0026-895X/94/040743-07$03.00/0

MOLECULAR PHARMACOLOGY, 46:743-749, 1994.

Identical Inhibitory Modulation of A-Type Potassium Currents by Dihydropyridine Calcium Channel Agonists and Antagonists

BORIS MLINAR[1] and JOHN J. ENYEART

Department of Pharmacology and the Neuroscience Program, The Ohio State University College of Medicine, Columbus, Ohio 43210-1239

Received April 15, 1994; Accepted July 6, 1994

SUMMARY

We have studied the interaction of dihydropyridine (DHP) Ca^{2+} channel agonists and antagonists with A-type K^+ channels in whole-cell patch-clamp recordings from bovine adrenal zona fasciculata cells. At concentrations from 1 to 100 μM, DHP antagonists [nimodipine and (+)-Bay K 8644] and agonists [(−)-Bay K 8644 and RS 30026] each reversibly reduced A-type K^+ current (I_A) amplitude and markedly accelerated the apparent rate of I_A inactivation. Unlike their actions on Ca^{2+} channels, the effects of DHP agonists and antagonists on I_A were qualitatively indistinguishable. Inhibition of I_A by DHPs was not accompanied by changes in the voltage-dependent steady state inactivation of I_A or the kinetics of recovery subsequent to repolarization. The effects of DHPs on peak I_A and inactivation kinetics were not use dependent. The DHPs were much less effective in cells where fast N-type inactivation had spontaneously diminished with time. These actions of DHPs on I_A are in marked contrast to their voltage-dependent modulation of L-type Ca^{2+} currents, indicating that fundamentally different mechanisms are involved. Rather than directly occluding A-type K^+ channels, the drugs may enhance the voltage-independent rate of inactivation. This could occur through interaction of the DHP with a site on the amino-terminal inactivation domain or the DHP binding site at the inner mouth of the channel. Regardless of the mechanism involved, the identical modulation by DHP agonists and antagonists is a distinctive feature of A-type K^+ channels in adrenal zona fasciculata cells.

DHP Ca^{2+} channel modulators include agents that inhibit or enhance Ca^{2+} entry through voltage-gated channels in various excitable cells. Among the major classes of organic Ca^{2+} channel antagonists, DHPs are, in general, the most potent and selective blockers of Ca^{2+} entry through L-type channels (1–4). The DHP (−)-Bay K 8644 is a pure Ca^{2+} channel agonist that promotes rather than inhibits Ca^{2+} entry through L-type channels in various excitable cells.

(−)-Bay K 8644 has been widely used to explore the function of L-type Ca^{2+} channels in cellular processes including secretion, muscle contraction, and gene expression (5–8). The specificity of the DHPs as Ca^{2+} channel modulators is highlighted by the observation that (+)-Bay K 8644, in contrast to its agonist enantiomer, is a pure Ca^{2+} channel antagonist and blocks rather than promotes Ca^{2+} entry through voltage-gated Ca^{2+} channels (5, 8).

Although multiple subtypes of voltage-gated Ca^{2+} channels have been identified, DHP Ca^{2+} channel antagonists are relatively selective for L-type Ca^{2+} channels. However, at somewhat higher concentrations, these agents have been reported to block low voltage-activated T-type Ca^{2+} channels in various cell types (9–11). Assessing the relative potency of the DHPs as Ca^{2+} channel blockers is complicated by the observation that inhibition by these agents is markedly voltage dependent and may vary by a factor of 1000 over a physiological range of membrane potentials (4, 12).

In addition to inhibiting Ca^{2+} channels, DHP Ca^{2+} channel antagonists also inhibit some types of K^+ channels in both vertebrate and invertebrate cells (13–15). In vertebrate cells, only delayed rectifier K^+ channels have been reported to be inhibited by DHP Ca^{2+} channel antagonists (15). Transient K^+ currents (I_A) in vertebrate neurons were unaffected by DHP antagonists at concentrations as high as 5 μM. However, the DHP nifedipine has been reported to block I_A in *Aplysia* bag cell neurons with an IC_{50} of 3–5 μM (14). In contrast to the DHP antagonists, the mixed DHP "agonist" (±)-Bay K 8644 has been reported to have no effect on transient or noninactivating K^+ currents in vertebrate and invertebrate cells (14, 15).

Bovine AZF cells express a single variety of voltage-gated I_A, which can be observed in isolation with whole-cell patch-clamp recording (16). We have studied the effect of DHP Ca^{2+} channel antagonists and agonists on I_A in bovine AZF cells. In contrast

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK40131 to J.J.E.

[1] Present address: Biomedical Imaging Group, University of Massachusetts Medical Center, Worcester, MA 01655.

ABBREVIATIONS: DHP, dihydropyridine; AZF, adrenal zona fasciculata; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; I_A, A-type K^+ current; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

to previous studies in other cells, all of these DHPs produced identical inhibitory effects on this transient K^+ current of the adrenal cortex.

Materials and Methods

Sources. Tissue culture media, antibiotics, fibronectin, and fetal calf serum were obtained from GIBCO (Grand Island, NY). Culture dishes were purchased from Corning (Corning, NY). Coverslips were from Bellco (Vineland, NJ). Enzymes and GTPγS were obtained from Sigma Chemical Co. (St. Louis, MO). Nimodipine, nisoldipine, and the isomers of Bay K 8644 were kindly provided by Dr. Alexander Scriabine, Miles Institute of Preclinical Pharmacology (West Haven, CT). The Ca^{2+} channel agonist RS 30026 (17) was a gift from Dr. Robert Greenhouse, Syntex Research (Palo Alto, CA).

Isolation and culture of AZF cells. Bovine adrenal glands were obtained from steers (age range, 1–3 years) within 15 min of slaughter at a local slaughterhouse. Fatty tissue was removed immediately and the glands were transported to the laboratory in ice-cold phosphate-buffered saline containing 0.2% dextrose. Isolated AZF cells were prepared as described previously (18), with some modifications. In a sterile tissue culture hood, the adrenal glands were cut in half lengthwise and the lighter medulla tissue was trimmed away from the cortex and discarded. The capsule and attached glomerulosa and thicker fasciculata-reticularis layer were then dissected into pieces of approximately 1.0 × 1.0 × 0.5 cm. A Stadie-Riggs tissue slicer (Thomas Scientific) was used to slice fasciculata-reticularis tissue from the glomerulosa layers, by slicing 0.3–0.5-mm slices from the larger pieces. Fasciculata tissue slices were diced into 0.5-mm^3 pieces, dissociated with 2 mg/ml (about 200 units/ml) type I collagenase and 0.2 mg/ml deoxyribonuclease in DMEM, containing 100 units/ml penicillin and 0.1 mg/ml streptomycin, for approximately 45 min at 37° in a shaking water bath, and triturated after 15 and 30 min with a sterile plastic transfer pipette. After incubation, the suspension was filtered through two layers of sterile cheesecloth and then centrifuged at 100 × *g* for 5 min to pellet cells. The cells were washed twice with DMEM/0.2% bovine serum albumin, with centrifugation as before to pellet. Cells were filtered through 200-μm stainless steel mesh to remove clumps after resuspension in DMEM/0.2% bovine serum albumin. Dispersed cells were again centrifuged and either were resuspended in DMEM/F-12 medium (1:1) with 10% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin and plated for immediate use or were resuspended in fetal calf serum/5% dimethylsulfoxide, divided into 1-ml aliquots (each containing about 1 × 10^7 cells), and stored in liquid nitrogen for future use. Cells were plated in 35-mm dishes containing 9-mm^2 glass coverslips that had been treated with fibronectin (10 μg/ml) at 37° for 30 min and then rinsed twice with warm sterile phosphate-buffered saline immediately before addition of cells. Dishes were maintained at 37° in a humidified atmosphere of 95% air/5% CO_2.

Patch-clamp experiments. Patch-clamp recordings of K^+ channel currents were made in the whole-cell configuration. The standard pipette solution contained 120 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 11 mM BAPTA, 200 μM GTP, 2 mM MgATP, and 200 μM GTPγS, with pH adjusted to 7.2 using KOH. GTPγS was added to the pipette solution to suppress the functional expression of a novel, noninactivating, voltage-independent K^+ current that we have identified in AZF cells (19). Apparently, the noninactivating, voltage-independent K^+ current exists under the inhibitory control of a G protein, because its functional expression is completely suppressed by the nonhydrolyzable GTP analog in the pipette solution. The external solution consisted of 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, and 5 mM glucose, pH 7.4 using NaOH. All solutions were filtered through 0.22-μm cellulose acetate filters.

For recording whole-cell Ca^{2+} currents, the standard electrode filling solution was 120 mM CsCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 11 mM BAPTA, 10 mM HEPES, and 1 mM MgATP, with pH titrated to 7.25 using CsOH. The standard external solution was 117 mM tetraethylammonium chloride, 5 mM CsCl, 10 mM $CaCl_2$, 5 mM HEPES, and 10 mM glucose, with pH adjusted to 7.3 using tetraethylammonium hydroxide.

AZF cells were used for patch-clamp experiments 2–24 hr after plating. Typically, cells with diameters of 15–20 μm and capacitances of 10–20 pF were selected. Coverslips were transferred from 35-mm culture dishes to the recording chamber (volume, 1.5 ml), which was continuously perfused by gravity at a rate of 3–5 ml/min. Patch electrodes with resistances of 1.0–2.0 MΩ were fabricated from Corning 0010 glass (Garner Glass Co., Claremont, CA). K^+ currents were recorded at room temperature (22–24°) following the procedure of Hamill *et al.* (20), using a List EPC-7 patch-clamp amplifier.

Pulse generation and data acquisition were done using a personal computer and pCLAMP software with an Axolab interface (Axon Instruments, Inc., Burlingame, CA). Currents were digitized at 1–10 kHz after filtering with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA). Linear leak and capacity currents were subtracted from current records using scaled hyperpolarizing steps of one-half to one-quarter amplitude. Data were analyzed and plotted using pCLAMP (CLAMPAN and CLAMPFIT) and GraphPAD InPLOT software. Drugs were applied by bath perfusion, controlled manually by a six-way rotary valve.

Results

In whole-cell patch-clamp recordings, it was discovered that the DHP Ca^{2+} channel agonists and antagonists modulated I_A with identical characteristics. These DHPs reversibly reduced peak current amplitude and accelerated the rate of decay (Fig. 1). At concentrations ranging from 0.2 to 20 μM, the Ca^{2+} channel antagonists nimodipine and (+)-Bay K 8644, as well as the Ca^{2+} channel agonists (−)-Bay K 8644 and RS 30026, reduced peak current amplitude with similar potencies. At a concentration of 5–20 μM, these agents reduced peak current by an average of 21.3–32.4% (Fig. 2A).

The effects of the DHPs on I_A decay were more pronounced. As reported previously, I_A in AZF cells inactivates with two voltage-independent time constants (16). After exposure of cells to 5 μM DHP, the more slowly inactivating component of I_A (approximately 25%) could no longer be measured, whereas the remaining faster component inactivated with markedly accelerated kinetics (Figs. 1, A and B, and 2B). The four DHPs reduced the rapidly inactivating time constant ($\tau_{in\text{-}f}$) by an average of 33.6 ± 3.7% to 59.6 ± 3.5% (Fig. 2B). For example, RS 30026, the most effective agent, reduced $\tau_{in\text{-}f}$ from 23.9 ± 1.06 to 9.65 ± 0.57 msec (n = 3).

The inhibition curves for DHP-mediated reduction of peak I_A amplitude and the time-dependent current integral emphasized that the most pronounced effect of these agents was to accelerate inactivation. (−)-Bay K 8644 and (+)-Bay K 8644 reduced the peak I_A with IC_{50} values of 27.4 and 25.2 μM, respectively. The DHPs inhibited the time-dependent I_A integral at significantly lower concentrations, with respective IC_{50} values of 6.3 and 10.1 μM (Fig. 3).

In modulating L-type Ca^{2+} channels in various cells, DHPs typically alter voltage-dependent gating properties, including activation, inactivation, and recovery. Within the framework of the "modulated receptor hypothesis" (21, 22), voltage-dependent properties of the DHPs reflect preferential binding of these agents to channels that are in conformations other than those corresponding to the resting state. We studied the effect of the DHPs on the voltage-dependent availability of I_A in AZF cells. The reduction of I_A amplitude by DHP did not stem from a shift in the voltage dependence of activation. Fig. 4A shows

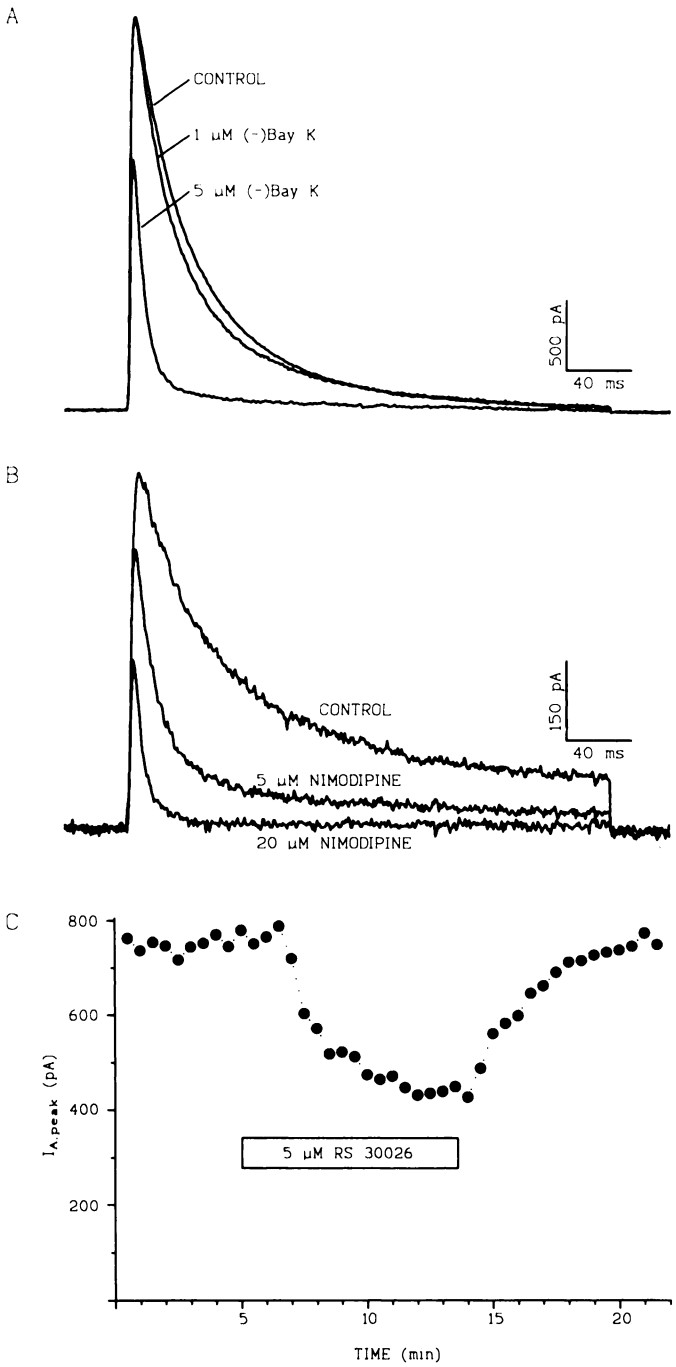

Fig. 1. Effect of DHP agonists and antagonists on I_A. Whole-cell I_A was recorded in bovine AZF cells in response to voltage-clamp steps to +20 mV applied at 30-sec intervals from a holding potential of −80 mV. After recording of currents in control saline solution, cells were superfused with either (−)-Bay K 8644 (A), nimodipine (B), or RS 30026 (C) at the indicated concentrations. Displayed current records in A and B were obtained after steady state block was achieved. In C, peak I_A is plotted against time.

that 5 μM nimodipine inhibited I_A to approximately the same extent at test potentials from −40 to +60 mV.

Preferential binding to and stabilization of inactivated L-type Ca^{2+} channels by DHPs lead to a significant hyperpolarizing shift in the steady state inactivation curve (4). To determine the effect of DHPs on voltage-dependent inactivation of I_A, peak I_A was measured during voltage steps to a common test potential of +20 mV, immediately after application of 10-sec conditioning pulses to voltages between −90 and −30 mV. The current was plotted as a function of the conditioning voltage and fitted with the equation $I/I_{max} = 1/[1 + \exp(v - v_{½})/k]$, where I_{max} is current activated from a holding potential of −90 mV, $v_{½}$ is the voltage at which half the channels are inactivated, and k is the slope factor. In the example shown in Fig. 4B, 5 μM (+)-Bay K 8644 reduced the peak I_A from 589 to 346 pA but produced no shift in the midpoint of the steady state availability curve, with $v_{½}$ values before and after exposure to the drug of −44.4 mV and −45.0 mV, respectively. Similar results were obtained in three other cells exposed to either isomer of Bay K 8644.

The DHP also failed to significantly alter the kinetics of inactivated A-type channels returning to the closed state. Recovery was studied by first voltage clamping cells at −20 mV to inactivate I_A and then switching to −80 mV for various periods up to 30 sec before applying an activating test pulse to +20 mV. Fig. 4C illustrates the temporal pattern of I_A recovery measured in four cells before and after exposure to 5 μM (−)-Bay K 8644. In control saline solution, I_A recovered with fast and slow time constants of 0.175 ± 0.012 and 13.57 ± 1.95 sec, respectively ($n = 4$). After exposure to (−)-Bay K 8644, I_A again recovered with two time constants, which were not significantly different from those observed in control saline solution (τ_{fast} = 0.216 ± 0.020 sec; τ_{slow} = 12.95 ± 2.72 sec).

Some antagonists of voltage-gated ion channels are effective only after channels have been opened by depolarization (23). Agents such as these often accelerate the apparent rate of macroscopic inactivation. Block by these same agents frequently displays use dependence and is greatly facilitated by depolarizing stimuli. The inhibitory actions of DHPs on I_A were not use dependent and developed maximally in the absence of channel activation by depolarizing stimuli. In the experiment illustrated in Fig. 5A, I_A was recorded in control saline solution before superfusion of the cell with 10 μM RS 30026 for 5 min in the absence of depolarizing stimuli. I_A peak amplitude was subsequently reduced by 57% in response to the first depolarizing test pulse. Additional depolarizations did not further reduce I_A amplitude or accelerate inactivation kinetics (Fig. 5A). Similar results were obtained in each of four experiments using RS 30026. Other DHPs, including (−)-Bay K 8644 and nimodipine, also did not show use dependence.

Bovine AZF cells express a low-voltage-activated transient T-type Ca^{2+} current that is inhibited by DHP Ca^{2+} channel antagonists over a concentration range similar to that which inhibits I_A (24). In contrast to actions on I_A, the inhibition of T-type Ca^{2+} current by DHPs was not accompanied by any change in inactivation kinetics. Fig. 5B shows an experiment in which 10 μM nisoldipine reduced peak Ca^{2+} current by 26% but did not change inactivation kinetics. Inactivation was described by single-exponential functions with time constants of 16.2 and 16.1 msec in control and nisoldipine-containing solutions, respectively. Nimodipine (1–20 μM) also inhibited T-type Ca^{2+} current but failed to alter inactivation kinetics ($n = 5$) (data not shown).

In a small percentage of cells (~10%), the rate and extent of I_A inactivation were observed to diminish continuously with time during prolonged whole-cell recording (16). DHPs were less effective at reducing I_A amplitude and accelerating decay kinetics in cells where inactivation had dramatically decreased.

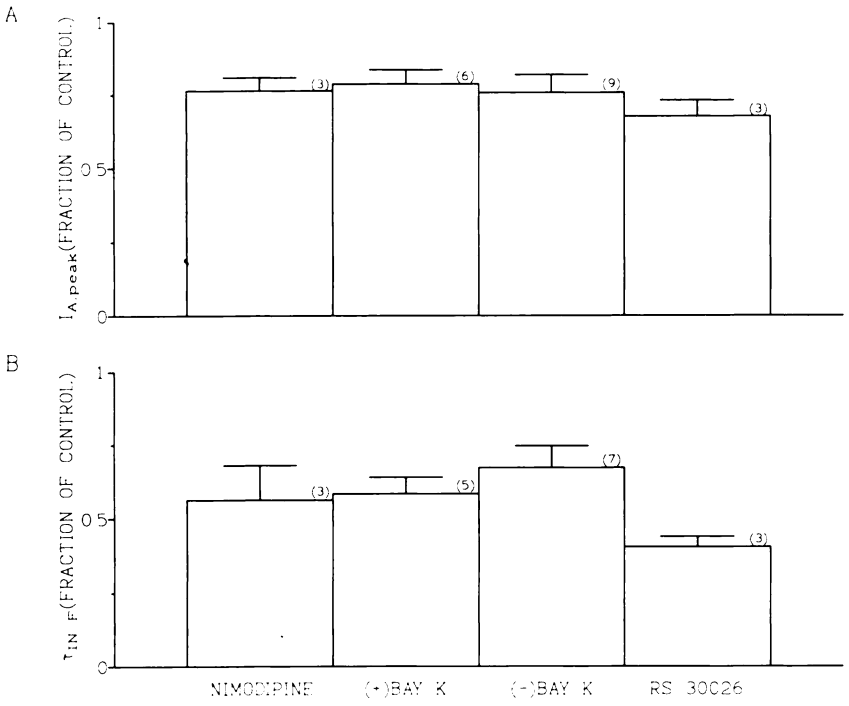

Fig. 2. Effect of DHPs on peak I_A amplitude and inactivation kinetics. I_A was recorded and cells were exposed to 5 μM DHP [nimodipine, (+)-Bay K 8644, (−)-Bay K 8644, or RS 30026] as described in the legend to Fig. 1. A, Peak current amplitudes recorded in the presence of each of the four DHPs, expressed as a fraction of the control value. Values are mean ± standard error (n = 3–9, as indicated in parentheses). B, Effect of DHPs on the fast inactivation time constant (τ_{in-f}). Decaying currents were fit with a two-exponential function. τ_{in-f} in the presence of DHP is expressed as a fraction of the control value. Values are mean ± standard error (n = 3–7, as indicated).

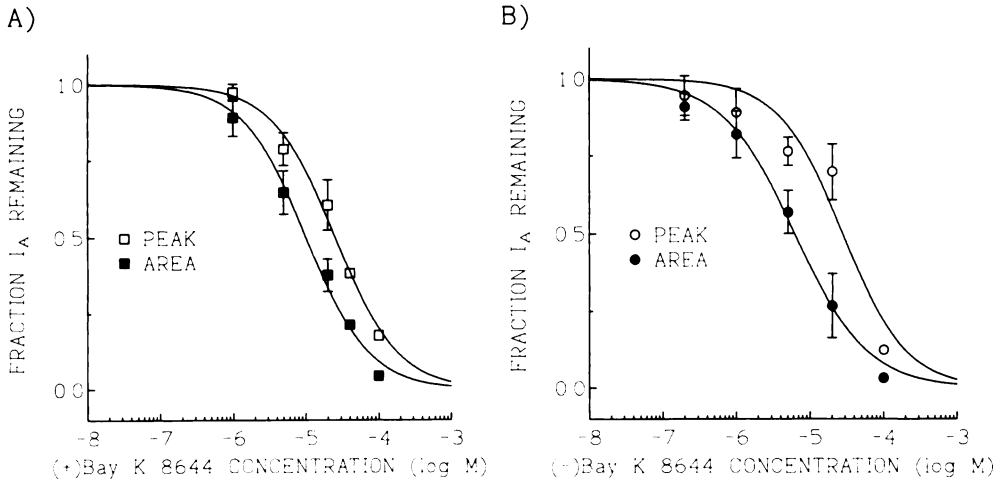

Fig. 3. Concentration-dependent inhibition of I_A by Bay K 8644 enantiomers. I_A in AZF cells was activated by 300-msec voltage steps to +20 mV applied at 30-sec intervals from a holding potential of −80 mV. Currents were measured in control saline solution and, after steady state block was reached, at various concentrations of either (+)-Bay K 8644 (A) or (−)-Bay K 8644 (B). For each enantiomer, maximum I_A (peak) or the time-dependent I_A integral (area) is plotted as a fraction of control I_A versus drug concentration. *Points* are fit with an equation of the form $I/I_{max} = 1/[1 + (B/K_d)]$, where B is the antagonist concentration and K_d is the equilibrium dissociation constant. Points are mean ± standard errors of two to nine separate determinations.

Fig. 6A shows I_A in a cell where inactivation had spontaneously slowed during 30 min of whole-cell recording (control). (+)-Bay K 8644 (10 μM) reduced peak I_A by only 15% in this cell, whereas 45% of I_A remained at the end of the 300-msec test pulse. Subtraction of I_A recorded in the presence of the DHP from the control current (Fig. 6B) also indicated that, during a 300-msec depolarizing pulse, the inhibitory effects of Bay K 8644 reached a maximum value in <50 msec and did not increase thereafter. Thus, in cells where inactivation diminished spontaneously with time, a component of I_A appeared to be insensitive to the DHP.

Discussion

In this study, we discovered that DHP Ca^{2+} channel agonists and antagonists produced identical inhibitory effects on I_A in bovine AZF cells. These included a reduction in the amplitude of the peak current and a marked increase in the rate of current decay after activation. The modulatory actions of the DHPs on I_A were not voltage or use dependent. With respect to potency, the DHPs inhibited I_A at 10–100-fold lower concentrations than did organic K^+ channel blockers such as 4-aminopyridine and tetraethylammonium (16, 23). Interestingly, in well polarized cells (e.g., at −80 mV), DHP Ca^{2+} channel antagonists inhibit AZF I_A and L-type Ca^{2+} current in various cells with similar potencies (4, 12).

The inhibitory effects of DHP Ca^{2+} channel modulators that we observed in AZF cells differ markedly from their previously reported actions on K^+ channels in both vertebrate and invertebrate cells. DHP Ca^{2+} channel antagonists failed to inhibit

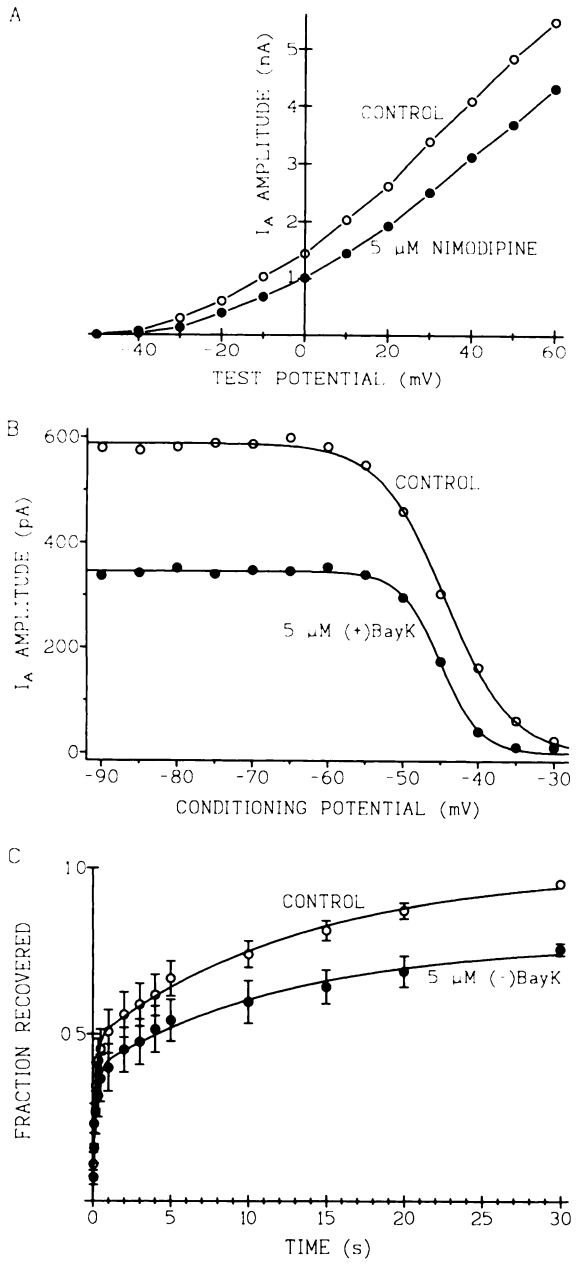

Fig. 4. Effect of DHPs on voltage-dependent availability of I_A. A, Current-voltage relationship. I_A was activated by depolarizing steps of increasing size from a holding potential of −90 mV, before and after superfusion of the cell with 5 μM nimodipine. Peak I_A amplitudes are plotted against test potential. B, (+)-Bay K 8644 and steady state inactivation. Voltage-dependent inactivation was measured by applying 10-sec prepulses to potentials between −100 and −0 mV, followed by activating test pulses to +20 mV. Peak current amplitudes activated from −90 mV are plotted against conditioning voltage and fitted with a Boltzmann function of the form $I/I_{max} = 1/[1 + \exp(v - v_{½})/K]$. *Curves* were obtained from a single cell before and after superfusion of 5 μM (+)-Bay K 8644 [control, I_{max} = 589 pA and $v_{½}$ = −44.4 mV; after (+)-Bay K 8644, I_{max} = 346 pA and $v_{½}$ = −45.0 mV]. C, (−)-Bay K 8644 and recovery kinetics. Time-dependent recovery of I_A (inactivated by a 30-sec prepulse to −20 mV) was monitored at a recovery potential of −80 mV for periods ranging from 0.02 to 30 sec, before and after exposure of cells to 5 μM (−)-Bay K 8644. Peak currents, normalized to the maximum current recorded in control saline solution (I/I_{max}), were plotted against time and fit with a function of the form $I/I_{max} = A(1 - e^{-T/\tau_f}) + B(1 - e^{-T/\tau_s})$. Normalized data points are mean ± standard error from four cells.

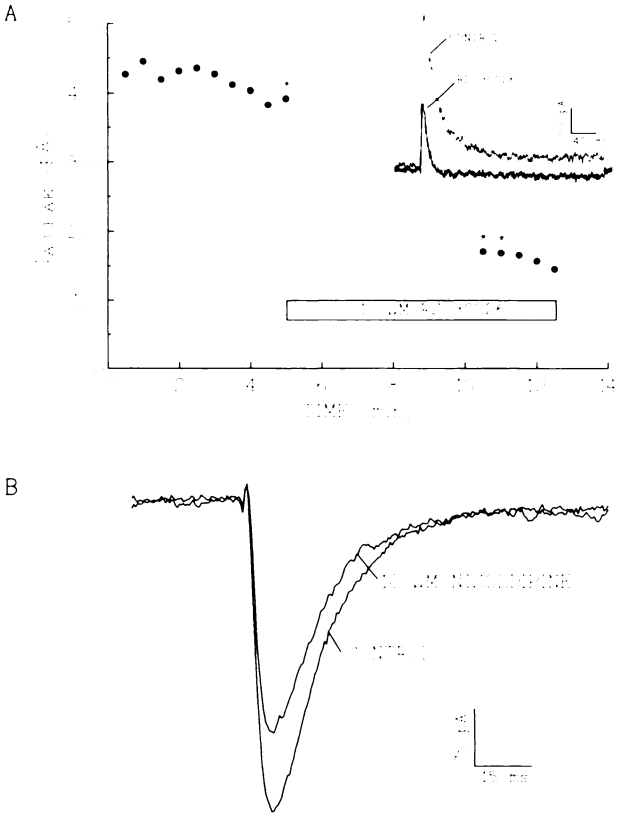

Fig. 5. Effect of DHPs on transient K^+ and Ca^{2+} currents. A, Absence of use dependence in block of I_A by RS 30026. I_A was recorded at 30-sec intervals in control saline solution before superfusion of 10 μM RS 30026 during a 5-min pulse-free period, after which depolarizing voltage steps were continued. Peak current is plotted against time. *, Times at which the three displayed current traces were recorded. B, Lack of effect of nisoldipine on T-type Ca^{2+} current inactivation kinetics. T-type Ca^{2+} currents were activated by depolarizing steps to 0 mV applied at 30-sec intervals from a holding potential of −80 mV. Current records in control saline solution and after steady state block with 10 μM nisoldipine are shown. The decaying component of current was fit with a single-exponential function.

any voltage-gated K^+ current in rat dorsal root ganglion neurons (14). At micromolar concentrations, DHPs did inhibit delayed rectifier-type K^+ channels in frog atrial cells (13) and in mouse embryonic sensory neurons (15). DHP antagonists have been reported to inhibit I_A only in *Aplysia* bag cell neurons, where nifedipine and nisoldipine inhibited an inactivating K^+ current with an IC_{50} of 3–5 μM (14). DHP antagonists accelerated I_A inactivation kinetics in *Aplysia* bag cells just as they did in AZF cells.

Modulatory effects of DHP Ca^{2+} channel agonists on voltage-gated K^+ current have not previously been reported. (±)-Bay K 8644 failed to inhibit or enhance any of the voltage-gated K^+ currents in vertebrate or invertebrate neurons, including those where DHP antagonists are effective (14, 15). Thus, A-type K^+ channels in bovine AZF cells are distinctive among K^+ channels described thus far, because they alone are sensitive to DHP Ca^{2+} channel agonists.

The interaction of DHP Ca^{2+} channel modulators with A-type K^+ channels is not surprising, in view of the structural and functional homologies that exist among voltage-gated ion channels (25). However, the modulatory effects of the DHPs on A-type K^+ channels differ markedly from their actions on Ca^{2+} channels in various cells. Block of L-type Ca^{2+} channels

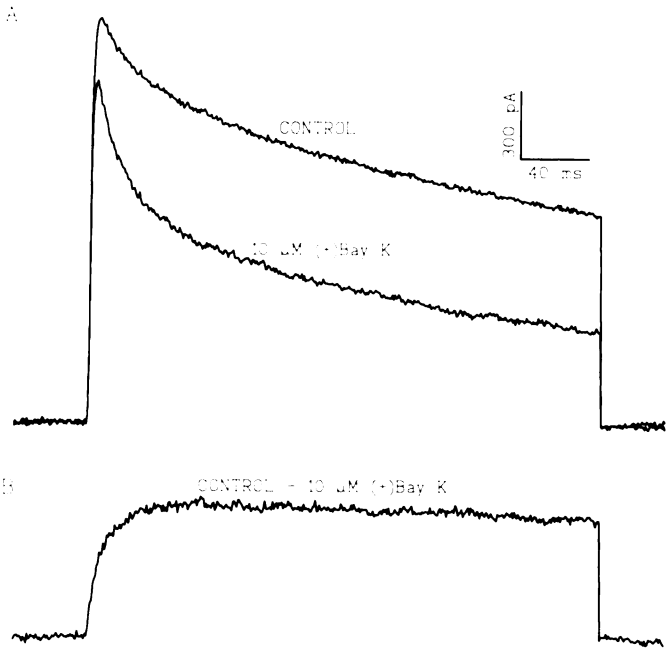

Fig. 6. Effect of (+)-Bay K 8644 on slowly inactivating I_A. I_A was activated at 30-sec intervals by voltage steps to +20 mV in a cell where activation decreased spontaneously with time. A, Control I_A recorded 35 min after the beginning of whole-cell recording and after superfusion of 10 μM (+)-Bay K 8644, as indicated. B, Difference current obtained by point by point digital subtraction of current after (+)-Bay K 8644 from control current.

by DHP antagonists is strongly voltage dependent and greatly enhanced at depolarized potentials (4, 12). This voltage-dependent block is accompanied by a significant hyperpolarizing shift in the steady state inactivation curve that, according to the modulated receptor hypothesis, reflects high affinity binding to and stabilization of inactivated channels (4, 21, 26). Because the DHP antagonists did not alter voltage-dependent steady state inactivation of I_A, it is unlikely that these agents inhibit A-type K^+ channels by a mechanism similar to that observed for L-type Ca^{2+} channels.

Agents such as local anesthetics, which also may act by stabilizing the inactivated state, slow the kinetics of recovery to the closed state (26). The failure of the DHPs to alter recovery kinetics of I_A provides additional proof that these agents do not preferentially interact with inactivated K^+ channels. This result provides additional evidence that DHPs act on Ca^{2+} and K^+ channels by distinct mechanisms.

Although the inhibition of I_A by DHPs does not appear to occur through stabilization of inactivated channels, the observed increase in the kinetics of current decay after activation is a common characteristic of open channel blockers (23). These agents, which gain access to their binding site through a hydrophilic pathway exposed upon channel opening, are frequently charged at physiological pH and include local anesthetics as well as some organic Ca^{2+} and K^+ channel antagonists (23, 26, 27). Block by these agents also displays use dependence and is greatly facilitated by depolarizing stimuli. Although the DHPs used in our study are largely uncharged at physiological pH, nimodipine has been reported to preferentially block open L-type Ca^{2+} channels in pituitary and smooth muscle cells (3, 28). However, the complete absence of use-dependent block by DHPs in our study indicates that DHPs do not inhibit I_A by preferential block of open channels.

The inhibitory effects of DHP Ca^{2+} channel agonists on A-type K^+ channel gating are in direct contrast to their well known promotion of L-type Ca^{2+} channel opening in response to depolarization. Inhibition of A-type K^+ channels by Bay K 8644 and RS 30026 is the most novel and distinctive action of DHPs on this transient current. The identical effects of DHP agonists and antagonists on I_A inactivation kinetics suggest that these agents share a common site and mechanism of action on A-type K^+ channels. These results do not exclude the possibility of separate binding sites for these agents.

The molecular mechanism by which DHP Ca^{2+} channel agonists and antagonists inhibit I_A is not clear. The evidence suggests a novel mechanism distinct from their action on Ca^{2+} channels. Specifically, in both vertebrate and invertebrate cells, fast inactivation involves the binding of an amino-terminal peptide, the "tethered ball," to a docking site that is exposed upon channel opening (29–31). A-type K^+ channels have been shown to reopen from the inactivated state upon repolarization, as though the blocking particle must exit the pore before the channel can close (30, 31). In this scheme, the amino-terminal tethered ball acts as an open channel blocker. Rather than directly occluding open channels, DHPs might speed inactivation by increasing the rate at which the tethered ball binds to its site on the cytoplasmic face of the channel. The decreased effectiveness of the DHPs in cells where I_A inactivation had diminished with time is consistent with such a mechanism. In contrast, the effectiveness of the open channel blocker 4-aminopyridine is not diminished in cells where inactivation has spontaneously slowed (16).

N-type gating kinetics depend on long range electrostatic interactions between the amino-terminal inactivation domain and its receptor sites in the pore. The "on" rate of the inactivation particle is strongly influenced by electrostatic forces (32–34). By binding to sites on the amino-terminal ball or pore region of the A-type channel, DHPs could alter electrostatic interactions between these regions, accelerating inactivation kinetics. Phosphorylation of Shaker A-type K^+ channels by cAMP-dependent protein kinase accelerates inactivation kinetics and reduces peak current, just as DHPs do in AZF cells (35). However, the DHPs used in our study are largely uncharged at physiological pH and therefore do not increase electrostatic interaction by the addition of negative charge. It will be interesting to determine whether DHPs that are permanently charged at physiological pH will enhance inactivation kinetics in the same way as the uncharged antagonists do.

It is less likely that the DHPs function through an action on activation kinetics. In some types of transient currents, inactivation kinetics are intrinsically voltage independent but become coupled to membrane potential through slower voltage-dependent activation kinetics (24, 36–38). Thus, agents that enhance activation kinetics could speed the decay of these currents. In bovine AZF cells, inactivation kinetics are voltage independent over a wide range of potentials. Strong depolarizations speed activation without affecting the relatively slower inactivation kinetics (16). These results indicate that DHPs do not produce their effects on I_A through an effect on kinetics of activation.

Combined with previous studies, our results indicate that considerable pharmacological diversity exists among K^+ channels in vertebrate cells, especially with respect to their sensitivity to DHP organic Ca^{2+} channel antagonists. These studies

also caution against using DHPs at micromolar concentrations in attempts to determine the function of L-type Ca^{2+} channels in cellular processes, such as secretion. The ability of the pure Ca^{2+} channel "agonist" Bay K 8644 is of particular interest in this regard, because it is routinely used at concentrations above 1 μM to demonstrate a role for L-type channels in processes ranging from transmitter and hormone release to gene expression. Our results indicate that this Ca^{2+} channel agonist might enhance secretion in some cells indirectly through an interaction with K^+ rather than Ca^{2+} channels. Bay K 8644 has been reported to stimulate cortisol secretion in bovine AZF cells, even though the great majority of these cells express only T-type Ca^{2+} channels (24, 39).

References

1. Bean, B. P. Classes of calcium channels in vertebrate cells. *Annu. Rev. Physiol.* **51**:367–384 (1989).
2. Enyeart, J. J., T. Aizawa, and P. M. Hinkle. Dihydropyridine Ca^{2+} antagonists: potent inhibitors of secretion from normal and transformed pituitary cells. *Am. J. Physiol.* **248**:C510–C519 (1985).
3. Cohen, C. J., and R. T. McCarthy. Nimodipine block of calcium channels in rat anterior pituitary cells. *J. Physiol. (Lond.)* **387**:195–225 (1987).
4. Sanguinetti, M. C., and R. S. Kass. Voltage-dependent block of calcium channel current in the calf cardiac Purkinje fiber by dihydropyridine calcium channel antagonists. *Circ. Res.* **55**:336–348 (1984).
5. Enyeart, J. J., B. A. Biagi, and R. N. Day. Opposing actions of Bay K8644 enantiomers on calcium current, prolactin secretion, and synthesis in pituitary cells. *Mol. Endocrinol.* **4**:727–735 (1990).
6. Duchemin, A.-M., J. A. Enyeart, B. A. Biagi, B. Mlinar, and J. J. Enyeart. Ca^{2+} channel modulation and kinase C activation in a pituitary cell line: induction of immediate early genes and inhibition of proliferation. *Mol. Endocrinol.* **6**:563–571 (1992).
7. Day, R. N., and R. A. Maurer. Pituitary calcium channel modulation and regulation of prolactin gene expression. *Mol. Endocrinol.* **4**:736–742 (1990).
8. Franckowiak, G., M. Bechem, M. Schramm, and G. Thomas. The optical isomers of the 1,4-dihydropyridine Bay K 8644 show opposite effects on Ca^{2+} channels. *Eur. J. Pharmacol.* **114**:223–226 (1985).
9. Akaike, N., P. G. Kostyuk, and Y. V. Osipchuk. Dihydropyridine-sensitive low-threshold calcium channels in isolated rat hypothalamic neurons. *J. Physiol. (Lond.)* **412**:181–195 (1989).
10. Enyeart, J. J., B. A. Biagi, and B. Mlinar. Preferential block of T-type calcium channels by neuroleptics in neural crest-derived rat and human thyroid C cell lines. *Mol. Pharmacol.* **42**:364–372 (1992).
11. Enyeart, J. J., B. Mlinar, and J. A. Enyeart. T-type Ca^{2+} channels are required for adrenocorticotropin-stimulated cortisol synthesis by bovine adrenal zona fasciculata cells. *Mol. Endocrinol.* **7**:1031–1040 (1993).
12. Bean, B. P. Nitrendipine block of cardiac calcium channels: high affinity binding to the inactivated state. *Proc. Natl. Acad. Sci. USA* **81**:6388–6392 (1984).
13. Hume, J. R. Comparative interaction of organic Ca^{2+} channel antagonists with myocardial Ca^{2+} and K^+ channels. *J. Pharmacol. Exp. Ther.* **234**:134–140 (1985).
14. Nerbonne, J. M., and A. M. Gurney. Blockade of Ca^{2+} and K^+ currents in bag cell neurons of *Aplysia californica* by dihydropyridine Ca^{2+} antagonists. *J. Neurosci.* **7**:882–893 (1987).
15. Valmier, J., S. Richard, E. Devic, J. Nargeot, M. Simonneau, and M. Baldy-Moulinier. Dihydropyridines interact with calcium-independent potassium currents in embryonic mammalian sensory neurons. *Pfluegers Arch.* **419**:281–287 (1991).
16. Mlinar, B., and J. J. Enyeart. Voltage-gated transient currents in bovine adrenal fasciculata cells. II. A-type K^+ current. *J. Gen. Physiol.* **102**:239–255 (1993).
17. Patmore, L., G. P. Duncan, B. Clarke, A. J. Anderson, R. Greenhouse, and J. R. Pfister. RS 30026: a potent and effective calcium agonist. *Br. J. Pharmacol.* **99**:687–694 (1990).
18. Gospodarowicz, D., C. R. Ill, P. J. Hornsby, and G. N. Gill. Control of bovine adrenal cortical cell proliferation by fibroblast growth factor: lack of effect of epidermal growth factor. *Endocrinology* **100**:1080–1089 (1977).
19. Mlinar, B., B. A. Biagi, and J. J. Enyeart. A novel K^+ current inhibited by ACTH and angiotensin II in adrenal cortical cells. *J. Biol. Chem.* **268**:8640–8644 (1993).
20. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:85–100 (1981).
21. Hille, B. Local anesthetics: hydrophilic and hydrophobic pathways for the drug receptor reaction. *J. Gen. Physiol.* **69**:497–515 (1977).
22. Hondeghem, L. M., and B. G. Katzung. Time- and voltage-dependent interactions of antiarrhythmic drugs with cardiac sodium channels. *Biochim. Biophys. Acta* **472**:373–398 (1977).
23. Hille, B. *Ionic Channels of Excitable Membranes*, Ed. 2. Sinauer Associates Inc., Sunderland, MA (1992).
24. Mlinar, B., B. A. Biagi, and J. J. Enyeart. Voltage-gated transient currents in bovine adrenal fasciculata cells. I. T-type Ca^{2+} current. *J. Gen. Physiol.* **102**:217–237 (1993).
25. Catterall, W. A. Structure and function of voltage-sensitive ion channels. *Science (Washington D. C.)* **242**:50–61 (1988).
26. Bean, B., C. J. Cohen, and R. W. Tsien. Lidocaine block of cardiac sodium channels. *J. Gen. Physiol.* **81**:613–642 (1983).
27. Lee, K. S., and R. W. Tsien. Mechanism of calcium channel blockade by verapamil, D600, diltiazem, and nitrendipine in single dialyzed heart cells. *Nature (Lond.)* **302**:790–794 (1983).
28. McCarthy, R. T., and C. J. Cohen. Nimodipine block of calcium channel currents in rat vascular smooth muscle cell lines. *J. Gen. Physiol.* **94**:669–692 (1989).
29. Hoshi, T., W. N. Zagotta, and R. W. Aldrich. Biophysical and molecular mechanisms of Shaker potassium channel inactivation. *Science (Washington D. C.)* **250**:533–538 (1990).
30. Ruppersberg, J. P., R. Frank, O. Pongs, and M. Stocker. Cloned neuronal I_K (A) channels reopen during recovery from inactivation. *Nature (Lond.)* **353**:657–660 (1991).
31. Demo, S. D., and G. Yellen. The inactivation gate of the Shaker K^+ channels behaves like an open-channel blocker. *Neuron* **7**:743–753 (1991).
32. Hoshi, T., W. N. Zagotta, and R. W. Aldrich. Two types of inactivation in Shaker K^+ channels: effects of alterations in the carboxy-terminal region. *Neuron* **7**:547–556 (1991).
33. Murrell-Lagnado, R. D., and R. W. Aldrich. Interactions of amino terminal domains of Shaker K^+ channels with a pore blocking site studied with synthetic peptides. *J. Gen. Physiol.* **102**:949–975 (1993).
34. Murrell-Lagnado, R. D., and R. W. Aldrich. Energetics of Shaker K^+ channel block by inactivation peptides. *J. Gen. Physiol.* **102**:977–1003 (1993).
35. Drain, P., A. E. Dubin, and R. W. Aldrich. Regulation of Shaker K^+ channel inactivation gating by the cAMP-dependent protein kinase. *Neuron* **12**:1097–1109 (1994).
36. Aldrich, R. W., D. P. Corey, and C. F. Stevens. A reinterpretation of mammalian sodium channel gating based on single channel recording. *Nature (Lond.)* **306**:436–441 (1983).
37. Chen, C., and P. Hess. Mechanisms of gating of T-type calcium channels. *J. Gen. Physiol.* **96**:603–630 (1990).
38. Belluzzi, O., O. Sacchi, and E. Wanke. A fast transient outward current in the rat sympathetic neurone studied under voltage-clamp conditions. *J. Physiol. (Lond.)* **358**:91–108 (1985).
39. Coyne, M. D., and L. Pinkney. Adrenocorticotropin activates barium-conducting channels from bovine zona fasciculata cells in lipid bilayers. *Endocrinology* **129**:263–269 (1991).

Send reprint requests to: John J. Enyeart, Department of Pharmacology, The Ohio State University College of Medicine, 5188 Graves Hall, 333 W. 10th Avenue, Columbus, OH 43210–1239.