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CONTROL OF ACTION POTENTIALS AND Ca^{2+} INFLUX BY THE Ca^{2+}-DEPENDENT CHLORIDE CURRENT IN MOUSE PITUITARY CELLS

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SUMMARY

1. Perforated patch recording was used to examine the influence of the calcium-dependent chloride current ($i_{Cl(Ca)}$) on Ca^{2+} action potentials in AtT-20 pituitary cells. The calculated chloride equilibrium potential (E_{Cl}) was adjusted by changing either intracellular or extracellular [Cl^-]. Action potential duration varied as a function of E_{Cl}. When E_{Cl} was set at -21 mV, both spontaneous and evoked action potentials displayed a long plateau phase between -20 and -25 mV, which typically lasted for several seconds. Setting E_{Cl} to more negative potentials resulted in briefer action potentials; at an E_{Cl} of -52 mV, no plateau phase was evident. Spontaneous depolarization and action potential firing still occurred when E_{Cl} was negative to firing threshold, which indicates that the slow depolarizing wave that precedes the firing of spontaneous action potentials does not require activation of $I_{Cl(Ca)}$.

2. In voltage clamp experiments the magnitude of $I_{Cl(Ca)}$ diminished slowly during a prolonged depolarization, over a time course that coincided with action potential termination.

3. Niflumic acid (100 μM) blocked $I_{Cl(Ca)}$ by 90% but had no effect on either K^+ or Ca^{2+} currents. This concentration of niflumic acid eliminated the plateau phase, but did not prevent the firing, of Ca^{2+} action potentials.

4. Internal [Ca^{2+}] was measured photometrically after loading cells with the Ca^{2+} indicator dye, Fura-2. Under voltage clamp conditions, concentrations of niflumic acid (30–100 μM) that blocked depolarization-evoked $I_{Cl(Ca)}$ had little or no effect on simultaneously recorded Ca^{2+} transients. Perforated patch recording from Fura-loaded cells showed that action potentials were temporally associated with transient increases in intracellular [Ca^{2+}]. Niflumic acid (30–100 μM) disrupted the rhythmic firing of spontaneous action potentials and associated intracellular Ca^{2+} transients.

5. Fluorescent measurements of Ca^{2+} transients were also made in cells unperturbed by patch recording, and were used as a measure of action potential duration in the absence of experimental alteration of internal [Cl^-]. Spontaneous Ca^{2+} transients were of long duration ($\sim$ 2 s), which suggests that intracellular [Cl^-]

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is relatively high (40–50 mM) in these cells. The spontaneous Ca^{2+} transients were inhibited by niflumic acid.

6. Niflumic acid up to 100 μM, had neglible effects on either basal or stimulated (by 2 μM-(±)-isoprenaline) hormone secretion, as shown by radioimmunoassay of adrenocortotrophic hormone release.

7. The results suggest that activation of $I_{Cl(Ca)}$ by Ca^{2+} influx during action potentials tend to maintain the membrane potential at a depolarized level, which enhances the Ca^{2+} influx through voltage-activated Ca^{2+} channels. Action potentials are terminated as the magnitude of $I_{Cl(Ca)}$ decreases. $I_{Cl(Ca)}$ thus acts to control the biphasic behaviour of membrane potential and Ca^{2+} influx, and appears to provide rhythmic control over the rise and fall of intracellular [Ca^{2+}]. Block of this rhythmic behaviour by niflumic acid had little effect on hormone secretion, which suggests that secretion is not tightly coupled to the transient nature of either action potentials or Ca^{2+} influx.

INTRODUCTION

Hormone secretion from anterior pituitary cells is usually accompanied by a rise in intracellular calcium, and is prevented by Ca^{2+} channel blockers or removal of extracellular Ca^{2+} (Luini, Lewis, Guild, Corda & Axelrod, 1985; Holl, Thorner & Leong, 1988). Both acutely dissociated and clonal anterior pituitary cells spontaneously fire Ca^{2+} action potentials (Kidokoro, 1975; Taraskevich & Douglas, 1987; Suprenant, 1982; Adler, Wong, Sabol, Busis, Jackson & Weight, 1983; Mollard, Vacher, Guerin, Rogowski & Dufy, 1987; Schlegel, Winiger, Mollard, Vacher, Wuarin, Zahnd, Wollheim & Dufy, 1987). One mechanism by which secretion is thought to be controlled is by modulation of Ca^{2+} influx though voltage-gated Ca^{2+} channels. For example, somatostatin, which inhibits secretion (Patel & Srikant, 1986), depresses intracellular Ca^{2+} transients that are associated with spontaneous action potentials (Schlegel *et al.* 1987). Conversely, β-adrenergic agonists, which stimulate secretion and cause a rise in intracellular Ca^{2+} (Luini *et al.* 1985), have been reported to increase the frequency of action potential firing (Suprenant, 1982; Adler *et al.* 1983).

The AtT-20 clonal cell line, derived from mouse anterior pituitary, has been used extensively as a model to study corticotrophin biochemistry and secretion (cf. Axelrod & Reisine, 1984). AtT-20 cells express a slowly activating, slowly deactivating chloride current ($I_{Cl(Ca)}$) that is gated primarily by intracellular Ca^{2+}, but is also mildly sensitive to membrane voltage (Korn & Weight, 1987). Although $I_{Cl(Ca)}$ has been observed in a variety of cell types (Bader, Bertrand & Schwartz, 1982; Evans & Marty, 1986; Owen, Segal & Barber, 1984; Mayer, 1985; Matthews, Neher & Penner, 1989; Hume & Thomas, 1989), little is known about its role in the regulation of cell excitability. In both rat dorsal root ganglion neurons and mouse spinal cord neurons $I_{Cl(Ca)}$ has been postulated to underlie action potential after-polarizations (Owen *et al.* 1984; Mayer, 1985). In developing chick skeletal muscle cells (Hume & Thomas, 1989), and in AtT-20 cells (Korn & Weight, 1987), $I_{Cl(Ca)}$ can be activated rapidly, and therefore may contribute to the shape of the action potential.

The experiments in this paper were designed to examine the function of $I_{Cl(Ca)}$ in

AtT-20 cells. The results indicate that $I_{Cl(Ca)}$ is not required for depolarization towards firing threshold, but that it contributes to the regulation of action potential duration, and consequently Ca^{2+} influx.

METHODS

Cells

AtT-20/D16-16 cells (kindly provided by the Laboratory of Cell Biology, National Institutes of Health) were grown in 75 mm tissue culture flasks, and maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (Gibco, Grand Island, NY, USA), as described previously (Korn & Horn, 1989). Cells were passed once weekly, and used for experiments 5–8 days following plating. At each passage, cells were plated in 35 mm Nunc dishes for electrophysiology, and in twelve-well plates for secretion studies. Plated cells (passage 18–35) were fed thrice weekly. Electrophysiological and secretion studies were done in parallel to insure that each type of experiment was done on the same batches of cells. Over the course of these experiments, no qualitative differences between batches of cells were observed.

Electrophysiology

Perforated patch recordings were made as described previously (Korn & Horn, 1989; Korn, Marty, Connor & Horn, 1991). In perforated patch recordings, nystatin (167 μg/ml) is placed into the pipette prior to formation of the high-resistance seal on the cell membrane. After seal formation, nystatin intercalates into the membrane and forms pores that are selectively permeable to monovalent ions; multivalent ions and large biomolecules do not permeate the membrane patch (Korn *et al.* 1991). Endogenous calcium buffering systems appear to remain intact (Korn & Horn, 1989), and exogenous Ca^{2+} buffers are not introduced to the cell interior. This technique thus facilitates control over intracellular $[Cl^-]$, $[K^+]$ and $[Na^+]$, but more accurately maintains the physiological time course of Ca^{2+}-mediated currents than standard whole-cell recordings. Series resistance (R_s) and membrane capacitance (C_m) were measured by cancellation of capacitive currents evoked by 20 ms, 10 mV voltage steps from -70 mV. R_s ranged from 5 to 30 MΩ in voltage clamp experiments, and was < 50 MΩ in current clamp experiments. Except where indicated, the electrophysiological experiments were performed at 21–23 °C. Stable patch clamp recordings could not be maintained for sufficient periods of time to conduct these experiments at 37 °C.

In most experiments the solution bathing the cell was changed by lowering a large bore (50–100μm) pipette that contained test solution near the cell. In the experiments involving fluorescent measurements, the solutions were changed by switching electronically among four Teflon, gravity-fed outlet tubes, nested within a 100 μm glass pipette. The 10–90% rise time for complete solution exchange was 3–5 s.

Membrane potential and fluorescence measurements were recorded on chart paper (Gould Inc., Cleveland, OH, USA) moving at 2·5–5 mm/s, and on a Panasonic VHS recorder (A. R. Vetter Co., Rebersburg, PA, USA). In voltage clamp experiments the currents and fluorescence signals were acquired and stored with pClamp (Axon Instruments Inc., Burlingame, CA, USA). Action potential parameters were measured from the chart paper with a resolution of 400 ms and 1 mV. Action potential duration was measured as the time between the positive and negative crossings of -25 mV. We used mean ± standard error of mean for statistical presentation of data.

Solutions

Except where indicated in the figure legends, the recording solutions were the standard external and internal solutions for perforated patch recording. The standard external solution contained (in mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, 0·8 $MgCl_2$, 10 HEPES–NaOH, 20 glucose, pH = 7·35, osmolality = 335 mosM. We changed external $[Cl^-]$ by substituting sodium isethionate, impermeant in $I_{Cl(Ca)}$ channels (Korn & Weight, 1987), for NaCl. The standard internal (pipette) solution for perforated patch recording contained (in mM): 55 KCl, 75 K_2SO_4, 8 $MgCl_2$, 10 HEPES–KOH, pH = 7·3, 310 mosM. Internal $[Cl^-]$ was changed by substitution of KCH_3SO_4 for KCl. Niflumic acid (Aldrich) was added to the external solution from a 10 mM stock solution in dimethyl sulphoxide (DMSO). The final concentration of DMSO never exceeded 1%, which has no observable effects on voltage-

activated membrane currents (S. J. Korn & R. Horn, unpublished). K^+ currents were blocked in Fig. 4 by substitution of Cs^+ for K^+ in the internal solution and substitution of tetraethylammonium (Aldrich) for K^+ in the bath. Where indicated, 1 μM-tetrodotoxin (TTX, Sigma) was added to the bath solution to block Na^+ currents.

Fluorescent measurements of intracellular $[Ca^{2+}]$

Intracellular $[Ca^{2+}]$ was measured photometrically from single cells, using the Ca^{2+} indicator dye Fura-2 (Molecular Probes, Inc., Eugene, OR, USA). The cells were typically incubated in 6·25 μg/ml of the membrane-permeant, acetoxymethyl ester derivative of Fura-2 (Fura-2 AM) for 10 min at 37 °C. Care was taken to avoid excessive loading of the dye, which in high concentrations can significantly buffer intracellular Ca^{2+} and thus diminish Ca^{2+}-activated currents in perforated patch recordings (Korn *et al.* 1991). Once deesterified, Fura-2 is a multivalent anion that remains trapped in the cell due to its impermeance through both the plasma membrane (Grynkiewicz, Poenie & Tsien, 1985) and through nystatin pores. The combined use of perforated patch recording and Fura-2 permitted simultaneous measurement of intracellular $[Ca^{2+}]$ and membrane currents (or potential) (Korn *et al.* 1991).

The $[Ca^{2+}]$ was determined by a modification of the ratio method (Grynkiewicz *et al.* 1985), using excitation wavelengths of $F_1 = 340$ nm and $F_2 = 380$ nm. The emitted light passed through a 500 nm bandpass filter and was collected by a photomultiplier tube mounted on the trinocular head of the inverted microscope. The fluorescent signal from the photomultiplier was amplified 20 × and low-pass filtered at 50–500 Hz before being stored, either directly into a pClamp file or onto the VHS recorder. In order to enhance the time resolution of the measurements of Ca^{2+} transients, we measured the fluorescence monochromatically at F_2, and determined $[Ca^{2+}]$ as follows.

Immediately preceding a test run the $[Ca^{2+}]$ concentration was determined by measuring the fluorescence during successive 400 ms periods at F_1 and F_2, using the formula (Grynkiewicz *et al.* 1985):

$$[Ca^{2+}] = K_d S(R - R_{min}/R_{max} - R), \quad (1)$$

where K_d (= 135 nM) is the effective dissociation constant for Ca^{2+}, S (= 14·5) is a factor determined from fluorescence intensities of our calibration solutions containing known concentrations of Ca^{2+}, $R = F_1/F_2$, R_{min} is the value of R at a $[Ca^{2+}]$ approaching 0, and R_{max} is the value of R at 10 mM $[Ca^{2+}]$. From the above measurements we calculated

$$Z = (K_d S + [Ca^{2+}])/\{F_2(K_d + [Ca^{2+}])\}. \quad (2)$$

The excitation wavelength was left at F_2, and fluorescence was then measured and recorded continuously during a test run of up to ~ 3 min. The values of $[Ca^{2+}]$ were determined from the recorded values of F_2 and the previously calculated factor Z, by the equation

$$[Ca^{2+}] = K_d(F_2 Z - S)/(1 - F_2 Z). \quad (3)$$

The validity of eqn (3) requires that several experimental factors remain constant during the test run, i.e. instrumental sensitivity, optical path length and effective dye concentration. The most problematic of these factors in our experiments was bleaching of the dye, which we minimized by decreasing the excitation light intensity to as low a level as possible, while preserving a manageable signal/noise. The amount of bleaching was determined by comparing the level of F_2 with a ratio measurement of $[Ca^{2+}]$ after each test run. Cells with greater than 10% changes in apparent dye concentration during a test run were not used for analysis.

Radioimmunoassay of hormone release

Adrenocorticotrophic hormone (ACTH) release was measured by radioimmunoassay (RIA). For secretion experiments, the growth medium was replaced with serum-free DMEM supplemented with $CaCl_2$ to bring $[Ca^{2+}]$ to 2 mM. Stock solutions of niflumic acid (10 mM in DMSO) and (±)-isoprenaline (20 μM in DMEM) were made fresh and added to appropriate wells to give the correct final concentration. Nimodipine (Biomol, Plymouth Meeting, PA, USA) was added from a frozen stock solution (10 mM in ethanol). After incubation for 3 h at 37 °C the medium was removed, rapidly chilled and centrifuged at 14000 r.p.m. in a Model 5415 Eppendorf centrifuge to remove cells and cellular debris. The clear supernatant was stored at −20 °C for future RIA. RIAs were performed using a modification of the procedure outlined by the supplier (Immuno Nuclear Corp., Stillwater, MN, USA). In order to increase the assay sensitivity, each assay contained 30000–40000 c.p.m. of ^{125}I-ACTH, a 2-fold increase above the level recommended by the supplier.

In addition, to prevent ACTH adsorption to glass, all assays were carried out in 1·5 ml Eppendorf tubes, which also allowed us to carry out the final centrifugation step at a higher speed. The range of sensitivity of the assay was 4–80 pg per tube. All assays were carried out in a minimum of triplicates, and each experiment was repeated at least three times.

RESULTS

Figure 1 illustrates the effect of changing intracellular [Cl^-] on action potential duration and frequency. Action potentials recorded with the standard external and internal solutions (71 mM $[Cl^-]_i$; $E_{Cl} = -21$ mV) displayed brief sharp spikes, followed by a slowly decaying plateau phase (Fig. 1*A a*). These action potentials terminated with a large hyperpolarizing wave followed by a depolarizing wave that triggered the next action potential. Action potentials recorded with 42 mM $[Cl^-]_i$ ($E_{Cl} = -34$ mV) exhibited the same general properties, but were briefer (Figs 1*B* and 2), and hyperpolarized slightly less following action potential termination (the membrane potential hyperpolarized to $-52{\cdot}9 \pm 1{\cdot}9$ *vs.* $-56{\cdot}8 \pm 2{\cdot}1$ mV; mean ± S.E.M., ten cells in each group). When recording pipettes were filled with 21 mM $[Cl^-]_i$ ($E_{Cl} = -52$ mV), action potentials consisted of brief spikes, with no plateau phase evident (Fig. 1*A b* and *B*). Under these conditions, the post-spike hyperpolarization was significantly smaller than for the other two groups (cells hyperpolarized to $-46 \pm 2{\cdot}1$ mV, $n = 5$). The shorter action potential duration with 21 mM $[Cl^-]_i$ was not a function of the more depolarized overall membrane potential, since action potentials were not prolonged by hyperpolarization of the cell following injection of negative current through the pipette (Fig. 1*A c*). As previously reported (Adler *et al.* 1983), firing frequency was decreased by hyperpolarization.

Spontaneous depolarization to firing threshold did not require activation of $I_{Cl(Ca)}$. With 21 mM $[Cl^-]_i$, spontaneous action potentials and the preceding depolarizing wave persisted, even though E_{Cl} was always negative to the cell membrane potential (Fig. 1*A b*).

Manipulation of E_{Cl} by changing external [Cl^-] resulted in similar effects on action potentials (Fig. 2). With an internal [Cl^-] of 42 mM, the external [Cl^-] was changed from 160 to 95 mM, thus shifting the calculated E_{Cl} from -34 to -21 mV. Action potentials were immediately prolonged, and post-spike hyperpolarizations became larger, indicative of greater Ca^{2+} influx. These effects were rapidly reversible (Fig. 2, bottom trace).

The effect of changing E_{Cl} on firing frequency was more complicated. If action potential frequency is regulated by $I_{Cl(Ca)}$, then a more negative E_{Cl} could move the resting potential away from firing threshold and thus decrease firing frequency. In contrast to this prediction, action potential frequency increased as E_{Cl} was made more negative (Fig. 1*C* inset; also compare Fig. 1*A a* to 1*A b*). However, this frequency increase appeared to be an indirect consequence of changing E_{Cl}. With more positive E_{Cl}, the long duration action potentials limited the maximum possible firing frequency. Indeed, firing frequency correlated highly with action potential duration, regardless of $[Cl^-]_i$ (Fig. 1*C*, open symbols). The smaller post-spike hyperpolarizations associated with shorter action potentials at more negative E_{Cl} would also facilitate an increase in firing frequency.

In order to examine the currents that were activated during action potentials, cells

were studied in voltage clamp with protocols that roughly mimicked the shape of action potentials. From a holding potential of −55 mV, cells were depolarized to −15 mV for 20 ms, stepped to −25 mV for different durations, and then repolarized to −60 mV. This protocol revealed the net current behaviour during prolonged

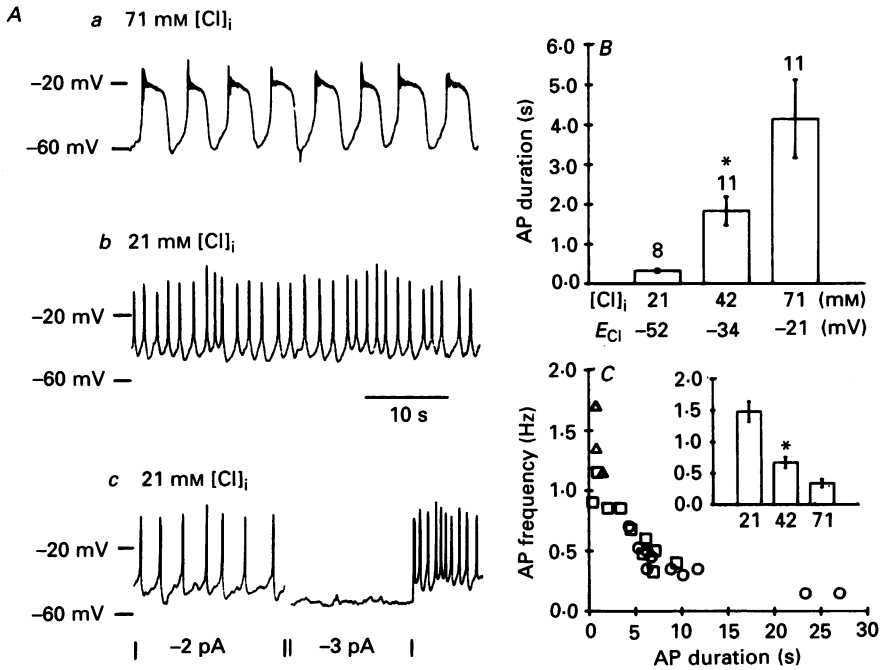

Fig. 1. Duration and frequency of spontaneous action potentials as a function of $[Cl^-]_i$. Representative examples of spontaneous action potentials with E_{Cl} set at −21 mV (*A a*) and −52 mV (*A b* and *A c*). In *A c*, the same cell as in *A b* was hyperpolarized by injection of −2 pA of current through the recording electrode (first panel) and then by −3 pA (second panel as marked). Current injection was termination in the second panel. *B*, action potential (AP) duration is plotted as a function of both $[Cl^-]_i$ and E_{Cl}. Values (mean ± S.E.M.) were obtained by averaging the duration of ten to twenty consecutive action potentials from the number of cells indicated. *C*, plot of action potential firing frequency as a function of action potential duration. Data are pooled from all $[Cl^-]_i$ values, and were obtained by counting the number of action potentials in a 20–80 s period. The circles, squares and triangles represent $[Cl^-]_i$ values of 71, 42 and 21 mM, respectively. Inset, plot of action potential frequency as a function of $[Cl^-]_i$. The asterisks indicate that the values at 42 mM $[Cl^-]_i$ were significantly different from those at 21 mM $[Cl^-]_i$ ($P < 0.01$) and 71 mM $[Cl^-]_i$ ($P < 0.05$). The standard external solution contained 1 μM-TTX.

depolarization at the average membrane potential of the action potential plateau phase. The magnitude of $I_{Cl(Ca)}$ during prolonged depolarization was determined by measuring the magnitude of the tail current following repolarization to −60 mV (Korn & Weight, 1987).

Depolarization to −25 mV activated $I_{Cl(Ca)}$, which then decreased in magnitude as the voltage pulse became more prolonged (Fig. 3; $[Cl^-]_i = 42$ mM, $E_{Cl} = -34$ mV). $I_{Cl(Ca)}$ began to decrease within 1 s, and became progressively smaller over the next several seconds. Concomitant with this decrease in $I_{Cl(Ca)}$, the net current at −25 mV

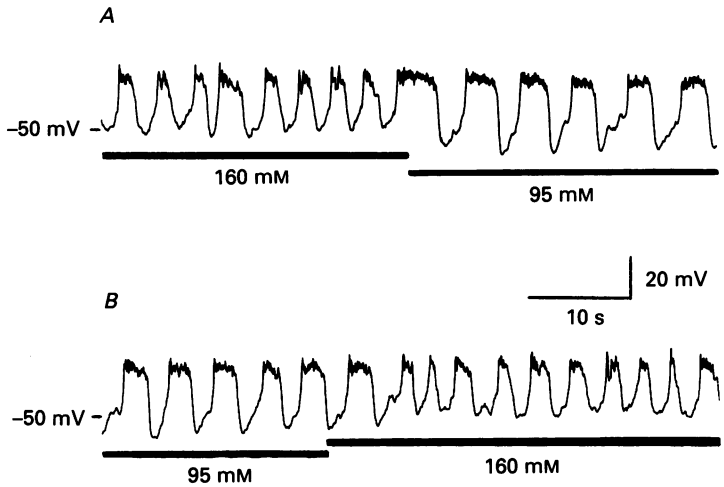

Fig. 2. Action potential changes as a function of extracellular $[Cl^-]$. *A* and *B* illustrate a continuous record of spontaneous action potentials recorded with 42 mM $[Cl^-]_i$. The external $[Cl^-]$ was changed from 160 to 95 mM and back to 160 mM as marked by the bars below the potential traces. The bath contained 1 μM-TTX.

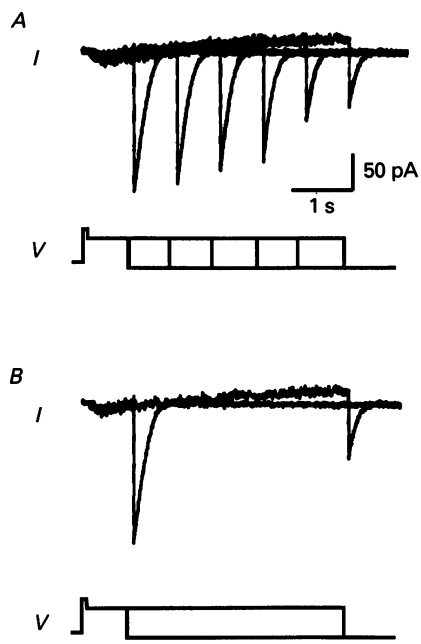

Fig. 3. Decrease in $I_{Cl(Ca)}$ and growth of outward current following prolonged depolarization. Voltage clamp conditions were designed to approximate the membrane potential changes that occur during an action potential. Cells were held at -55 mV. Voltage was then stepped to -15 mV for 20 ms, and then to -25 mV for steps of increasing duration (applied over 15 s). *A*, voltage was stepped to -25 mV for 500–3000 ms in 500 ms increments. Cells were repolarized to -60 mV. The voltage protocol (*V*) is illustrated below the superimposed current traces (*I*). *B*, the responses to the shortest and longest duration pulses from *A* are plotted. The bath contained 1 μM-TTX.

reversed from inward to outward and slowly increased in magnitude. These data support the hypothesis that $I_{Cl(Ca)}$ is activated during action potentials, and that under these conditions action potential termination is at least partly due to the decrease in Cl^- channel conductance.

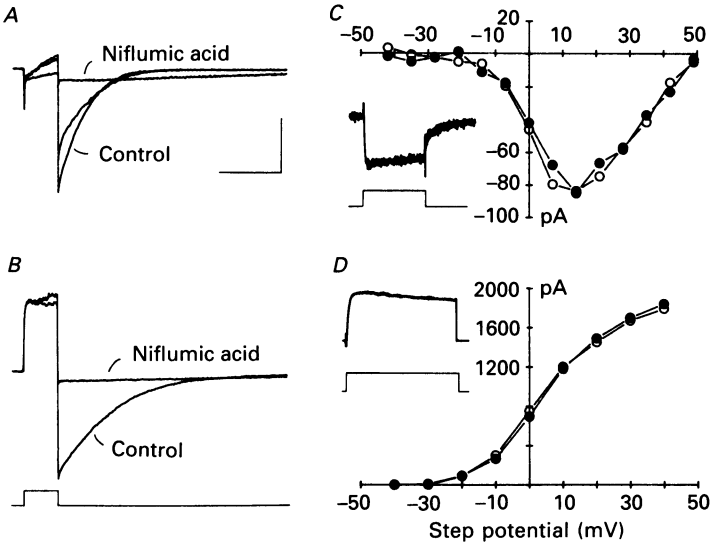

Fig. 4. Effect of 100 μM-niflumic acid on currents under voltage clamp. *A* and *B* are perforated patch recordings using either Cs^+ (*A*) or K^+ (*B*) in the pipette solution. *C* and *D* are whole-cell recordings. The whole-cell pipette solutions were (in mM): 125 XCl, 2 $MgCl_2$, 1 $CaCl_2$, 2 Na-ATP, 11 X-EGTA, 5 creatine phosphate, 10 HEPES, pH = 7·3, where X = Cs^+ (*C*) or K^+ (*D*). In *A* and *C* the KCl in the bath was replaced by tetraethylammonium chloride. 1 μM-TTX was used in all bath solutions except in *A*. *A* shows the effect, and partial recovery, of niflumic acid on $I_{Cl(Ca)}$ for a pulse from −70 to 0 mV. Inward and outward current were blocked. *B* shows the effect of niflumic acid on both K^+ and Cl^- currents for a pulse to +20 mV from −70 mV. The $I_{Cl(Ca)}$ tail is blocked with less effect on the outward current carried by both Cl^- and K^+. *C* shows Ca^{2+} current from a holding potential of −90 mV. $I_{Cl(Ca)}$ is not activated due to the buffering action of EGTA in the pipette solution. The inset shows superimposed currents, activated by a 100 ms pulse to +14 mV, in the presence and absence of niflumic acid. The graph shows the lack of effect of niflumic acid on the peak Ca^{2+} current. *vs.* voltage. Control, open symbols. *D* shows the lack of effect of niflumic acid on K^+ current. The inset shows superimposed currents, activated by 90 ms pulses to +30 mV from a holding potential of −70 mV, in the presence and absence of niflumic acid. Note that Ca^{2+}-activated K^+ currents are reduced in *D* by EGTA in the pipette solution. Calibration bars: *A*, 260 pA and 370 ms; *B*, 600 pA and 370 ms; *C*, 85 pA and 100 ms; *D*, 2300 pA and 55 ms.

Effect of niflumic acid on action potentials and membrane currents

In order to examine directly the role of $I_{Cl(Ca)}$ on action potentials and Ca^{2+} influx, we used niflumic acid to selectively block $I_{Cl(Ca)}$. In accord with the findings of White & Aylwin (1990), 100 μM-niflumic acid inhibited $I_{Cl(Ca)}$ tail currents by 89·2 ± 1·3 % ($n = 4$) (Figs 4*A*, 4*B* and 6*Bb*). Niflumic acid did not affect Ca^{2+}-dependent or independent K^+ currents (Figs 4*B*, 4*D* and 6*Bb*) or voltage-activated Ca^{2+} currents (Fig. 4*C*).

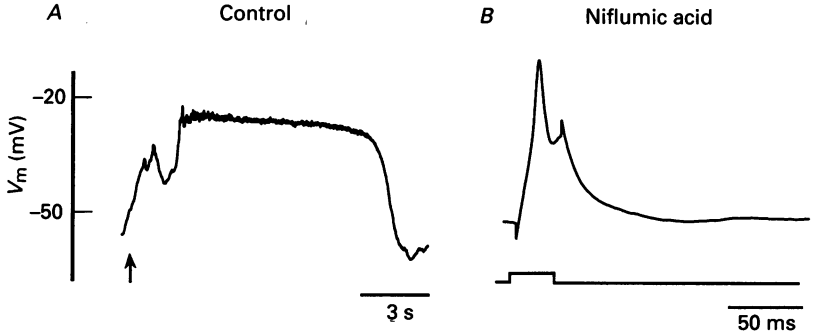

Fig. 5. Effect of niflumic acid on stimulated action potentials. *A*, an action potential triggered by a 65 ms depolarizing stimulus (arrow) of 70 pA. Note the prolonged plateau. *B*, an action potential stimulated in the same cell in the presence of 100 μM-niflumic acid. The 70 pA stimulus (lower trace) failed to elicit a plateau. Note the change in time scale between *A* and *B*.

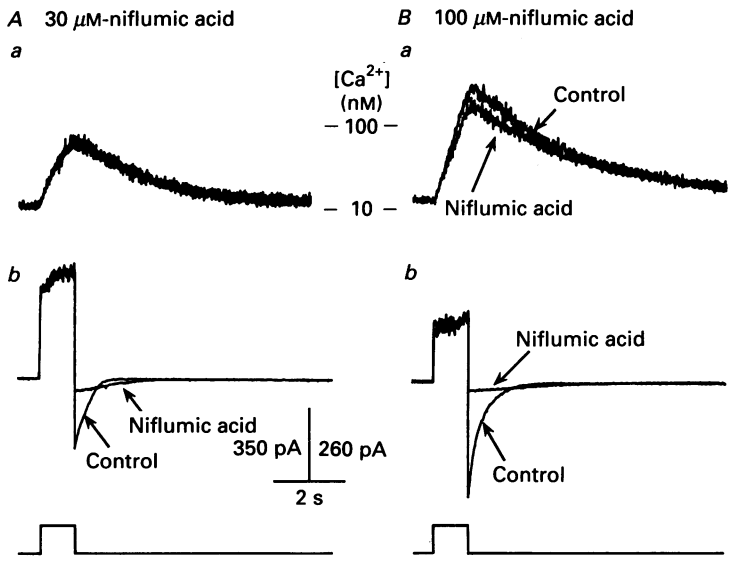

Fig. 6. Effect of niflumic acid on Ca^{2+} transients and membrane currents. The cells were loaded with Fura-2 AM and examined under voltage clamp using perforated patch recording. Holding potential was −70 mV, and currents were activated by 1 s steps to 0 mV every 60 s. *A*, superimposed traces of membrane currents and $[Ca^{2+}]$ and the effects of 30 μM-niflumic acid. R_s = 14 MΩ, C_m = 16 pF. *B*, currents and $[Ca^{2+}]$ from another cell show the effects of 100 μM-niflumic acid. R_s = 21 MΩ, C_m = 18 pF.

The data in Figs 1–3 suggested that the action potential plateau was maintained largely by the Cl^- conductance (g_{Cl}). Consequently, a reduction of g_{Cl} should decrease the action potential duration by reduction or abolition of the plateau phase. To test this, action potentials were evoked by a 70 pA depolarizing current pulse before (Fig. 5*A*) and after (Fig. 5*B*) exposure of a cell to niflumic acid (100 μM). The plateau phase, but not the Ca^{2+} spike, was abolished by niflumic acid, even following a larger depolarization.

Effect of niflumic acid on Ca^{2+} transients

Although niflumic acid did not block Ca^{2+} currents, it might have had effects on internal [Ca^{2+}] transients, perhaps by alterations in Ca^{2+} buffering or transport. To examine this possibility, cells were loaded with Fura-2, and then examined

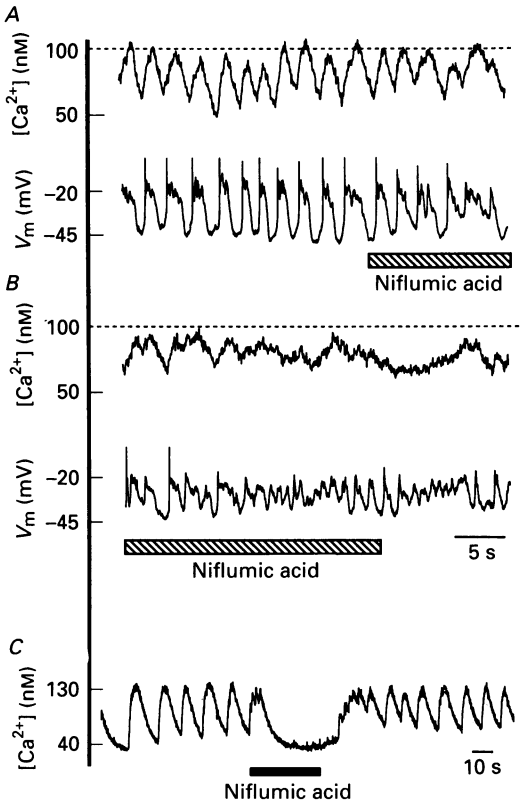

Fig. 7. Effect of niflumic acid on spontaneous action potentials and associated Ca^{2+} transients. *A* and *B*, simultaneous measurement of membrane potential (lower trace) and intracellular [Ca^{2+}]. *B* is a continuation of the traces in *A*. 30 μM-niflumic acid (hatched bar) blocked action potentials and Ca^{2+} transients. *C*, fluorescent measurement of Ca^{2+} transients from another cell in the absence of patch recording. 100 μM-niflumic acid (filled bar) reversibly blocked Ca^{2+} transients.

simultaneously with perforated patch recording and fluorescence photometry. Figure 6 demonstrates that 30 μM- (Fig. 6*A*) and 100 μM (Fig. 6*B*) niflumic acid inhibited the inward $I_{Cl(Ca)}$ tail current but had little or no effect on the depolarization-evoked Ca^{2+} transients.

As expected, spontaneous action potentials in AtT-20 cells were accompanied by temporally associated Ca^{2+} transients. Figure 7*A* and *B* illustrates the typical effect (eighty cells examined) of 30 μM-niflumic acid on spontaneous action potentials and Ca^{2+} transients. In niflumic acid, the rhythmic behaviour of membrane potential and Ca^{2+} influx was abolished, and replaced by small erratic voltage fluctuations and

poorly controlled intracellular [Ca^{2+}]. Higher concentrations of niflumic acid had similar effects, although at 100 μM, the voltage and Ca^{2+} transients were occasionally shut down (Fig. 7*C*).

In perforated patch experiments (as with standard whole-cell recording), the internal [Cl^-] is determined by the pipette solution (Horn & Marty, 1988). As shown above (Fig. 1), this [Cl^-] can affect action potential duration. Since the physiological intracellular [Cl^-] is unknown in these (and in other) pituitary cells, we wished to determine whether the prolonged action potentials were due to an unphysiologically high [Cl^-] (71 mM) in our standard internal solution. The experiment of Fig. 7*A* and *B* shows that prolonged action potentials were accompanied by Ca^{2+} transients. Therefore, we examined action potential duration indirectly by monitoring Ca^{2+} transients in single Fura-2-loaded cells without accompanying patch recording. Figure 7*C* illustrates the results of a typical experiment ($n = 22$ cells), in which cells at room temperature displayed prolonged Ca^{2+} transients, which were blocked by 100 μM-niflumic acid. Following brief application, effects of niflumic acid were reversible (Fig. 7*C*; see also Fig. 4*A*), whereas effects produced by prolonged application of niflumic acid were often irreversible (Fig. 7*A* and *B*). The mean duration of the Ca^{2+} transients in the absence of blocker was $2{\cdot}20 \pm 0{\cdot}21$ s ($n = 52$ cells). The Ca^{2+} transient duration was strongly dependent on temperature. At an elevated temperature of 28 °C, the Ca^{2+} transients had a duration of $0{\cdot}93 \pm 0{\cdot}21$ s ($n = 7$), giving a $Q_{10} \cong 3{\cdot}4$.

A rank-sum test showed that the Ca^{2+} transient durations in these experiments were significantly shorter ($P < 0{\cdot}001$) than the durations of action potentials recorded with 71 mM [Cl^-] in non-Fura-2-loaded cells ($4{\cdot}14 \pm 0{\cdot}93$ s; $n = 11$). The difference in action potential durations in the two types of measurements could be due to differences in $[Cl^-]_i$ and/or to the fact that Fura-2-loaded cells have a higher concentration of Ca^{2+} buffer (Korn *et al.* 1991). This was tested by examining the action potential duration in Fura-2-loaded cells (e.g. Fig. 7*A* and *B*). The action potential durations in such cells average $3{\cdot}32 \pm 0{\cdot}70$ s (nine cells examined), not significantly different from the durations in electrophysiological experiments in non-Fura-2-loaded cells. Thus, the fura-2 loading itself did not significantly affect action potential duration. Together with the experiments of Fig. 1, these experiments suggest that the longer durations observed in perforated patch experiments were caused by the fact that the [Cl^-] in our standard internal solution (71 mM) was higher than found normally in these cells.

Effect of niflumic acid on hormone secretion

The above experiments show that inhibition of $I_{Cl(Ca)}$ disrupts the rhythmic firing pattern of Ca^{2+} action potentials and reduces the duration of associated Ca^{2+} transients. Since Ca^{2+} influx is important for hormone release from these cells, we examined the effect of blocking g_{Cl} on ACTH release, measured by radioimmunoassay. Since there is little secretion at room temperature, these experiments were done at 37 °C. Figure 8*A* shows a typical experiment and the effects of niflumic acid at concentrations of 0·3–100 μM. Isoprenaline was added at a concentration of 2 μM (○) to stimulate ACTH release. Niflumic acid had insignificant effects on either basal (▽) or stimulated secretion up to a concentration of 30 μM, and caused a

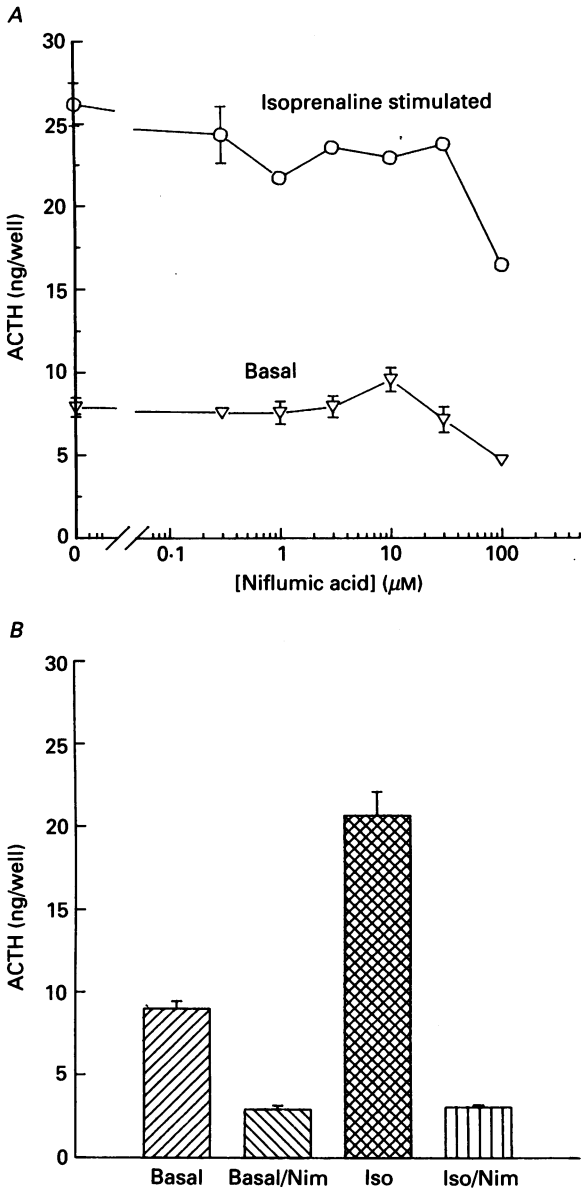

Fig. 8. Effect of niflumic acid and nimodipine on ACTH release measured by radioimmunoassay. *A*, basal (▽) and 2 μM-isoprenaline-stimulated (○) release are plotted as a function of [niflumic acid]. *B*, basal and 2 μM-isoprenaline-(Iso) stimulated release of ACTH in the presence and absence of 10 μM-nimodipine (Nim), showing that block of Ca^{2+} channels blocks secretion.

modest reduction at 100 μM. In six experiments, 100 μM-niflumic acid reduced stimulated secretion to 76% (range: 38–122%) of control. Similar results (data not shown) were obtained with niflumic acid when secretion studies were done on cells bathed in DMEM buffered with 40 mM-HEPES and incubated at 37 °C in air ($n = 2$). In agreement with an earlier study (Luini *et al.* 1985), 10 μM-nimodipine, a

Ca^{2+} channel blocker, reduced isoprenaline-stimulated secretion by 99% and basal secretion by 68% (Fig. 8*B*). Therefore Ca^{2+} influx through Ca^{2+} channels is necessary for isoprenaline-stimulated secretion in these cells.

DISCUSSION

The data in this study suggest that, in AtT-20 cells, $I_{Cl(Ca)}$ does not underlie the spontaneous depolarization that leads to action potential discharge, but plays a role in determining the duration of Ca^{2+}-mediated action potentials and the associated $[Ca^{2+}]_i$ transients. The $[Cl^-]_i$ in intact pituitary cells, including AtT-20 cells, is presently unknown. However, the experiments illustrated in Figs 1 and 7*C*, taken together, suggest that $[Cl^-]_i$ is rather high in an intact cell. The mean duration of 2·2 s for Ca^{2+} transients in the absence of patch recording (Fig. 7*C*) corresponds with a $[Cl^-]_i$ between 40 and 50 mM (see Fig. 1). $[Cl^-]_i$ may be somewhat lower at 37 °C. However, such a high $[Cl^-]_i$ is not surprising, since AtT-20 cells spend much of their lives depolarized, thus causing g_{Cl} to be activated much of the time. In addition, the depolarized membrane potential itself would also contribute to high $[Cl^-]_i$, because a passive influx of Cl^- would be enhanced by positive intracellular potentials.

Three conductances (g_{Cl}, g_{Ca} and g_K), controlled by voltage and $[Ca^{2+}]_i$, are likely to play a role in the plateau of the action potential of these cells. The amplitude of the $I_{Cl(Ca)}$ tail current (e.g. Fig. 3) indicates that g_{Cl} is approximately 3–4 nS during the plateau. g_{Ca} is typically at least 4 times smaller at voltages between −20 and −30 mV (Korn & Horn, 1990). The contribution of g_K can be estimated in the presence of 100 μM-niflumic acid, which blocks g_{Cl} (Fig. 4*B* and *D*). At a typical plateau potential of −25 mV, g_K is < 1·5 nS. Thus, g_{Cl} is the dominant conductance during the plateau, and its decline (presumably due to a decrease of $[Ca^{2+}]_i$ near the surface of the plasma membrane) correlates well with the termination of the action potential (Fig. 3). Thus, the relatively positive E_{Cl} creates a positive feedback cycle. Upon depolarization, Ca^{2+} enters the cell via voltage-activated Ca^{2+} channels and activates g_{Cl}, which maintains the depolarization and thus facilitates additional Ca^{2+} entry. Since $I_{Cl(Ca)}$ does not inactivate (Korn & Weight, 1987), the cycle is probably terminated by inactivation of the Ca^{2+} current, which leads to hyperpolarization as g_K becomes larger than g_{Cl}. Activation of a Cl^- conductance may also potentiate Ca^{2+} entry into mast cells following stimulation by secretagogues (Matthews *et al.* 1989). In contrast to AtT-20 cells, however, the rise in mast cell $[Ca^{2+}]_i$ causes activation of a hyperpolarizing Cl^- conductance, which is postulated to enhance Ca^{2+} influx by increasing the driving force on Ca^{2+}.

The control of action potential duration by a Cl^- conductance has been examined in two other cell types, both from muscle. A Cl^- conductance appears to play a role in the termination of the action potentials in mammalian cardiac myocytes (Harvey, Clark & Hume, 1990), but this current is not dependent on $[Ca^{2+}]_i$. In developing chick skeletal muscle however, long duration action potentials seem to be controlled by a Ca^{2+}-activated Cl^- conductance similar to that found in AtT-20 cells (Hume & Thomas, 1989).

Although $I_{Cl(Ca)}$ plays a significant role in both action potential duration and Ca^{2+} transients, it is not clear what role it has in hormone secretion in AtT-20 cells. Block

of $I_{Cl(Ca)}$ and spontaneous Ca^{2+} transients had little effect on either basal or stimulated secretion of ACTH (Fig. 8*A*). The interpretation of these data is complicated, however, by the different conditions of the electrophysiological and secretion experiments. The latter were performed at 37 °C in DMEM over a 3 h period, whereas electrophysiological experiments were usually performed at 21–23 °C in a buffered salt solution over a period of seconds to minutes. However, 100 μM-niflumic acid blocked Ca^{2+} transients in each of seven cells at 28 °C, and cells displayed rhythmic long-lasting Ca^{2+} transients at 30 °C (data not shown). Although it is clear that some Ca^{2+} influx is important for secretion from pituitary cells (Fig. 8*B*; Luini *et al.* 1985; Holl *et al.* 1988), the present studies suggest that secretion does not depend on the occurrence of rhythmic, long-duration action potentials. This is not unexpected, since bathing cells in high extracellular $[K^+]$ or Ca^{2+} ionophores stimulates secretion (Axelrod & Reisine, 1984). These treatments would tend to eliminate the transient nature of intracellular Ca^{2+} rises.

The control exerted by $I_{Cl(Ca)}$ over membrane potential and intracellular $[Ca^{2+}]$ may in itself be the more important physiological role of g_{Cl}. These cells, like many other endocrine cells, have a very high resting input resistance. Consequently, random fluctuations in the gating of a few ion channels can have a dramatic effect on the membrane potential. $I_{Cl(Ca)}$ appears to have an important impact on the control of rhythmic membrane potential activity, which in turn controls intracellular Ca^{2+} levels. When $I_{Cl(Ca)}$ was blocked by niflumic acid (Fig. 7), intracellular $[Ca^{2+}]$ and membrane potential fluctuated arhythmically at levels intermediate between those attained before block.

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