40- to 150-ms duration. A paired t test indicated that there was no effect on the resting conductance or on the value of the conductances activated by depolarization or hyperpolarization.

Spikes. As described, spikes occurred in response to nerve stimulation. However, they appeared only after a delay of 2.44 ± 0.1 s (38 preparations). Most often the spikes were variable in amplitude and time course, with some having a simple rise and fall while others were more complex, with humps occurring on the rising or falling phase or both (Fig. 5). The spikes were of small amplitude, the average for the largest being 22 ± 3 mV

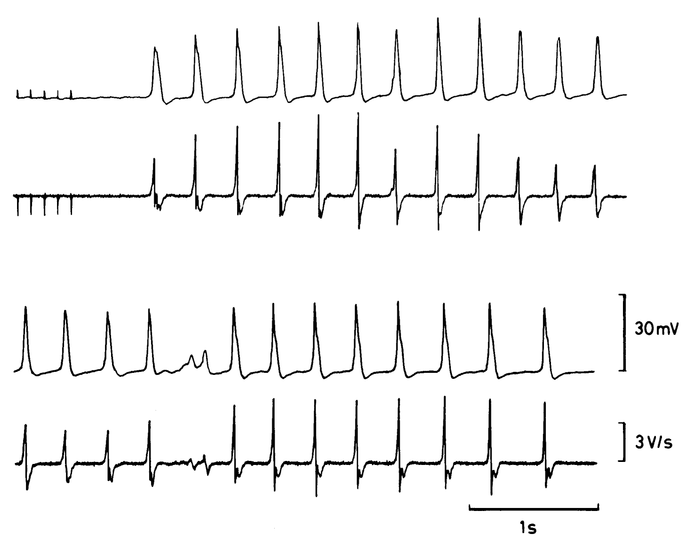

FIG. 5. Spikes observed in response to nerve stimulation were variable in amplitude (*top*) and maximum rate of rise (*bottom*). Humps occurred on rising or falling phases or both.

(39 preparations). Their time course was slow with a maximum rate of rise of 1.9 ± 0.3 V/s and maximum rate of fall of 1.7 ± 0.2 V/s.

Depolarizing current steps, with durations from 1 ms to 2 s, were injected into the cells, but regenerative action potentials could not be elicited despite the membrane being depolarized by up to 60 mV (see Fig. 2). Depolarizing current steps produced a peak, spikelike response only at depolarizations in excess of ~20 mV and was not affected by 10^{-7} or 10^{-6} M nifedipine. The amplitude was graded with the amplitude of the current step and was not considered to represent a regenerative response.

In a further attempt to elicit regenerative action potentials the membrane of the entire cell (soma and processes) was depolarized by raising the external concentration of potassium to 10, 15, 20, 40, or 60 mM, but the only spikes observed were those that could be blocked by the α-adrenoceptor antagonist, prazosin. These results suggested that the membrane of the cell might not be capable of being activated by depolarization alone.

It seemed possible that the spikes observed in response to nerve stimulation might reflect active responses initiated on the processes of the pinealocytes or on the small bleb that terminates at least one of these processes and might cause passive depolarization of the soma. If this were the case then the responses might be expected to become attenuated as they propagate along the fine processes and be recorded as spikes in the soma, accounting for their small size. The possibility of a passive soma was tested further.

1) Small hyperpolarizing or depolarizing current steps of 3- to 5-ms duration were applied to six cells during (Fig. 6*B*) and between (Fig. 6*A*) the spikes induced by nerve stimulation. Spikes with electrotonic potentials on the peak were digitally subtracted from spikes without electrotonics (Fig. 6*C*). The amplitude and time course of the electrotonic potential did not change when it occurred during the peak of a spike compared with its amplitude and time course between spikes.

2) Spikes were induced with phenylephrine, and, when the soma was voltage clamped at the resting membrane potential, fluctuations were observed in the current (Fig. 7). The responses to voltage steps applied (third step) or terminated (fourth step) at the peak of the inward currents were not different from the responses to voltage steps applied between the fluctuations in current (first and second steps). This indicated that there was no change in membrane conductance in the soma during the spike and that the fluctuations in current reflected inadequate voltage clamping of the membrane at the site of initiation of the responses.

Ionic basis of the spike. To study their ionic basis, spikes were elicited by including the α-adrenoceptor agonist, phenylephrine, in the perfusing solution in order to circumvent possible effects of drugs and ionic manipulations on neural transmission.

These spikes were not affected by tetrodotoxin (10^{-7} or 10^{-6} M). When the sodium in the perfusing solution was lowered to 20 or 4 mM by substituting tris(hydroxymethyl)aminomethane (Tris) or choline, spike activity stopped abruptly after ~5 min, but the amplitude and maximum rate of rise of the last few spikes were not substantially changed. This suggests that sodium may be required for the initiation of spike activity but is unlikely to carry the inward current during the upstroke of the spike.

The amplitude and maximum rate of rise of spikes was progressively reduced when increasing concentrations of nifedipine or verapamil were added to the perfusing solution, and spikes were abolished when the concentrations of these blockers reached 10^{-6} and 10^{-5} M, respectively.

A fast peak appeared during the early phase of the spike in the presence of 10 mM TEA (Fig. 8). However, TEA did not seem to increase the duration of the spike and had no effect on the after hyperpolarization.

DISCUSSION

The results in this paper describe some of the passive and active electrical properties of cells within intact pineal glands of guinea pigs. These cells are the type I cells of the previous study (14). Small spikes can be recorded in response to nerve stimulation, and similar events are evoked by the exogenous application of α-adrenoceptor agonists. In the present study these spikes were found to be resistant to block by tetrodotoxin but were blocked by nifedipine, suggesting that a calcium current underlies the spike.

The spikelike events that appeared early during responses to depolarizing current steps were not considered to be regenerative. First, they occurred only when the membrane was depolarized beyond around −30 mV, the level of depolarization attained by only the largest spikes in response to nerve stimulation. Second, they were unaffected by nifedipine, which abolished the spikes in response to nerve stimulation or to perfusion with phenylephrine. Third, no inward current corresponding to a regenerative action potential was observed under voltage clamp. The peak events can be explained in terms of the

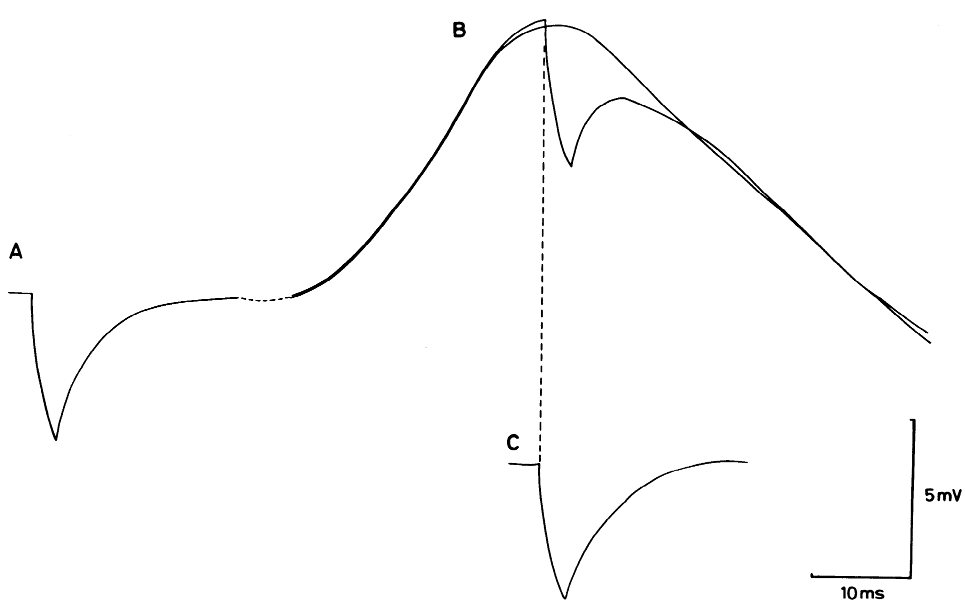

FIG. 6. Hyperpolarizing current steps were applied between (*A*) and during (*B*) spikes evoked by nerve stimulation. Spikes with electrotonic potentials on peak were digitally subtracted from spikes with no electrotonics, and resulting electrotonic potential (*C*) was not different in amplitude or time course from that occurring between spikes (*A*).

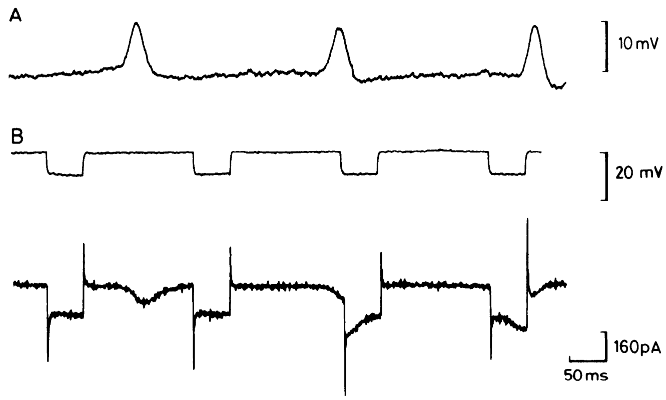

FIG. 7. *A*: spikes occurred in pineal gland when 10^{-6} M phenylephrine was included in perfusing solution. *B*: membrane of soma was clamped at resting potential in continued presence of phenylephrine. Membrane potential was hyperpolarized by 7 mV every 200 ms. Steps coinciding with spontaneous inward currents were identical to those that occurred between currents, indicating that there was no change in conductance in soma. (Both *A* and *B* in same cell.)

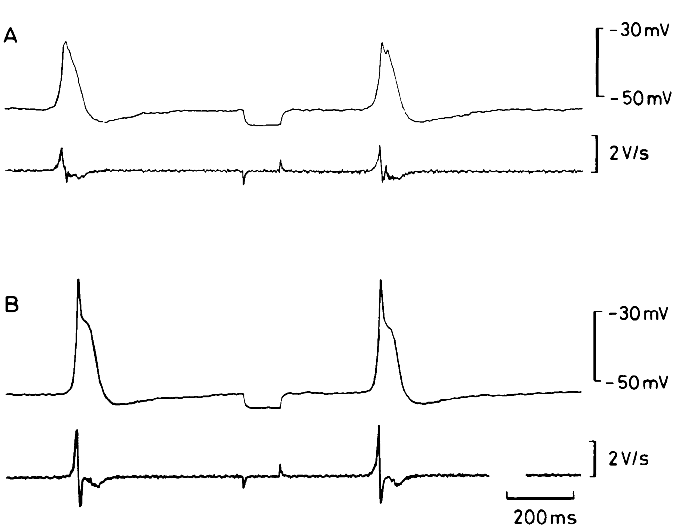

FIG. 8. *A*: spikes and an electrotonic potential recorded in presence of 10^{-6} M phenylephrine. *B*: when 10 mM TEA was also included in perfusate, a fast peak appeared early during spike. Duration of spike was not different nor was time course of electrotonic potential.

time- and voltage-dependent outward current recorded under voltage clamp. The activation of this conductance would result in a decrease in membrane resistance, and therefore the membrane potential would relax back in the direction of the reversal potential for the outward current. Large depolarizing current steps resulted in a small "trough" after the initial peak in the membrane potential (Fig. 2). The cause of this trough is not clear, but it can be explained in terms of a partial inactivation of the outward current. The underlying ionic mechanism was not manifest in the voltage clamp results (Fig. 3*A*). This apparent anomaly is most likely due to the fact that the membrane was not depolarized to the same level under voltage clamp as is the peak potential resulting from large depolarizing current steps. The limitations of the single-electrode voltage clamp precluded the use of large voltage steps to study this phenomenon in greater detail.

TEA has little effect on the conductances in the soma of these cells. However, in its presence the rising phase of the spike recorded in the soma became more peaked. It is tempting to suggest that TEA may result in an increase in the amplitude of the spike at its site of initiation and that the larger passive potential that results in the soma, now more than 20–25 mV in amplitude, activates the outward current and is rapidly attenuated in a fashion similar to the potential resulting from injected current.

Spikes could not be evoked in the soma despite large depolarizations of the membrane produced by passing current through the microelectrode or during perfusion with high potassium or TEA. Furthermore, electrotonic potentials evoked at the peak of the spikes indicated that there was no significant change in the membrane resistance of the soma during the spikes. These observations suggest that the spikes recorded from the soma are passive potentials. Additional evidence indicating that spikes may be generated at sites remote from the soma was obtained under voltage clamp. Depolarizing voltage steps of short duration did not activate an inward current, whereas when the membrane was clamped at the resting potential during nerve stimulation or during the application of α-adrenoceptor agonists, trains of transient inward currents were recorded that were not associated with a change in conductance in the soma. Thus it would appear that the membrane potential at the site of origin of the spikes was not clamped. These results all suggest that spikes originate at sites electrically distant from the soma, that the single-electrode voltage clamp used here cannot control the membrane potential at these sites, and that the soma cannot support the spikes as active responses. A somewhat similar inability to clamp responses initiated at electrically distant sites has been described in sympathetic ganglion cells (9). Such a heterogeneous distribution of ion channels is not unique to pinealocytes, and in this regard the pineal cells are only a more extreme case of observations in some neurons. The axon of the giant neuron of *Aplysia* is more excitable than the soma, and the soma is not essential for synaptic transmission and the subsequent orthodromic propagation of an action potential down the axon (22). In retinal ganglion cells the labeling of sodium channels indicates that the soma has a far lower density of these channels than does the initial segment (23). Tetrodotoxin-sensitive sodium channels are restricted to the area of the nodes of Ranvier in motoneurons and are not present in internodal regions (13). Microfluorometric imaging indicates that voltage-dependent calcium channels are localized on the dendrites of Purkinje cells, rather than occurring on the soma (21).

Nonetheless, an unusual aspect of the present results is that the soma does not support spikes. Despite this, it is not devoid of voltage-dependent channels. Depolarization activates a set of channels that carries an outward current, the reversal potential of which is close to the resting potential, suggesting that potassium is a permeating ion but that other ions may be involved. The time course of the current is reminiscent of the delayed rectifier, but the channels are not blocked by TEA. A similar TEA-resistant outward current has been recorded in cultured cells from pineal glands of rats (8). The lack of either a detectable inward current or any effect of calcium channel blockers during the early phase of depolarization does not exclude the possibility that depolarization-activated channels that carry an inward current are present in the somatic membrane. However, such observations indicate that if such channels do occur on the somatic membrane, they must carry a current that is small compared with the outward current.

Another set of channels in the soma is activated by hyperpolarization of the membrane and results in an inward current that activates with relatively slow kinetics. This current may be similar to the currents that are activated by hyperpolarization in other preparations insofar as it may be carried by channels that are not specific for a single species of cation. It is unlikely to be the anomalous rectifier, since its kinetics are too slow and it is resistant to TEA. It is qualitatively similar to the current (i_f) in cells of sinoatrial node (6), although its time course appears to be quicker in pineal cells.

The intracellular injection of the fluorescent dye Lucifer yellow has shown morphologically that these pineal cells have processes (14). In the present study small electrotonic potentials in 41% of the cells had biphasic time courses with an initially fast component and a final decay that could be described by a single exponential. Intercellular coupling is unlikely to be the explanation because Lucifer yellow always remained in the cell into which it was injected (14). The most likely explanation is that the processes of the pineal cells are electrically significant.

The small size of the spikes is consistent with their initiation in the cell processes, since a quick transient electrical event, such as an action potential, would be expected to be severely attenuated in amplitude by passive propagation along a cable. Action potentials originating in different cell processes and propagating to a passive soma might be expected to summate in the soma, and this could explain the variation in amplitude of the spikes and the occurrence of humps and shoulders on the spikes. Thus it is likely that action potentials can be generated on more than one of the processes. Active events in cell processes are not unusual, since considerable evidence has accumulated indicating that the processes of various neurons, such as Purkinje cells (3, 21) and sympathetic ganglion cells (4, 9), can support active responses.

The final exponential component to electrotonic potentials suggests that at the end of a current step the processes are equipotential with the soma. It is surprising therefore that a large depolarizing current or voltage-clamp steps did not appear to activate the channels carrying the inward current that underlies the upstroke of the action potential. Furthermore, depolarization with high potassium also failed to elicit action potentials. It is most likely that the channels through which current flows during the depolarizing phase of the action potentials are voltage dependent, yet depolarization alone appears to be insufficient to activate the channels. From these observations we suggest that stimulation of α-adrenoceptors in pinealocytes sets in motion a train of biochemical events within the cell that takes time and is essential for the response that is observed. In this regard it may be significant that the appearance of spikes after nerve stimulation has the unusually long delay of 2.4 s. This phenomenon appears very similar to that occurring in the circular myometrium of guinea pigs in early pregnancy. In the latter case the calcium channels that underly the action potential must first be transformed by the prostaglandins into an excitable state before they can be activated by depolarization (5). Somewhat similarly, in bag cell neurons of *Aplysia* a class of calcium channel only becomes evident after the cells have been exposed to agents that activate protein kinase C (17). Demonstrations of modulation of calcium channels by intracellular messengers are becoming commonplace (5, 10).

It has been found that activation of α-adrenoceptors in pinealocytes of rats mobilizes phospholipid in the membrane (16, 18). It may be that the resulting kinase is required for transformation of the calcium channels that conduct the action potential current. The results of this study present indirect evidence suggesting that the action potential current is likely to be through calcium channels that are voltage dependent; they are blocked by nifedipine and verapamil and appear to be enhanced by TEA.

Although the lack of activation of the action potentials by depolarization can be explained by a requisite transformation of the channels, it is not clear why transient currents were recorded under voltage clamp during the application of phenylephrine. A possible explanation is that not all processes are electrically short structures. Some processes may be electrically long, such as the long process that terminates in a bleb (14), and may have such a small input conductance that they have little effect on the electrotonic potential recorded from the soma. For such processes the membrane may well not be clamped and therefore may give rise to regenerative action potentials.

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