Correlation between Electrical Activity and ACTH/β-Endorphin Secretion in Mouse Pituitary Tumor Cells

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ABSTRACT The electrical and secretory activities of mouse pituitary tumor cells (AtT-20/D-16v), which contain and release the ACTH/β-endorphin family of peptides, were studied by means of intracellular recordings and radioimmunoassays. Injection of depolarizing current pulses evoked action potentials in all cells and the majority (82%) displayed spontaneous action potential activity. Action potentials were found to be calcium-dependent. Barium increased membrane resistance, action potential amplitude and duration, and release of ACTH and β-endorphin immunoactivity. Isoproterenol increased both action potential frequency and hormone secretion. Raising the external calcium concentration increased the frequency and amplitude of the action potentials and stimulated secretion of ACTH and β-endorphin immunoactivity. Thus, stimulation of secretory activity in AtT-20 cells was closely correlated with increased electrical activity. However, a complete blockade of action potential activity had no effect on basal hormone secretion in these cells. These results suggest that the mechanisms underlying stimulated hormone secretion are different from those responsible for basal secretory activity. It is proposed that the increased influx of calcium due to the increased action potential frequency initiates the stimulated release of hormone from these cells.

The mechanisms underlying secretion of hormones from endocrine gland cells in response to physiological stimuli are as yet unclear. Douglas (7, 8) has proposed that the stimulus for hormone secretion may be an influx of calcium ions as a consequence of action potential activity or depolarization of the plasma membrane, leading to an increased free calcium concentration intracellularly, and initiation of the exocytotic release of hormones from secretory granules. There is now considerable support for such stimulus-secretion coupling of hormone release in insulin-secreting β-cells of the pancreas (2, 26, 30) as well as chromaffin cells of the adrenal medulla (4, 7). Recent findings that mammalian pituitary cells and pituitary-derived tumor cells are capable of generating action potentials suggest that such electrical activity may provide a means for stimulus-secretion coupling of pituitary hormone release (1, 5, 9, 21, 27–30, 34, 35, 37). Support for a direct effect of action potentials on pituitary secretion is provided by the findings that the frequency of action potentials recorded from normal anterior pituitary cells can be increased by the application of a stimulatory hypophysiotropic peptide, thyrotropin-releasing hormone (TRH) (34).

The AtT-20/D-16v pituitary tumor cell line, originally derived from mouse anterior pituitary tissue, has provided an excellent model system for studies on the biosynthesis and release of adrenocorticotropic hormone (ACTH) and β-endorphin (cf. 11, 17, 18). AtT-20 cells, like corticotropic cells of the anterior pituitary and intermediate pituitary cells, secrete end-products derived from a single precursor molecule (pro-ACTH/endorphin). ACTH and β-endorphin released from AtT-20 cells are biologically active and the forms secreted by this cell line are very similar to those secreted by corticotropic cells of normal murine anterior pituitary (10–12, 18, 31). Corticotrophs comprise only about 2–5% of the total cell population in normal anterior pituitaries. Since AtT-20 cells comprise a homogeneous hormone secreting cell population, this cell line provides a good model system for studying the relationship between electrical activity and ACTH/β-endorphin secretion in anterior pituitary corticotropic cells. In these studies an electrophysiological investigation using intracellular recording techniques was carried out on AtT-20 tumor cells; parallel secretion studies using immunoassays were also performed.

MATERIALS AND METHODS

Cultures of AtT-20/D-16v mouse pituitary tumor cells were maintained in Dulbecco's modified Eagle's medium (DME) containing 2.5% horse serum,

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glucose, glutamine, and antibiotics (10). The cells were maintained by weekly serial passage, and culture medium was changed every 2 d.

Intracellular Recordings

Dishes containing the cultured cells were placed on the stage of microscope equipped with Nomarski differential phase contrast optics (320-times magnification). In some experiments an inverted microscope with phase contrast optics was used, allowing observation at 800-times magnification. Microelectrodes pulled from microfilament glass were filled with 3 M potassium acetate and had resistances of 120–180 MΩ. A single electrode was used for passing current and measuring membrane potential by means of a high input impedance amplifier with bridge circuit. The bathing solution contained 10% DME (vol/vol) without bicarbonate and the following: NaCl, 130 mM; KCl, 4.5 mM; $CaCl_2$, 2 mM; $MgCl_2$, 0.9 mM; glucose, 5 mM; HEPES, 1.5 mM, and tricine buffer, 1.5 mM (pH 7.35). Sodium-free solutions were prepared by replacing NaCl with an equiosmotic amount of choline chloride. Calcium-free solutions were prepared by substituting an equimolar amount of $MgCl_2$ or $BaCl_2$. In most experiments drugs were added to the perfusion solution, but in some experiments drugs were ejected under mild pressure via a micropipette (tip diameter ~15–20 μm) lowered into the solution and positioned close (~20 μm) to the recording electrode just before pressure application. Pressure was applied for 2–5 s, control experiments showed this procedure ejected 2–3.5 μl of pipette contents. Drug concentrations were expressed as concentration present in the micropipette; presumably the concentration of drug reaching the cell under study would be less. Temperature in the organ bath was maintained at 35–36°C. Records were photographed from the oscilloscope or displayed on a pen recorder.

Several preliminary experiments were done in an attempt to determine adequate criteria for an acceptable impalement since AtT-20 cells are quite small (cells used in this study were ~5–10 μm wide × 10–20 μm long × 2–3 μm thick) and susceptible to damage due to microelectrode penetration. Impalements resulting in apparent resting membrane potentials (E_m) of −20 to −60 mV were obtained in over 200 cells. Recordings made from AtT-20 cells which showed E_m values <−40 mV exhibited low input resistance (R_{IN}) (<250 MΩ, see results), a reduced or absent response to injection of depolarizing current, and were usually not maintained for more than a few minutes. Most cells (>60% of all impalements) gradually depolarized from an initial E_m more negative than −40 mV over a period of 10 min after penetration; this was associated with a decrease in R_{IN} and reduction in action potential amplitude. Such observations are typical of damage due to microelectrode insertion. In ~10% of all impalements, a gradual, irreversible hyperpolarization (to levels of −70 to −75 mV) was associated with a marked decrease in R_{IN} and complete loss of action potential activity was observed. A similar sequence of events has been observed in a small proportion of GH3 cells (28). These cells were not further investigated. The final criteria, which are similar to those of Dichter and Fischbach (6), used for accepting a recording were: (*a*) that a stable recording be maintained at E_m more negative than −40 mV for at least 10 min in control solution with no decrease in R_{IN}, and (*b*) that the impalement from a single cell be maintained for the duration of any test including a period of drug washout. The results reported in this study were obtained from a total of 39 cells which met these conditions.

Secretion Studies

Cells were grown in 16-mm microwells (Costar, Cambridge, MA) and maintained in DME as described above. 30 to 60 min before the beginning of an experiment the medium was replaced with DME containing bovine serum albumin (BSA; 2.5 mg/ml), lima bean trypsin inhibitor (0.1 mg/ml), and aprotonin (0.17 TIU/ml) without serum. All test solutions were made up in this medium. Medium was removed from the cells at 10-min intervals and replaced with fresh culture medium. The collected medium was treated with protease inhibitors (phenylmethylsulfonyl fluoride and iodoacetamide; 0.3 mg/ml) and secreted hormones were measured by immunoassay. These incubation conditions prevent hormone degradation after secretion (23, 25). At the end of an experiment cells were extracted with 5 N acetic acid plus protease inhibitors, lyophilized, redissolved in buffer plus protease inhibitors, and assayed for total hormone content. In further experiments cells were grown on Cytodex beads (33), placed in a column and perfused at 36°C with the same solution used for electrophysiological studies (with the addition of BSA, lima bean trypsin inhibitor, and aprotonin) at a rate of 0.09 ml/min. Fractions were collected every 10 min and analyzed by immunoassay. All experiments were carried out at least in triplicate. If basal secretion rates fell by >20% during the course of an experiment, this was considered indicative of a general deterioration in cell viability (see results); results from such experiments were discarded.

Immunoassays

ACTH immunoassays were performed using antiserum Bertha (specific for ACTH(17–24)); β-endorphin immunoassays were carried out using antiserum Danielle (specific for β-endorphin(10–19)) (22, 24). Synthetic human ACTH(1–39) Ciba-Geigy, Summit, NJ and synthetic camel β-endorphin (Bachem, Bubendorf, Switzerland) were used as standards. A standard (ten serial twofold dilutions) was assayed with each group of unknowns; four or five serial threefold dilutions were assayed for each unknown. Assays were performed on the medium alone and all test media, and showed <3.0 pM apparent cross reactivity with ACTH or β-endorphin.

Drugs

The following drugs were used: tetrodotoxin (TTX) (Calbiochem-Behring Corp., San Diego, CA); l-isoproterenol-D-bitartrate; l-norepinephrine (l-arterenol bitartrate); and dl-propranolol (Sigma Chemical Co., St. Louis, MO); and tetraethylammonium chloride (TEA) (Eastman Kodak Co., Rochester, NY).

Differences of means were assessed using a paired Student's *t*-test.

RESULTS

Resting Membrane Properties

The resting membrane potentials recorded from AtT-20 cells ranged from −45 to −55 mV (mean ± SD = −49 ± 4 mV, N = 39). Input resistance (R_{IN}) and time constant were measured by passing inward current pulses through the electrode and recording the resulting changes in membrane potential. Input resistances were determined by plotting the steady-state membrane potential against current, and measuring the relation at the resting potential. These ranged from 400 to 850 MΩ with a mean of 680 MΩ. The time constant calculated from the exponential time course of the potential change produced by small current pulses ranged from 12 to 28 ms. No consistent or conspicuous changes in either the resting or active electrical properties of these cells were noted with time from plating (that is, from day 0.5 through day 6 after passage).

Active Membrane Properties

In all cells examined, depolarizing current pulses elicited single or multiple action potentials of 35–40 mV in amplitude (Fig. 1*a*). In the majority of cells (36 of 39) an afterhyperpolarization of some 5–10 mV was observed on cessation of the depolarizing current pulse. Spontaneous electrical activity was recorded in 82% (32 of 39) of the cells. It is of interest that the cells exhibiting spontaneous activity showed the highest R_{IN} values (>500 MΩ), suggesting that high input resistance and

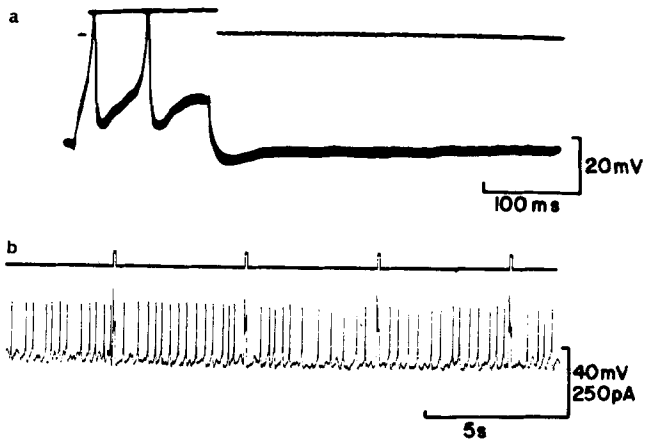

FIGURE 1 Intracellular recordings made from AtT-20 pituitary tumor cells. (*a*) Action potentials evoked in response to injection of depolarizing current pulse of 120-ms duration. Note prominent afterhyperpolarization. Resting potential was −54 mV. (*b*) Spontaneous action potentials recorded from another cell; frequency was ~3/s. Resting potential was −48 mV. In each record, upper trace is current, lower trace is voltage.

spontaneous electrical activity probably represent the best criteria for a successful impalement. This is supported by the observation that >90% of the extracellular recordings made from AtT-20 cells showed spontaneous activity (1). A typical example of the spontaneous action potentials recorded from these cells is shown in Fig. 1 *b*. In most cases, spontaneous activity occurred as action potentials of 35–40 mV in amplitude (as in Fig. 1 *b*), although in two of these cells only small amplitude (4–6 mV) oscillations in membrane potential were recorded. The spontaneous action potentials had rise times of 10–15 ms and were followed by an afterhyperpolarization of 5–12 mV. The spontaneous activity continued throughout the duration of any stable impalement (up to 60 min). The frequency of the spontaneous activity varied between cells from irregularly occurring spikes of <1/s to a maintained rhythm of 3–4/s. In all of these cells, application of hyperpolarizing current through the recording electrode decreased the frequency of the action potentials while application of depolarizing current increased the frequency of action potentials. Hyperpolarizing the membrane beyond ~−70 mV produced complete cessation of action potential activity, which resumed when the hyperpolarization was terminated.

Ionic Basis of Electrical Activity

The effects of tetrodotoxin (TTX) on the electrical activity of AtT-20 cells were examined. Fig. 2 *a* shows the records obtained from one cell in control solution, after the application of TTX (10 μM) and after washout of the TTX; no depression of action potential amplitude is apparent. A similar lack of effect of TTX (0.5–50 μM) was observed in six out of eight cells examined. The addition of TTX also had no effect on the amplitude, time course, or frequency of the spontaneous action potentials recorded from these cells. Tetrodotoxin (10 μM) appeared to cause a block of the action potentials within 2–5 min in the remaining two cells. However, washout of the drug for longer than 15 min did not reverse this apparent blockade. In view of the inability to evoke action potentials in these cells after washout, it is not clear whether the blockade was due to TTX or to coincidental cell damage. The same preparation of TTX (15 μM) reversibly blocked all evoked action potentials in cultured chick spinal cord neurons within 10 min (figure not shown).

Replacing the external sodium with choline produced no change in the response to injection of depolarizing current in four cells examined. An example is shown in Fig. 2 *b*. This cell exhibited spontaneous activity which also did not change during the 10-min perfusion of the sodium-free solution. No reversible change in the resting membrane potential was observed when sodium was removed from the bathing solution. These results suggest that sodium ions play little, if any, role in the generation of action potentials in these pituitary tumor cells.

The addition of cobalt (5 mM) to the bathing solution abolished action potential activity as did the removal of calcium from the perfusion fluid. Fig. 2 *c* shows intracellular recordings obtained from one cell before, during, and after the addition of 5 mM cobalt. This concentration of cobalt completely blocked the depolarization-induced action potentials in all five cells examined; however this effect was fully reversible (within 20 min) in only two of the cells.

When the external calcium concentration was reduced to 0.1 normal (0.2 mM) or zero, action potential activity was decreased or completely abolished within 2–5 min and these

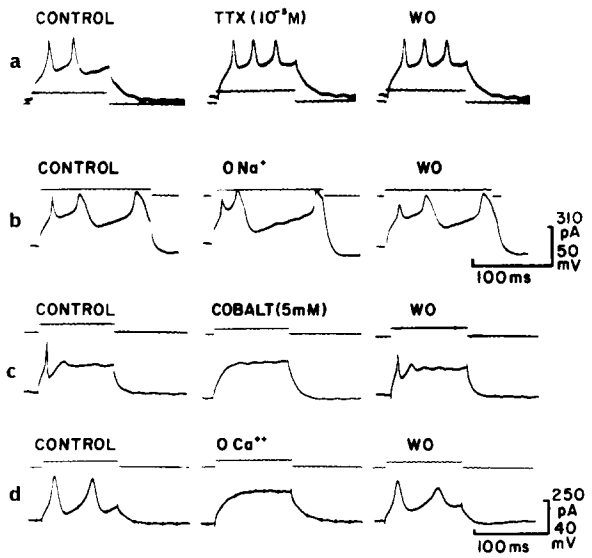

FIGURE 2 Effects of tetrodotoxin (TTX, *a*), sodium-free (*b*), cobalt (*c*), and calcium-free (*d*) solutions on evoked action potentials in AtT-20 cells. (*a*) Records obtained from one cell in control solution, 12 min after introduction of 10 μm TTX and 8 min after washout of TTX. (*b*) Removal of external sodium also had no effect on action potentials; recordings made from a second cell. (*c*) Recordings obtained from a third cell before, during, and after the addition of 5 mM cobalt to the perfusion fluid. This concentration of cobalt abolished action potentials in all cells examined (N = 5). (*d*) Records from a fourth cell showing the reversible blockade of action potentials in calcium-free solution. Calibrations in *b* refer to *a* and *b*; those in *d* refer to *c* and *d*. In *a*, lower trace is current, upper is voltage; in *b–d*, upper trace is current, lower is voltage.

effects were readily reversible in all five cells examined. Blockade of the action potential normally evoked by depolarizing current is shown in Fig. 2 *d*. Fig. 3 shows recordings made from another cell where the spontaneous activity (frequency ~0.8/s) was abolished when the low calcium solution was perfused through the system. On reintroduction of normal calcium (2 mM) spontaneous activity returned; in fact, immediately after return to normal calcium the amplitude and frequency of the spontaneous action potentials were significantly increased over control values (Fig. 3, Table I). This increased frequency and amplitude continued for ~5–10 min after calcium was reintroduced and then gradually returned to control levels (Table I).

Increasing the calcium concentration in the bathing fluid to 5 mM also caused an increase in the frequency and amplitude of action potentials in both cells examined. Fig. 4 shows records obtained from one of these cells. In this cell, the average frequency in control solution was 1.6/s; upon addition of 5 mM calcium the frequency increased to 2.7/s; this effect was reversible within 5–8 min after returning to normal calcium (Fig. 4). No change in resting membrane potential was apparent on increasing (or decreasing) the external calcium concentration. The increased amplitude of the action potentials is most consistent with an increased calcium conductance and increase in the equilibrium potential for calcium due to the higher external calcium concentration. However the mechanism underlying the increased action potential frequency, in the absence of any apparent membrane potential change, is unclear. In control solution spontaneous activity was characterized by membrane potential oscillations of 5–10 mV which did not evoke action potentials (e.g. Fig. 3, *inset*). It may be that these potential changes did not generate action potentials because of

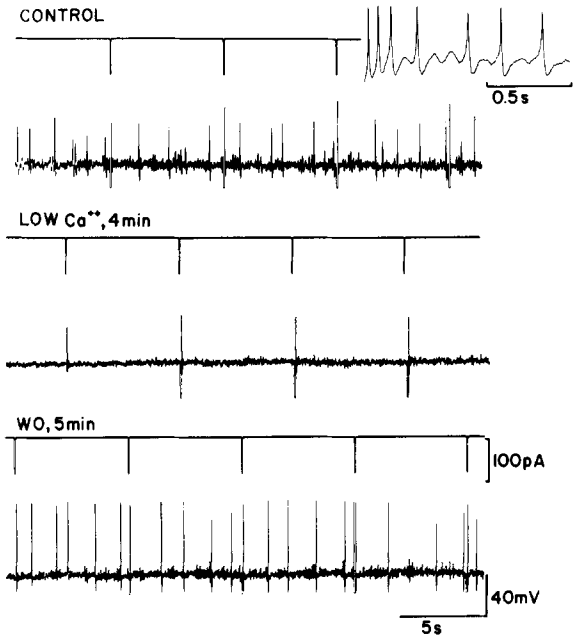

FIGURE 3 Effects of lowering external calcium on spontaneous electrical activity. Records obtained from the same cell. Lowered external calcium (0.2 mM) abolished spontaneous action potentials and decreased the amplitude of spikes evoked on cessation of hyperpolarizing current pulses (middle trace). On reintroduction of normal calcium (2 mM) the amplitude of the action potentials increased (from 40 mV in control to 60 mV on reintroduction of calcium) and the average frequency increased (from 0.8/s to 1.1/s). This "rebound" effect was transient; after 10 min the amplitude and frequency of action potentials had returned to control levels. Insert in upper right shows spontaneous action potentials recorded in control solution from this cell at faster sweep speed. Resting membrane potential was −50 mV throughout.

TABLE I

Summary of Ca^{2+} on Action Potential Frequency and Secretion in AtT-20 Tumor Cells

	Normal	Test		Normal
Cell	Action Potentials/Second			
Ca^{2+} (mM)	*2*	*0*	*5*	*2*
1	3.0	0		4.2
2	0.8	0		1.2
3	1.8	0		2.3
4	2.6	0		2.9
5	3.1		6.0	3.2
6	1.6		2.7	2.0
Preparation	Secretion			
	pmol/10 min			
1	2.5	2.8		5.2
2	0.8	0.7		1.8
3	1.8	1.7		2.1
4	8.5		16.0	6.0
5	1.6		5.4	2.2
6	3.4		4.8	2.7

an ongoing partial inactivation mechanism; if so, an increased calcium concentration might well be expected to lead to an increased frequency by raising the threshold for the inactivation process.

One of the commonly accepted criteria used in identifying calcium spikes in excitable membranes is that other divalent cations, such as barium and strontium, can sibstitute for calcium in the generation of action potentials (15, 16). In the AtT-20 cells, as in a variety of other tissues that possess calcium-dependent action potentials, replacing calcium with barium resulted in an increase in the amplitude and duration of the action potential evoked by depolarizing current pulses (Fig. 5*a*). Barium (2 mM) also lowered the threshold potential required for initiation of evoked action potentials in all cells examined (N = 5). It is apparent from the records shown in Fig. 5 that barium markedly increased the input resistance of these cells. This barium-induced increase in R_{IN} is shown in Fig. 5*b* where the current-voltage relation for hyperpolarizing current pulses obtained in one cell in normal solution and in the presence of 2 mM barium is plotted. At this concentration the increased R_{IN} was not associated with any change in E_m. Higher concentrations of barium (5–10 mM) depolarized the

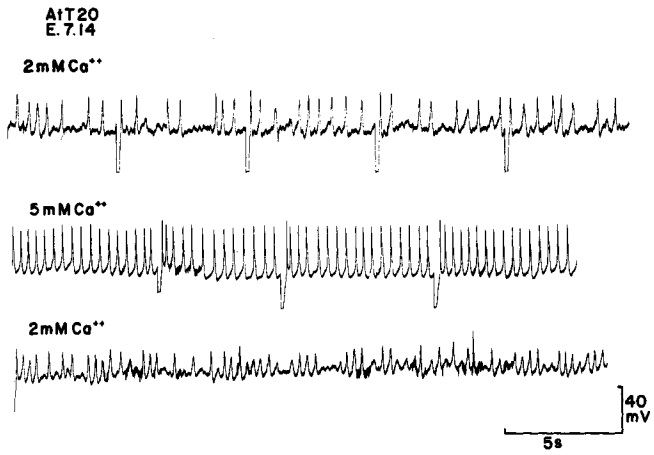

FIGURE 4 Raising external calcium (to 5 mM) increased action potential frequency and amplitude in AtT-20 cells. Records obtained from the same cell. Current traces not shown. In this cell the average frequency in 2 mM calcium was 1.4/s; frequency in 5 mM calcium was 2.2/s. Electrotonic potentials evoked by hyperpolarizing current pulses were off the screen in this recording.

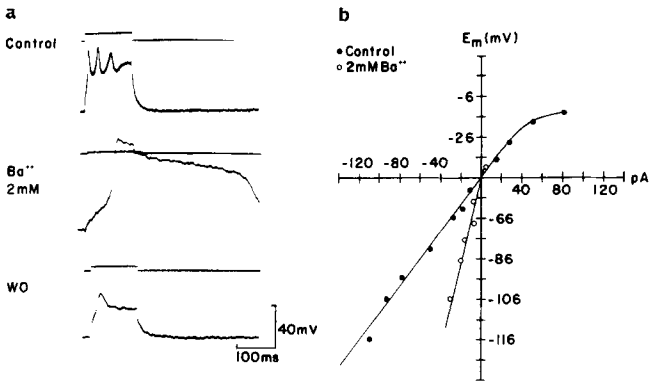

FIGURE 5 Effects of barium on evoked action potentials. (*a*) Records from the same cell in control solution 3 min after barium was substituted for calcium in the perfusion solution and 10 min after return to control solution. Note the large amplitude action potential evoked by a very weak depolarizing current pulse in the presence of barium and the prolongation of the action potential. (*b*) Current-voltage relation obtained in another cell by application of hyperpolarizing current pulses in control solution (●) and ~6 min after the introduction of 2 mM barium (replacing calcium) into the bathing fluid (○). Input resistances obtained from the slopes of the lines were 625 MΩ in control and 1,650 MΩ in barium in this cell.

resting potential by 10–35 mV. These results indicate that barium not only can substitute for calcium in the generation of the action potential, but also that barium affects both resting and voltage-dependent membrane conductances. These results are consonant with findings in several types of cells where barium is known to block various types of potassium conductances (15). It is of interest in this regard that tetraethylammonium ions (TEA) (10–25 mM) had no effect on the resting potential, passive membrane properties, or threshold for action potential initiation in these cells. These concentrations of TEA did, however, increase the duration of both evoked and spontaneous action potentials (four cells examined), consistent with its well-known ability to block the voltage-dependent potassium conductance in excitable cells (figure not shown).

Effects of Isoproterenol on Electrical Activity

Isoproterenol (ISO), a synthetic β-adrenergic agonist, has been reported to stimulate the release of ACTH and β-endorphin from AtT-20 cells (25, see below). Isoproterenol (1–10 μM) significantly increased the frequency of the spontaneous action potentials in all three cells examined (Figs. 6 and 8). (In another three cells, the frequency of the action potentials approximately doubled within 5 min after the introduction of ISO [10 μM] into the bathing fluid; however, these impalements were not maintained during washout of the drug.) The increase in action potential frequency was apparent within 5–15 s after ISO was injected into the bathing fluid via a micropipette placed close to the impaled cell (Fig. 7). Isoproterenol had no apparent effect on the resting membrane potential or input resistance of these cells; similarly, ISO did not affect the amplitude or time course of the action potentials evoked by depolarizing current pulses.

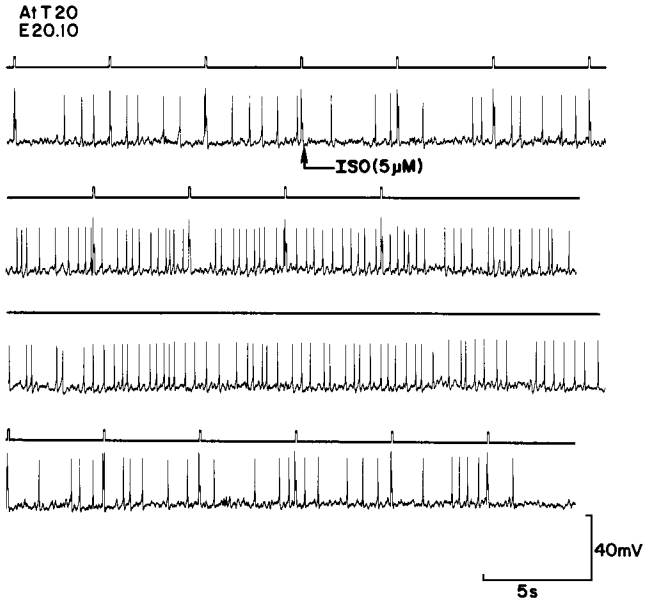

FIGURE 6 Effects of ISO on action potential activity. Records obtained from one cell: top three traces are continuous. ISO was injected into the bath for 3 s via a micropipette placed close to the recording electrode. Injection of ISO doubled the frequency (from ~0.9/s to 2.0/s) of action potentials within ~10 s. Bottom record begins 2 min after end of previous trace; frequency had returned to control levels at this time. Resting membrane potential was −45 mV throughout.

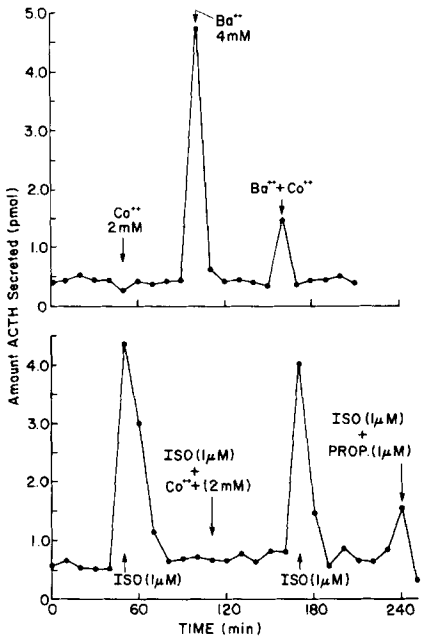

FIGURE 7 Effects of barium, cobalt, and ISO on secretion of ACTH by AtT-20 cells. Culture medium was removed at 10-min intervals, replaced with fresh medium and amounts of ACTH present in the collected medium measured by immunoassay. Each point represents the immunoactive ACTH released into the culture medium during the preceding 10-min period. Arrows indicate the 10-min time period during which each "test" solution was present. Although cobalt (2–5 mM) did not significantly depress basal release of hormones (N = 5), cobalt (2 mM) was effective in depressing the barium-induced stimulation of hormone release (upper graph). Barium alone (2–4 mM) stimulated secretion by two- to eightfold over basal (N = 10). Cobalt as well as propranolol (*PROP*) inhibited the ISO-induced stimulation of hormone secretion (lower graph). This experiment also illustrates that the ISO-induced stimulation of ACTH secretion was reproducible within the same preparation.

Secretion of ACTH and β-Endorphin from AtT-20 Cells

Several workers have shown that the rate of basal release of ACTH and β-endorphin-related molecules from AtT-20 cells is constant over several hours and that ACTH and β-endorphin are secreted in equimolar amounts (18, 25, 36, 38). Results from the present study are in agreement with these findings: the immunoassayable amount of ACTH and/or β-endorphin secreted into the medium per 10-min time periods under basal condition remained unchanged (±12%) for 8 h (the longest time examined); within the limits of the methods used (25), secretion of immunoactive ACTH and β-endorphin was found to be equimolar in all preparations examined (±15%, $N = 7$). The basal rate of secretion of the tumor cells ranged from 8–18% of their cellular hormone content per hour, which is within the range found by other workers (10, 18, 22).

In all experiments barium (N = 8), ISO (N = 10), and norepinephrine (NE) (N = 6) caused a dose-dependent increase in the amount of ACTH and β-endorphin secreted into the incubation medium (Figs. 7, 8, and 9). At each concentration tested, ISO (0.1 nM–100 μM) was a more potent secretagogue than NE (0.1–100 μM). Propranolol (1 μM), a β-adrenergic receptor antagonist, was effective in depressing the ISO-induced stimulation of hormone release (Fig. 7).

In five experiments, the addition of cobalt to the medium

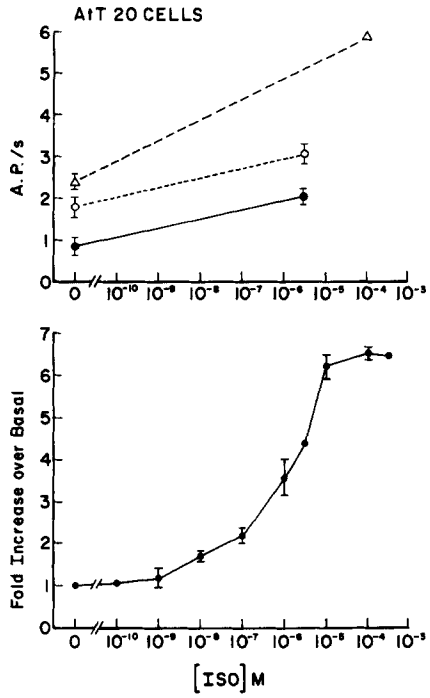

FIGURE 8 Correlation between ISO-induced stimulation of action potential frequency and ACTH/β-endorphin secretion. Upper graph illustrates the average (±SD) action potential frequency obtained for the three cells examined in control solution at the ISO concentration present in the perfusion solution or in the ejection micropipette. Lines drawn to indicate each cell. Lower graph is the dose-response curve obtained for all preparations; relative secretion (where 1 = basal release) is plotted as a function of ISO concentration in the culture or perfusion media. Each point is the mean ± SE obtained from four to six preparations, except where no SE bar is present, in which case N = 1. In all experiments ISO was present for 10 min.

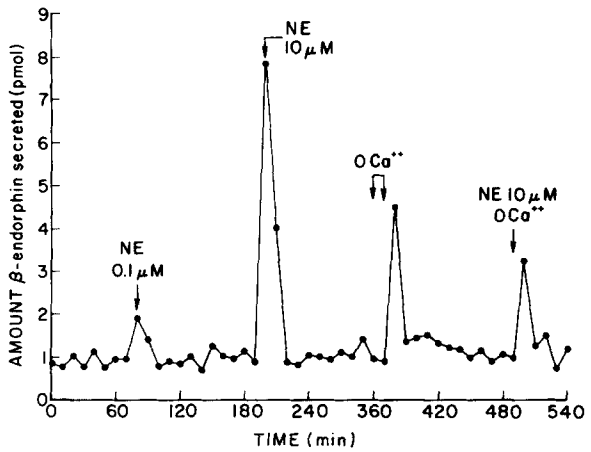

FIGURE 9 Effects of removal of external calcium on basal and stimulated β-endorphin secretion by AtT-20 cells. Norepinephrine (0.1 and 10 μM), present in the culture medium for 10 min, stimulated β-endorphin release in a dose-dependent manner. When cells were bathed in calcium-free medium for 20 min (double arrows) no depression of basal release of β-endorphin was observed. However, on reintroducing calcium (2 mM) into the culture medium a large (threefold) increase in hormone release was observed. The NE-induced (10 μM) increase in β-endorphin secretion was completely prevented in the absence of external calcium (at arrow). Again a "rebound" increase in hormone release occurred in the 10-min time period after reintroduction of calcium (without NE) into the medium.

did not significantly inhibit the basal rate of hormone secretion at concentrations (2–5 mM) that blocked action potential activity (Fig. 7). However the stimulation of release caused by NE, ISO, and barium was depressed or abolished in the presence of 2–5 mM cobalt. Similar results have been reported by Mains and Eipper (25). These experiments would suggest that the mechanisms responsible for basal release of hormone from AtT-20 cells differ from those responsible for stimulation of secretion.

Fig. 8 summarizes the effects of ISO on both the electrophysiological and the secretory activity obtained in these experiments. In Fig. 8*a* the average action potential frequency is plotted for concentrations of ISO present for the three cells examined. Fig. 8*b* shows the relative secretion of ACTH or β-endorphin as a function of ISO concentration. Although the numbers of intracellular recordings are not sufficient to allow any statistical comparison, there is a positive correlation in the ability of ISO to increase both electrical and secretory activity in AtT-20 pituitary tumor cells.

In three expeirments, removal of calcium from the incubation or perfusion medium had no significant effect on the basal rate of hormone secretion (Fig. 9, Table I). However, on reintroducing calcium a "rebound" effect was observed in all cases; that is, a large (1.5- to 3-fold) increase over basal rate of release occurred and release then returned to control levels within 10–15 min. One such experiment is shown in Fig. 9. Fig. 9 also shows that removal of calcium from the external medium prevented the NE-induced increase in hormone release (N = 3). In the absence of external calcium, ISO (1–5 μM) also failed to stimulate secretion of ACTH and β-endorphin (N = 3). It should be noted that bathing the cells in calcium-free solution for periods longer than ~20 min caused irreversible damage to the cells (i.e. basal and stimulated release were markedly depressed and sometimes the cells floated off the dishes).

Increasing the concentration of external calcium stimulated the release of ACTH and β-endorphin (Fig. 10). Fig. 10 shows that this increased rate of release was not maintained during prolonged exposure to increased concentrations of calcium; after an initial increase secretion returned to approximately control conditions within 20–40 min. The results of the effects of external calcium concentration on the action potential frequency and on the amount of hormone secreted obtained from all experiments are shown in Table I. From the data of Table I it is clear that an increase in the action potential frequency is closely correlated with an increase in secretion of hormones. However, a complete blockade of action potentials does not result in a change in the basal secretory activity of these cells.

DISCUSSION

Electrical Properties

These experiments demonstrate that AtT-20 tumor cells are electrically excitable. All of the cells produced action potentials on injection of depolarizing current and the majority also displayed spontaneous action potential activity. Action potentials persisted in the presence of tetrodotoxin-containing and sodium-free solutions but were depressed or abolished in the presence of cobalt or calcium-free solutions (Figs. 2 and 3). These observations provide good evidence for the role of calcium as the major inward-carrying current source during the generation of action potentials in AtT-20 cells. The observations that the amplitude of the spontaneous spikes was

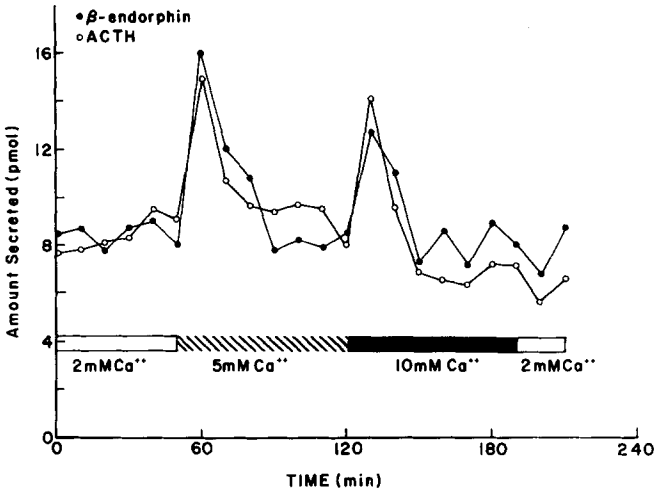

FIGURE 10 Increasing external calcium transiently increases ACTH and β-endorphin secretion by AtT-20 cells. Immunoactive ACTH (○) and β-endorphin (●) secreted into the culture medium per 10-min time intervals obtained from one preparation are shown. Perfusion with 5 and 10 mM calcium solutions for the times indicated by stippled (5 mM calcium) and filled (10 mM calcium) bars resulted in an increased rate of hormone release for the first 20–30 min. Hormone release returned to basal levels in the continued presence of raised extracellular calcium. Note that ACTH and β-endorphin were released in approximately equimolar amounts throughout the experiment.

increased when the external calcium concentration was raised and that barium could substitute for calcium in supporting the action potential are further indications that the action potentials recorded from these cells are calcium-dependent. Similar results have been reported for the prolactin/growth hormone-secreting GH3 tumor cell lines and many other cells which show "calcium-spikes" (9, 15, 16, 21, 29, 36, 38). AtT-20 cells, like the GH cells, have been derived from anterior pituitary tissue. The electrophysiological studies on normal anterior pituitary cells have suggested a predominant calcium component to the action potentials recorded (30, 35). Thus, it seems reasonable that the electrical properties of AtT-20 cells may reflect the existence of similar characteristics in normal anterior pituitary corticotropic cells. Continued studies on the electrical activities of AtT-20 cells should provide insight into the mechanisms involved in ACTH/β-endorphin secretion from the anterior pituitary.

Stimulated Secretion of ACTH and β-Endorphin is Correlated with Increased Electrical Activity

The present studies have shown that concentrations of ISO and calcium that increase the frequency of action potentials also increase the amount of ACTH and β-endorphin secreted by AtT-20 cells. Since the action potentials appear to be due to an influx of calcium ions, an increased action potential frequency should lead to an increased intracellular calcium concentration that could trigger the exocytosis of hormones from secretory granules and thus account for the elevated levels of hormones in the culture medium. The mechanism whereby these agents induce increased action potential activity remains to be elucidated; the increased frequency of action potentials induced by ISO was not associated with any apparent change in the resting potential or input resistance. Perhaps β-receptor activation lowers the membrane potential required for spike initiation by altering the potential at which activation of the voltage-dependent conductances underlying the action potential takes place, as has been reported for the actions of enkephalinergic peptides (3).

The actions of barium on the electrical properties of AtT-20 cells are obviously complex; nevertheless the results of this study would indicate that barium acts to increase membrane excitability (i.e. barium lowered the threshold for initiation of action potentials and increased their amplitude and duration). Studies on the release of ACTH and β-endorphin showed barium to be a very potent secretagogue in these cells (Fig. 7). The finding that barium stimulated secretion even in the absence of external calcium suggests that barium can substitute for calcium in evoking hormone release as well as in the generation of action potentials.

Calcium itself was found to be a potent secretagogue in AtT-20 cells. As the experiment in Fig. 10 indicates, the stimulatory action of raising external calcium was transient; levels of hormone release returned to control levels in the continued presence of raised extracellular calcium. Similarly, a transient increase in levels of ACTH and β-endorphin released was observed when calcium was returned to the medium of a calcium-deficient culture (Fig. 9). A parallel sequence of events was seen in the electrical activity, where raising external calcium or reintroducing calcium into a calcium-free perfusate resulted in a transient increase in action potential frequency (Table I). Both the secretory and electrical changes were observed over the same time intervals (i.e. within 3–10 min).

It is proposed that an increase in the frequency of action potentials over any previously maintained level is the trigger that initiates the stimulated release of hormones from these cells. It is now clear that an increased influx of calcium would occur due to the increased frequency of action potentials.

Basal Release of Hormone Differs from Stimulated Secretion

On the other hand, the electrophysiological and biochemical data obtained in these experiments show quite clearly that a complete blockade of electrical activity has little effect on the basal secretory activity of AtT-20 cells. The most likely explanation for these results would appear to be that the mechanisms underlying stimulated secretion of hormones from AtT-20 cells are different from those underlying basal hormone release. A similar conclusion was reached by Mains and Eipper (25) and Sabol (32) who found that NE increased only the smaller forms of ACTH and β-endorphin released from AtT-20 cells. These smaller forms of the ACTH/endorphin molecule have been shown to be contained predominantly in secretory granules (13, 14). In light of these findings and results presented in this study, it is suggested that hormones released from AtT-20 cells during basal conditions originate mainly from cellular components other than secretory granules. This could explain the unaltered basal release of ACTH and β-endorphin in the absence of action potential activity. Calcium influx due to increased action potential activity would be expected to stimulate mainly exocytosis of secretory granules, as is the case in neurons and neurosecretory cells (7, 19, 20) and as proposed by the stimulus-secretion coupling mechanism of hormone release (7, 8). If basal secretion is, however due primarily to nongranular release, it may be expected to be relatively independent of extracellular calcium and therefore independent of action potential activity.

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