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MOLECULAR PHARMACOLOGY, 38:524-530

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Nordihydroguaiaretic Acid Inhibits Voltage-Activated Ca^{2+} Currents Independently of Lipoxygenase Inhibition

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Received April 27, 1990; Accepted July 5, 1990

SUMMARY

The effects of nordihydroguaiaretic acid (NDGA), a widely used lipoxygenase inhibitor, were examined on voltage-activated Ca^{2+} channel currents in GH_3 and AtT-20 pituitary cells. NDGA (10–100 μM) produced a reversible, dose-dependent inhibition of Ca^{2+} channel currents, with half-maximal inhibition occurring at 18.6 μM. Inhibition by NDGA developed relatively slowly, did not exhibit use dependence or voltage dependence, and did not require access of NDGA to the extracellular domain of the Ca^{2+} channel. The maximum inhibition of macroscopic currents by 30 μM NDGA was equivalent in the presence of 5 and 50 mM extracellular Ca^{2+} and 5 mM Ba^{2+}. NDGA inhibited Ca^{2+} channel currents in excised, outside-out patches, in the absence of intra- and extracellular Ca^{2+} (with Ba^{2+} as the charge carrier), and following preincubation of the cells with the phospholipase A_2 inhibitor 4-bromophenacylbromide. Of five other lipoxygenase inhibitors tested, only one inhibited Ca^{2+} currents. These results suggest that NDGA inhibits Ca^{2+} channel currents by a mechanism distinct from that of other known Ca^{2+} channel antagonists and that, when influx of Ca^{2+} through voltage-gated channels is involved, inhibition of Ca^{2+}-dependent cell functions by NDGA (>10 μM) may be independent of effects on arachidonic acid metabolism.

The role of AA and its metabolites in endocrine and neuronal function has recently received much attention (1–6). In many cases, support for the role of AA metabolism in cell function has come from studies that utilized the compound NDGA. At low concentrations (<5 μM), NDGA is a relatively selective inhibitor of lipoxygenase enzymes (see Ref. 7), which catalyze the conversion of AA to several biologically active products, including hydroxyperoxyeicosatetraenoic acids and leukotrienes. At higher concentrations, NDGA also inhibits cyclooxygenase (7) and possibly phospholipase A_2 (Ref. 8, but see also Ref. 9).

At concentrations between 1 and 100 μM, NDGA inhibits hormone secretion in endocrine cells (1, 3, 4, 10) and certain excitatory events in neuronal cells (11, 12). Inhibition by NDGA of insulin secretion from pancreatic islets appears to be independent of effects on intracellular Ca^{2+} levels (10). However, effects of NDGA on pituitary hormone secretion suggest that NDGA interferes with Ca^{2+} entry into the cell. For example, stimulated pituitary hormone secretion that depends on entry of extracellular Ca^{2+} is inhibited by NDGA (3, 4), whereas phorbol ester-stimulated secretion, which appears not to require a rise in intracellular Ca^{2+}, is not inhibited by NDGA (3). Further, the inhibition of Ca^{2+}-dependent pituitary hormone secretion by NDGA can be overcome by addition of the Ca^{2+} ionophores A23187 and ionomycin (3, 4).

NDGA has also been used to implicate AA or its metabolites in synaptic function (11, 12). In the hippocampus, application of high concentrations of NDGA reduced or abolished several Ca^{2+}-dependent events, including population excitatory postsynaptic potentials, tetanus-induced increases in glutamate release, and the generation but not maintenance of long term potentiation of the excitatory postsynaptic potential.

The observations described above are consistent with the hypothesis that NDGA, either through interaction with AA metabolism or via some other mechanism, inhibits Ca^{2+} entry through plasma membrane Ca^{2+} channels. To test this hypothesis, we directly examined the effects of NDGA on voltage-gated Ca^{2+} channels. We utilized two anterior pituitary cell lines, AtT-20 cells, which are used extensively to study mechanisms of corticotropin secretion (13), and GH_3 cells, which have been used extensively to study the properties of transient and sustained voltage-activated Ca^{2+} currents (see Refs. 14 and 15). The results indicate that, at concentrations equal to or greater than 10 μM, NDGA inhibits both transient and sustained Ca^{2+} currents and that this inhibition occurs independently of effects on AA metabolism.

S.J.K. was supported in part by National Institutes of Health Grant NS-08117.

ABBREVIATIONS: AA, arachidonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NDGA, nordihydroguaiaretic acid; 4BPB, 4-bromophenacylbromide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; NMG, *N*-methylglucamine; TEA, tetraethyl ammonium.

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Experimental Procedures

Cell culture and materials. Sources and maintenance conditions for the cells used (GH_3 and AtT-20 anterior pituitary cell lines) were described previously (16). Lipoxygenase inhibitors, indomethacin, and BAY K-8644 were obtained from Biomol (Plymouth Meeting, PA), enzymes, creatine phosphate, and adenosine triphosphate were obtained from Calbiochem (La Jolla, CA), and the tetramethyl ether derivative of NDGA (RO7-6302) was kindly provided by Dr. Felicia Cochran at Hoffmann La-Roche (Nutley, NJ). All other reagents and chemicals were obtained from Sigma (St. Louis, MO). Stock solutions (10 mM in dimethyl sulfoxide) of AA, indomethacin, lipoxygenase inhibitors, and ascorbyl palmitate were made up immediately before use and diluted to the concentrations indicated. The final dimethyl sulfoxide concentration never exceeded 1% and did not affect Ca^{2+} currents.

Patch-clamp recording and solutions. Standard whole-cell and perforated patch recordings were made as described previously (16). All data were collected at room temperature, with a filter frequency of 2 KHz (internal Axopatch filter). Data were acquired and macroscopic current magnitudes were analyzed with PCLAMP (Axon Instruments, Burlingame, CA).

To facilitate the study of single L-type Ca^{2+} channels, cells were preincubated for 30 min and then bathed during the experiment in a solution containing 300 μM isobutylmethylxanthine to phosphorylate the Ca^{2+} channels (15) and 1 μM Bay K 8644 to prolong the mean Ca^{2+} channel open time (17). For studies of single channels in cell-attached patches, cells were bathed in 155 mM KCl to bring the membrane potential to near 0 mV. Membrane patches were held at −50 mV, and Ba^{2+} currents were evoked by 70-msec steps to −25 mV.

The recording chamber contained 2 ml of static bathing solution. Solution changes were accomplished (except in Fig. 3, A and B) by manual placement of a large-bore pipet that contained the drug or test solution into the bath near the cell under study. Application of test solution was terminated by removal of the large-bore pipet from the bath. With this technique, solutions bathing the cell were exchanged within 5 sec. For the experiments illustrated in Fig. 3, A and B, a magnetically driven stepping device was used to apply solutions with a latency of tens of milliseconds (16). The bath and pipet solutions, listed in Table 1, are cited in the figure legends.

Analysis of concentration-response relationship. We fit our data for the effect of NDGA on macroscopic Ca^{2+} currents (Fig. 1D) with a model that assumed a cooperative action of n molecules of NDGA on each channel. The percentage block, Y, depends on [NDGA], according to

$$Y = 100\{[\text{NDGA}]^n/(K^n + [\text{NDGA}]^n)\} \quad (1)$$

where K is the inhibition constant. The parameters n and K were estimated by minimization of least squares of error between the data and the theory.

Display and analysis of single-channel data. Single-channel data were digitized at a sample rate of 10 KHz (2-KHz filter) and digitally refiltered at 1 KHz for display. Capacity transients were cancelled by the averaging of 10–25 blank traces (most of which occurred during the NDGA treatment period) and subtraction of this averaged trace from each record. Each response to a test voltage was designated an episode, and the number of single-channel openings/episode was counted manually, with transitions between open and closed states determined using the crossing of half-maximum thresholds. Although 30 μM NDGA clearly reduced the opening probability in every patch, the recovery was less obvious. Because Ca^{2+} channel activity in an excised patch can diminish due to washout, it was critical to determine whether the effects of NDGA (reduction in channel activity) were reversible. We, therefore, used a rank sum test (18) to ask whether the number of openings/episode changed after removal of the NDGA from the bath. The level of significance was set at 0.05. Single-channel current amplitudes were determined by fitting gaussian curves to amplitude histograms (using PCLAMP from Axon Instruments, Inc.), which included 90–150 traces of 1024 data points.

TABLE 1

Solutions used

Bath solution 1 contained 1 μM tetrodotoxin. Pipet solutions 1, 2, and 5 contained 5–20 mM creatine phosphate, 2–4 mM ATP/Na, 50 units/ml creatine kinase, and 100 μM leupeptin. Pipet solution 4 and bath solution 6 contained 300 μM isobutylmethylxanthine, 1 μM Bay K 8644, and 1 μM tetrodotoxin. Dashes indicate that chemical wasn't added.

Bath solution (pH 7.35, osmolality 330–340)	NaCl	TEA-Cl	KCl	$CaCl_2$	$MgCl_2$	$BaCl_2$	HEPES	Glucose
				mM				
1	155	—	—	—	1	5	10	20
2	150	—	5	5	0.8	—	10	20
3	—	165	—	5	1	—	10	20
4	—	160	5	5	1	—	10	20
5	—	—	155	1	2	—	10	20
6	—	55	—	—	—	90	10	—
7	—	87.5	—	50	1	—	10	20

Pipet solution (pH 7.32, osmolality 300–315)	CsCl	NMG-Cl	Cs_2SO_4	$CaCl_2$	$MgCl_2$	$BaCl_2$	TEA-Cl	EGTA	HEPES
				mM					
1	—	100	—	—	4	—	—	10	10
2	110–125	—	—	1	4	—	—	11	10
3	55	—	75	—	8	—	—	—	10
4	—	—	—	—	—	90	50	—	10
5	125	—	—	—	2	—	—	5	40

Results

Inhibition of Ca^{2+} currents by NDGA. In GH_3 cells, 10 to 100 μM NDGA produced a reversible dose-dependent inhibition of calcium channel currents (Fig. 1). GH_3 cells contain at least two classes of Ca^{2+} channel. Activation of the low threshold T-type channel results in an early transient current, whereas activation of the high threshold L-type channel results in a sustained, slowly inactivating current (14). Voltage steps to 0 mV from a holding potential of −90 mV evoke Ca^{2+} currents through both channel types. Both transient and sustained Ca^{2+} currents in GH_3 cells were inhibited by NDGA (Fig. 1C). The concentration-response curve was quite steep; reversible Ca^{2+} current inhibition was detectable with 10 μM NDGA, and 100% inhibition was always achieved with 100 μM NDGA (Fig. 1D). On average, 30 μM NDGA inhibited Ca^{2+} currents, measured at their peak, by 80.1 ± 3.8% (10 cells; Fig. 1D). The smooth curve in Fig. 1D was calculated from the best fit parameters for Eq. 1. For these data, the value of the parameter n was 3.02, which for this model implies a cooperative interaction of at least three molecules of NDGA with a receptor. The half-maximal inhibition (parameter K) occurred at a concentration of 18.6 μM. Similar results were obtained with AtT-20 cells (data not shown). Although it was not rigorously studied, the degree of reversibility generally depended on the duration of NDGA exposure; longer exposures (>30–60 sec) usually resulted in incomplete recovery.

Production of AA via phospholipase A_2 and AA metabolism via the 5-lipoxygenase pathway require Ca^{2+} (19, 20). To determine whether Ca^{2+} was required for the action of NDGA, Ca^{2+} channel currents were studied in Ca^{2+}-free solutions, using Ba^{2+} as the charge carrier. Cells were perfused internally with a solution containing 10 mM EGTA and no added Ca^{2+} (AtT-20

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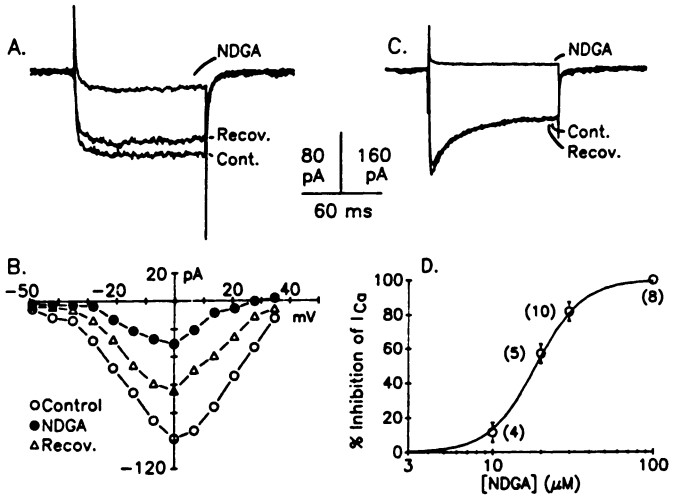

Fig. 1. Inhibition of Ca^{2+} and Ba^{2+} currents by NDGA. A, Ba^{2+} currents evoked in an AtT-20 cell by 100-msec depolarizations from −90 mV to 0 mV, before (*Cont.*), during (*NDGA*), and after removal of (*Recov.*) 30 μM NDGA. B, Ba^{2+} current I-V curve from a different AtT-20 cell, held at −90 mV and stepped incrementally to potentials between −49 and +35 mV. Briefer exposure to NDGA in A resulted in greater recovery. Bath solution 1; pipet solution 1. C, Ca^{2+} currents evoked as in A, in a GH_3 cell, before, during and after application of 30 μM NDGA. D, Plot of GH_3 cell Ca^{2+} current inhibition as a function of NDGA concentration. NDGA was applied until a constant current magnitude was obtained, which usually occurred between 15 and 80 sec after the start of NDGA application, depending on the concentration (see Fig. 3C). *Points* represent the mean ± standard error of the number of cells in *parentheses*. *Smooth curve*, best fit of Eq. 1, with parameters $n = 3.02$ and $K = 18.6$ μM. Holding potential, −90 mV; bath solution 3; pipet solution 2.

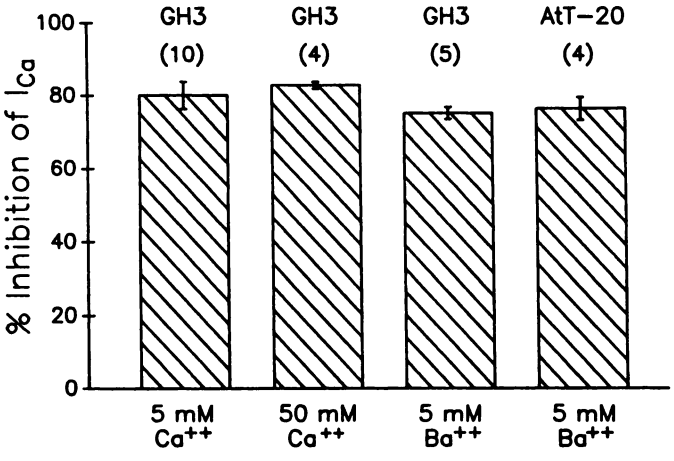

Fig. 2. Comparison of NDGA-induced inhibition at different $[Ca^{2+}]$ and with Ba^{2+} substituted for Ca^{2+}. Ca^{2+} currents were evoked as in Fig. 1A. NDGA (30 μM) was applied until maximum inhibition was achieved. GH_3 cells were bathed in either 5 or 50 mM Ca^{2+} or 5 mM Ba^{2+}. AtT-20 cells were bathed in 5 mM Ba^{2+}. *Error bars*, standard errors, *numbers in parentheses*, number of cells tested. In 50 mM Ca^{2+}, the peak of the Ca^{2+} current occurred following a voltage jump to between +28 and +35 mV (not shown), due to the well known charge-screening effect of Ca^{2+}. Currents were, therefore, elicited by voltage jumps from −60 mV to +30 mV. *Bar 1*, same data as in Fig. 1D. Other solutions were: bath 1 (*bars 3* and *4*); bath 7 (*bar 2*); pipet 1 (*bar 3*); pipet 2 (*bars 2* and *4*).

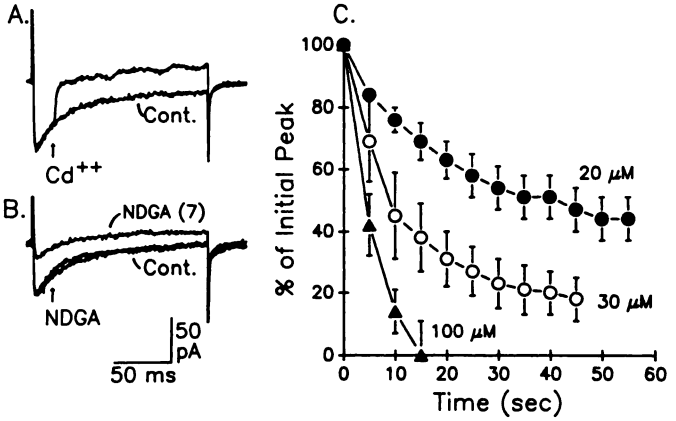

Fig. 3. Latency of inhibition by NDGA and Cd^{2+}. A and B, The perforated patch technique was used to record GH_3 cell Ca^{2+} currents, evoked by 160-msec voltage steps from −70 mV to +10 mV. A, Two superimposed currents, one in control solution (*Cont.*). During the second evoked current, the bathing solution was rapidly switched (*arrow*) to one containing 300 μM Cd^{2+}. B, Three superimposed currents evoked from the same cell by the same stimulus, 5 min after the currents in A. During the second evoked current in B, the bathing solution was switched (*arrow*) to one containing 100 μM NDGA. Application of this solution was continued, and 7 sec later the next voltage step was applied [*NDGA (7)*]. Bath solution 3; pipet solution 3. C, Percentage of inhibition of the peak Ca^{2+} current as a function of time for three different concentrations of NDGA, applied starting at time 0. Cells were the same as in Fig. 1D.

cells) or a solution in which $[Ca^{2+}]$ was buffered to less than 0.01 μM (GH_3 cells). The external solution contained 5 mM Ba^{2+} and no added Ca^{2+}. NDGA (30 μM) inhibited Ba^{2+} currents in both AtT-20 and GH_3 cells to the same extent as it inhibited Ca^{2+} currents (Figs. 1A and 2), which suggests that Ca^{2+} was not required for NDGA-induced inhibition.

Fig. 1B illustrates current-voltage (I-V) plots of Ba^{2+} currents in an AtT-20 cell before, during, and after exposure to NDGA. NDGA inhibited Ba^{2+} currents regardless of the step potential and did not shift the I-V relationship. In order to maximize reversibility of the inhibitory effects of NDGA, most experiments studied Ca^{2+} (or Ba^{2+}) currents evoked by steps to just a single voltage (0 mV unless stated otherwise).

$[Ca^{2+}]$ dependence of NDGA-induced inhibition. The observation that currents were inhibited equally whether Ca^{2+} or Ba^{2+} was the charge carrier suggested that NDGA did not block open Ca^{2+} channels by competing with permeant cations within the channel pore (see Discussion). To further test this hypothesis, the dependence of NDGA-induced inhibition on extracellular $[Ca^{2+}]$ was examined. If NDGA competed with Ca^{2+}, elevation of extracellular $[Ca^{2+}]$ should reduce the inhibition by NDGA (see Ref. 21). The maximal inhibition produced by 30 μM NDGA in the presence of 50 mM extracellular $[Ca^{2+}]$ was virtually identical to that produced in the presence of 5 mM Ca^{2+} (Fig. 2), which suggests that Ca^{2+} and NDGA did not compete either for a common binding site within the pore or for entrance into the channel.

Time course of block. The inhibitory effect of NDGA also differed from that of other Ca^{2+} channel antagonists in its time course. The experiments in Fig. 3 compared the onset latencies of effects of NDGA and Cd^{2+}. Calcium currents were recorded with the perforated patch technique to retard rundown, and a magnetically driven stepping device was used to rapidly (within tens of milliseconds) replace the solution bathing the cell with the test solution (16). Fig. 3A illustrates two superimposed Ca^{2+} currents evoked, 7 sec apart, by steps to +10 mV. The first was evoked in the control solution. During the second, 8 msec after the onset of the voltage step to +10 mV (Fig. 3A, *arrow*), the solution surrounding the cell was rapidly switched to one containing 300 μM Cd^{2+}. Within 20 msec, the Ca^{2+} current was

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completely blocked. The Cd^{2+} solution was removed at the end of the voltage step, and within 7 sec the Ca^{2+} current was fully recovered (not shown). The solution in the drug application pipet was then replaced with one containing 100 μM NDGA (Cd^{2+}-free). With a protocol identical to that used in Fig. 3A, NDGA applied rapidly to the same cell did not rapidly affect the magnitude of the Ca^{2+} current (Fig. 3B, *NDGA*). The application of NDGA was continued without interruption to determine whether NDGA would, in fact, inhibit the Ca^{2+} current in this cell. Seven seconds later, the Ca^{2+} current was inhibited [Fig. 3B, *NDGA* (*7*)]. Indeed, the onset of Ca^{2+} current inhibition was gradual and dose related (Fig. 3C). Higher concentrations of NDGA inhibited Ca^{2+} currents more quickly.

There are at least three possible explanations for the gradual onset of inhibition by NDGA with successive pulses (Fig. 3C). First, the action of NDGA may have required repetitive depolarization. This would manifest itself as a use-dependent channel block, as observed with classical organic Ca^{2+} channel antagonists (e.g., verapamil, diltiazem) or local anesthetic Na^{+} channel blockers (21, 22). Second, the action of NDGA may have been slow because it had to partition into the membrane to reach its site of action. Third, NDGA may not have been acting directly on the channel but rather by interference in a biochemical pathway (e.g., as a lipoxygenase inhibitor) to cause the gradual build-up or depletion of an endogenous Ca^{2+} channel modulator.

The experiment illustrated in Fig. 4 tested the hypothesis that Ca^{2+} current inhibition by NDGA was use dependent. In Fig. 4A, Ca^{2+} currents were evoked in control solution and then 45 sec following application of 30 μM NDGA. NDGA inhibited the current by about 43% (Fig. 4C, *triangle*). NDGA was removed, and a Ca^{2+} current was evoked every 30 sec to follow the recovery. After 5 min, the Ca^{2+} current reached a constant magnitude, and the protocol in Fig. 4B was begun. Following a stimulus in control solution, Ca^{2+} currents were evoked in the presence of NDGA at 10-sec intervals, beginning 5 sec after the start of NDGA application. After 45 sec (five stimuli in NDGA), the magnitude of Ca^{2+} current inhibition was virtually identical (47%) (Fig. 4C, *circles*) to that obtained with the protocol of Fig. 4A. These results suggest that NDGA activity was not use dependent and, further, that Ca^{2+} channels need not open for NDGA to act.

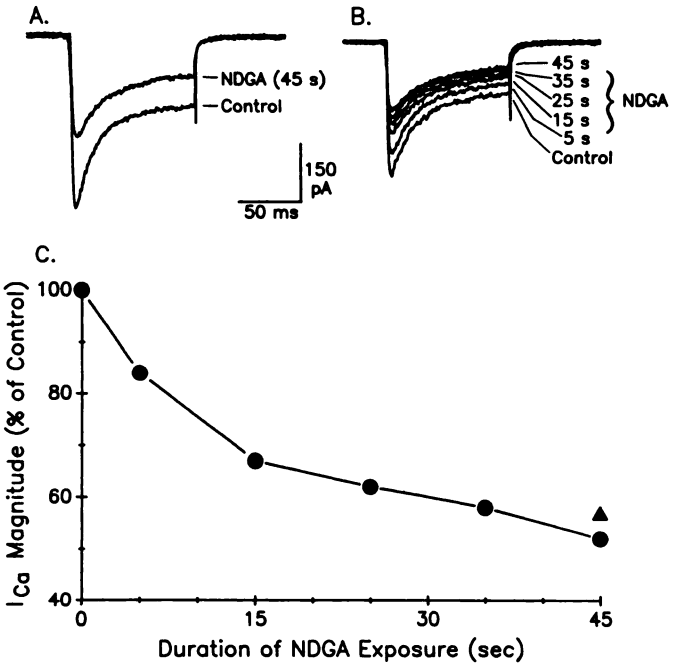

Fig. 4. Lack of use dependence of inhibition by NDGA. Ca^{2+} currents were evoked in a GH_3 cell by 100-msec depolarizing pulses from −90 mV to 0 mV. A, Two currents were evoked, one as control and one 45 sec after application of 30 μM NDGA. B, In the same cell, following a 5-min recovery period, currents were evoked every 10 sec in control conditions and then starting 5 sec after application of NDGA. C, Plot of percentage of inhibition of Ca^{2+} current as a function of the duration of NDGA exposure. ▲, Percentage of inhibition using protocol A above; ●, time course of inhibition using protocol B above. Bath solution 4; pipet solution 1.

Effect of NDGA on single Ca^{2+} channel currents studied in cell-attached membrane patches. The remaining two hypotheses described above, that NDGA either partitioned into the membrane to reach its site of action or interfered in an endogenous biochemical pathway, predict that NDGA should inhibit Ca^{2+} channel currents even if it does not have direct access to the extracellular channel domain. This prediction was tested by the study of single Ca^{2+} channel currents in the cell-attached configuration. NDGA was applied to the cell in the bath solution, where it had access to the entire cell except for the external face of the membrane patch that contained the Ca^{2+} channels under study.

Fig. 5A illustrates single Ba^{2+} currents evoked before, during, and after bath application of NDGA (30 μM). NDGA dramatically reduced the probability of the channel being open (Fig. 5B), while having no effect on the single-channel current (1.29 ± 0.13 pA in control, 1.29 ± 0.15 pA with NDGA). NDGA inhibited channel activity in five of five patches; statistically significant (see Experimental Procedures) but incomplete recovery occurred in four of four patches (one patch became too noisy in recovery to analyze).

Effect of NDGA on single Ca^{2+} channel currents studied in excised patches. The observation that NDGA blocked Ca^{2+} currents in standard whole-cell recordings suggested that soluble cytoplasmic biochemicals were not required for its action. To further investigate this hypothesis, the effect of NDGA on Ca^{2+} channels was examined on excised, outside-out, membrane patches. As with cell-attached patches, NDGA (30 μM) dramatically reduced the probability of channel opening (Fig. 6; nine of nine patches). Statistically significant recovery occurred in four of eight patches (one patch became too noisy in recovery to analyze). In two of four patches, a smaller current amplitude (0.8–0.9 pA at −25 mV) (Fig. 6A) was observed for some openings during recovery. Amplitude histograms revealed that this component was also present in control records. However, due to the small number of events in the presence of NDGA, neither this nor the effects of NDGA on single-channel current amplitude were studied in detail.

Under these conditions, the only cellular biochemicals available for interaction with NDGA are those that were bound to the excised membrane patch. Because lipoxygenase is not active as a membrane-bound enzyme (23, 24), these data indicate that NDGA inhibited Ca^{2+} channel opening independently of lipoxygenase inhibition.

Effects of antioxidants on Ca^{2+} currents. NDGA is a symmetric compound containing two terminal catechols separated by a six-carbon chain. This structure makes it both

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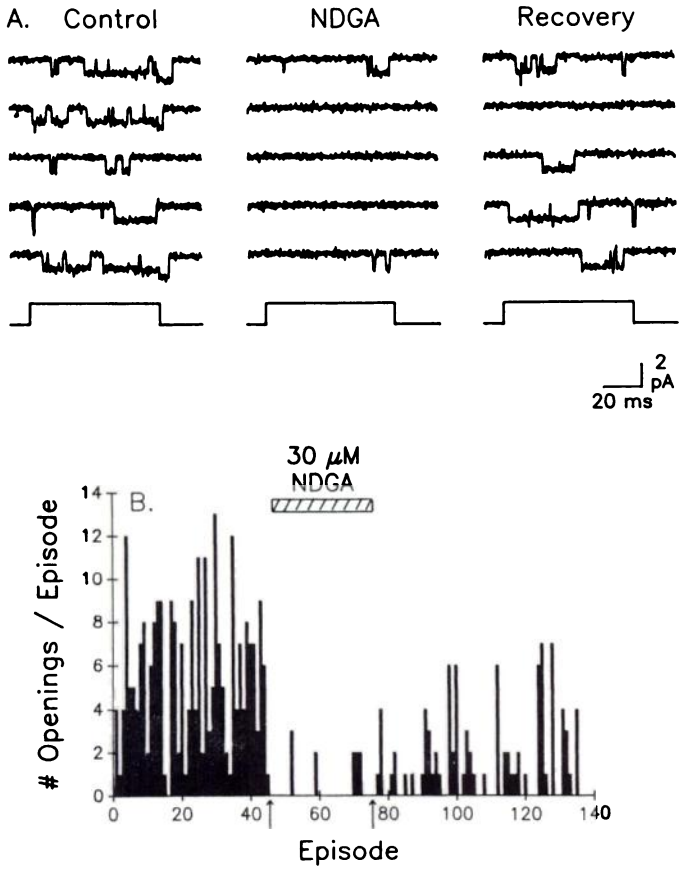

Fig. 5. Inhibition by NDGA of single Ca^{2+} channel currents recorded in the cell-attached patch configuration. Single Ca^{2+} channel currents (with Ba^{2+} as the charge carrier) were evoked by 70-msec depolarizations from −50 mV to −25 mV. A, Selected current traces from a GH_3 cell patch in control solution (*left*), 30–180 sec after application of 30 μM NDGA (*middle*), and 30–180 sec after removal of the NDGA (*right*). Downward deflections reflect channel opening. B, Histogram of data from experiment in A, showing number of openings/trace before, during (*bar* and *arrows*), and after application of NDGA. Bath solution 5; pipet solution 4.

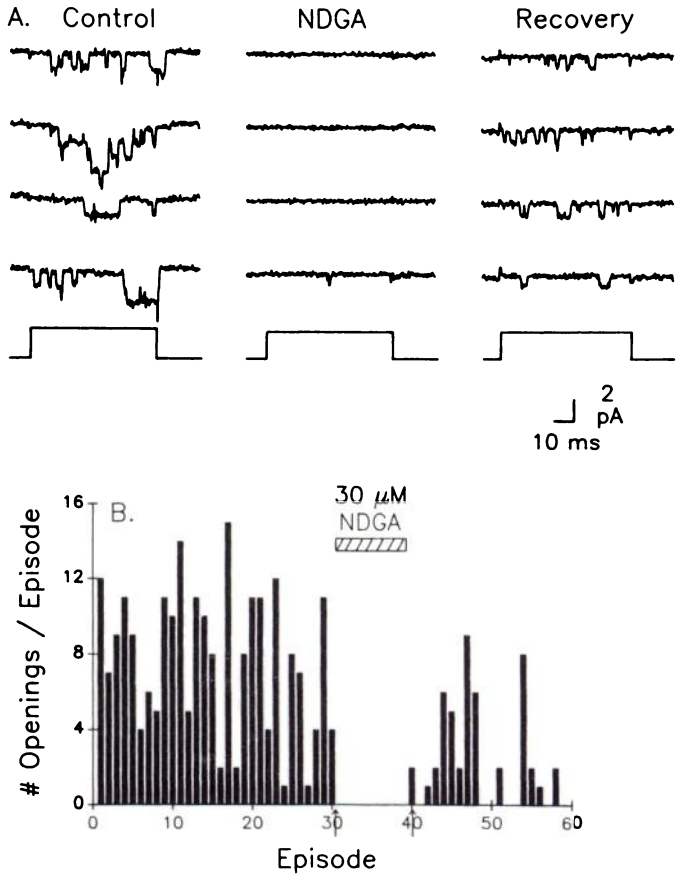

Fig. 6. Inhibition by NDGA of single Ca^{2+} channel currents recorded in an excised, outside-out membrane patch. Following formation of the outside-out patch from a GH_3 cell, the protocol in Fig. 5 was used to evoke single Ba^{2+} currents. Bath solution 6; pipet solution 5.

lipophilic and a potent antioxidant and radical scavenger (25). Application of the hydrophilic antioxidants catechol (200 μM) or ascorbate (1 mM) or the lipophilic antioxidant ascorbyl palmitate (100 μM) (26) did not influence Ca^{2+} currents. Thus, it appears that NDGA did not influence Ca^{2+} currents by antioxidant or radical-scavenging activity, either on the membrane surface or from within the membrane. This conclusion is supported by the finding that the lipoxygenase inhibitors phenidone, esculetin, and caffeic acid (100 μM), all of which have antioxidant activity (7, 9, 26), did not inhibit Ca^{2+} currents. However, the hydroxyl groups on the NDGA catechols apparently were important for activity, because the tetramethyl ether derivative of NDGA (RO7-6302) (100 μM), in which ether-linked methyl groups are substituted for the four catechol hydroxyls, was also without effect on Ca^{2+} currents.

Inhibition of Ca^{2+} currents by AA. Although NDGA appeared to inhibit Ca^{2+} channel currents independently of effects on AA metabolism, it was still of interest to determine whether a buildup of AA or depletion of eicosanoid products could influence Ca^{2+} channel function.

In GH_3 and AtT-20 cells, AA had multiple effects on membrane currents. At concentrations between 10 and 50 μM, AA inhibited Ca^{2+} currents by approximately 10 to 50% (Fig. 7A). This inhibition was partially reversible. Prolonged exposure (20 sec to 2 min, depending on the concentration) to >10 μM AA also made the cells very leaky, from which they rarely recovered (not shown). We never observed greater than 30% inhibition by AA without the appearance of 'membrane breakdown'-like activity. These data demonstrate that AA could inhibit Ca^{2+} currents, but they also suggest that a simple buildup of free AA in the plasma membrane would create very leaky membranes at concentrations that produce a substantial Ca^{2+} current inhibition.

To further examine the possibility that free endogenous AA or AA metabolites tonically influenced GH_3 cell Ca^{2+} currents, 4BPB, a phospholipase A_2 inhibitor, was applied to the cell. 4BPB (10 μM) did not affect Ca^{2+} currents when applied acutely for a duration of 3 min (Fig. 7B). In addition, a 28-min preincubation with 10 μM 4BPB, which prevents putative AA-mediated responses in *Aplysia* neurons (27), did not prevent the effects of subsequently applied NDGA (Fig. 7C).

Several other lipoxygenase inhibitors were examined to determine whether this class of compounds inhibited Ca^{2+} currents. Of five compounds tested at the high concentration of 100 μM, only ketoconizole inhibited Ca^{2+} currents (by 60 ± 7.5%; seven cells). Phenidone, esculetin, caffeic acid, and 5,8,11,14-eicosatetraynoic acid were without effect. Application of the cyclooxygenase inhibitor indomethacin (100 μM for ≥60 sec) inhibited whole-cell Ca^{2+} currents by 15.3 ± 2.6% (three

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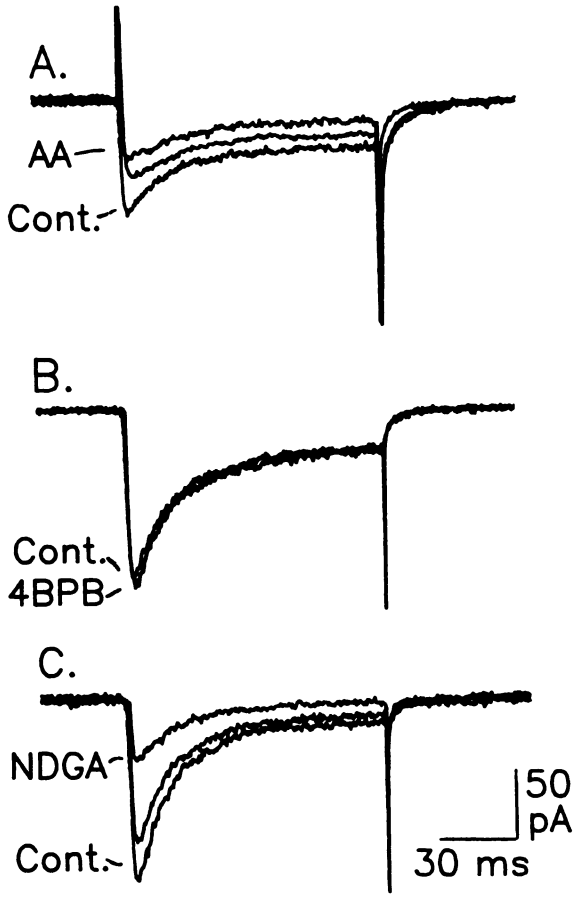

Fig. 7. Effects of AA and 4BPB on Ca^{2+} currents. Perforated patch (A) and standard whole-cell (B and C) recordings of GH_3 cell Ca^{2+} currents. Protocol as in Fig. 1C. A, AA (50 μM) was applied for 20 sec and removed. *Middle trace*, recovery 2 min after removal of AA. Bath solution 2; pipet solution 3. B, 4BPB (10 μM) was applied for 3 min. C, Cells were preincubated for 28 min with 10 μM 4BPB at 37° in a CO_2 incubator. *Traces*, Ca^{2+} currents in control solution (*Cont.*), following application of 30 μM NDGA (*NDGA*), and in recovery (*middle trace*). 4BPB (10 μM) was present throughout the experiment. Bath solution 3; pipet solution 2.

cells). This effect did not reverse, however, and could not be distinguished from rundown.

Discussion

NDGA is commonly used at concentrations between 10 and 100 μM to study the role of AA, and particularly its lipoxygenase metabolites, in cell function (see Refs. 1, 3–5, 11, and 12). The results of this study indicate that NDGA, at concentrations of 10 μM or more, inhibits voltage-activated Ca^{2+} currents independently of its effects on AA metabolism.

The strongest evidence that Ca^{2+} current inhibition by NDGA was independent of lipoxygenase is that NDGA inhibited single Ca^{2+} channel currents recorded in excised, outside-out, membrane patches. Lipoxygenase is a soluble cytoplasmic enzyme and is not active if it becomes membrane bound (23, 24). Thus, it is reasonable to assume that active lipoxygenase was not present in these excised patches. In addition, AA production and metabolism require Ca^{2+}, and NDGA inhibited currents through Ca^{2+} channels in the virtual absence of either internal or external Ca^{2+} (Figs. 1, A and B, and 6).

Whether lipoxygenase products influence Ca^{2+} channel function was not tested in this study. However, several lipoxygenase and cyclooxygenase inhibitors, including phenidone, esculetin, caffeic acid, and indomethacin, did not inhibit Ca^{2+} currents, which suggests that tonic AA metabolism by these pathways was not a prominent influence on Ca^{2+} channel function. It is interesting, however, that ketoconizole, which inhibits lipoxygenase but is structurally unrelated to NDGA (7), also inhibited Ca^{2+} currents in a similar concentration range. Whether ketoconizole and NDGA act via similar mechanisms awaits further study.

Effects of AA. As reported recently by others (28, 29), Ca^{2+} currents were reversibly inhibited by application of AA (Fig. 7). However, several observations suggest that NDGA did not inhibit Ca^{2+} currents via a build-up of AA. First, prolonged exposure of cells to greater than 10 μM AA produced 'detergent-like' breakdown of cell membranes but inhibited Ca^{2+} currents by less than 50%. In many cells, prolonged exposure to high concentrations (>30 μM) of NDGA also produced this apparent membrane breakdown, but only after Ca^{2+} currents were inhibited by nearly 100%. Second, AA production via the phospholipase A_2 pathway is Ca^{2+} dependent (19), yet NDGA inhibited Ca^{2+} channel currents when extracellular Ca^{2+} was replaced by Ba^{2+} and intracellular Ca^{2+} was reduced to less than 0.01 μM by addition of 10 mM EGTA. Third, preincubation of cells with 4BPB, a phospholipase A_2 inhibitor, did not prevent the action of NDGA. Although these findings cannot rule out a possible involvement of AA in the action of NDGA, any proposed mechanism of AA build-up would have to occur in an excised patch, where cytoplasmic factors are absent and free AA that leaves the cell membrane would, due to infinite dilution, be unlikely to influence events in the excised patch.

Comparison of NDGA with Cd^{2+}. Ca^{2+} channel block by Cd^{2+} and other inorganic divalent cations is believed to occur when the blocker enters the open channel via the same route as Ca^{2+} and competes with Ca^{2+} for a binding site within the channel but does not permeate the channel (30). Inhibition of Ca^{2+} currents by NDGA differed from that by Cd^{2+} in several ways. For example, the dose-response relationship (Fig. 1D) for NDGA block is steeper than that found for divalent Ca^{2+} channel blockers (30). NDGA apparently did not compete with Ca^{2+} for entry into the open channel or for a Ca^{2+} binding site, because 1) NDGA inhibited Ca^{2+} currents even when it did not have access to the external mouth of the open channel (Fig. 5), 2) the action of NDGA was very slow compared with that of Cd^{2+} (Fig. 3), and 3) inhibition was not reduced by a 10-fold increase in extracellular Ca^{2+} concentration (Fig. 2). Furthermore, it is predicted that a compound that competes for the hypothesized cation binding site within the channel will more effectively inhibit currents carried by Ba^{2+} than by Ca^{2+} (21), because Ba^{2+} has a lower affinity for the binding site (30). Whereas Cd^{2+} inhibits Ba^{2+} currents more effectively than Ca^{2+} currents (21), NDGA inhibited Ca^{2+} and Ba^{2+} currents equally. Together, these data suggest that NDGA does not act by a mechanism similar to that of inorganic divalent cations. In addition, NDGA did not produce single-channel flickering or reduce the single-channel conductance, which are characteristics of "fast" open channel blockers (see Ref. 31). Our data do not exclude the possibility, however, that NDGA entered the channel pore via a lipophilic pathway and plugged it by a mechanism not subject to competition with permeant cations.

Comparison of NDGA with organic Ca^{2+} channel antagonists. NDGA shares some structural similarity with the

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phenylalkylamine verapamil. Although both NDGA and verapamil are lipophilic, their mechanisms of channel block appear to be different. First, channel block by verapamil is use dependent (21) and that by NDGA is not (Fig. 4). Second, in contrast to the action of NDGA, the verapamil analog D600, and presumably also verapamil, inhibits currents carried by Ba^{2+} less effectively than those carried by Ca^{2+} (21). Third, substitution of ether-linked methyl groups for the hydroxyl groups on NDGA, which makes the structure even more similar to verapamil, eliminated the activity of NDGA. The action of NDGA also differed from that of the organic Ca^{2+} channel blocker diltiazem, in that the latter displays a marked use dependence and differential effectiveness at blocking Ca^{2+} and Ba^{2+} currents (21). Although we did not explore it in detail, inhibition of Ca^{2+} currents by NDGA did not share two of the hallmark characteristics of inhibition by the 1,4-dihydropyridine Ca^{2+} channel antagonists. In contrast to the actions of 1,4-dihydropyridines (see Ref. 32), NDGA inhibited both transient and sustained Ca^{2+} currents with equal potency and inhibited sustained Ca^{2+} currents equally regardless of the holding potential.

In summary, NDGA inhibited Ca^{2+} channel activity by a mechanism that required neither cytoplasmic molecules nor Ca^{2+}-dependent biochemical reactions. Although its mechanism remains unclear, NDGA apparently did not compete with Ca^{2+} for a cation binding site within the channel, nor did it act similarly to the other organic Ca^{2+} channel antagonists. The slow time course, lack of use dependence, and action without access to the external surface of the channel suggest that, to block Ca^{2+} currents, NDGA partitioned into the membrane and interacted either with the channel protein directly or with another membrane-bound Ca^{2+} channel modulator. Because NDGA appears to have a different mechanism of action than other known Ca^{2+} channel antagonists, studies of its properties may provide insight into the functioning of the channel. More importantly, these studies clearly indicate that NDGA cannot be used to investigate the role of AA metabolism in cell function without accounting for possible actions on Ca^{2+} channel activity. Indeed, of the lipoxygenase inhibitors tested, only those that inhibit Ca^{2+} currents inhibit adrenocorticotropin secretion from AtT-20 cells.[1]

Acknowledgments

We thank Arthur Bolden for maintaining the cell cultures, Drs. Felicia Cochran and Ronald LaMahieu for gifts of compounds and enlightening discussions, Dr. David Armstrong for helpful suggestions about recording single Ca^{2+} channel currents, and Drs. David Linden and James Morgan for reading the manuscript.

References

1. Abou-Samra, A.-B., K. J. Catt, and G. Aguilera. Role of arachidonic acid in the regulation of adrenocorticotropin release from rat anterior pituitary cell cultures. *Endocrinology* **119**:1427–1431 (1986).
2. Barkai, A. I., and N. G. Bazan, eds. *Arachidonic Acid Metabolism in the Nervous System*. New York Academy of Sciences, New York (1989).
3. Chang, J. P., J. Graeter, and K. J. Catt. Dynamic actions of arachidonic acid and protein kinase C in pituitary stimulation by gonadotropin-releasing hormone. *Endocrinology* **120**:1837–1845 (1987).
4. Luini, A. G., and J. Axelrod. Inhibitors of cytochrome P-450 enzymes block the secretagogue-induced release of corticotropin in mouse pituitary tumor cells. *Proc. Natl. Acad. Sci. USA* **82**:1012–1014 (1985).
5. Negro-Vilar, A., D. Conte, and M. Valenca. Transmembrane signals mediating neural peptide secretion: role of protein kinase C activators and arachidonic acid metabolites in leuteinizing hormone-releasing hormone secretion. *Endocrinology* **119**:2796–2802 (1986).
6. Stern, N., R. Natarajan, M. L. Tuck, E. Laird, and J. L. Nadler. Selective inhibition of angiotensin-II-mediated aldosterone secretion by 5-hydroxyeicosatetraenoic acid. *Endocrinology* **125**:3090–3095 (1989).
7. Beetens, J. R., W. Loots, Y. Somers, M. C. Coene, and F. De Clerck. Ketoconazole inhibits the biosynthesis of leukotrienes *in vitro* and *in vivo*. *Biochem. Pharmacol.* **35**:883–891 (1986).
8. Billah, M. M., R. W. Bryant, and M. I. Siegel. Lypoxygenase products of arachidonic acid modulate biosynthesis of platelet activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) by human neutrophils via phospholipase A2. *J. Biol. Chem.* **260**:6899–6906 (1985).
9. Ozaki, Y., T. Ohashi, and Y. Niwa. A comparative study on the effects of inhibitors of the lipoxygenase pathway on neutrophil function. *Biochem. Pharmacol.* **35**:3481–3488 (1986).
10. Metz, S. A. Lipoxygenase inhibitors reduce insulin secretion without impairing calcium mobilization. *Endocrinology* **120**:2534–2546 (1987).
11. Lynch, M. A., M. L. Errington, and T. V. P. Bliss. Nordihydroguaiaretic acid blocks the synaptic component of long-term potentiation and the associated increases in release of glutamate and arachidonate: an *in vivo* study in the dentate gyrus of the rat. *Neuroscience* **30**:693–701 (1989).
12. Williams, J. H., and T. V. P. Bliss. Induction but not maintenance of calcium-induced long-term potentiation in dentate gyrus and area CA1 of the hippocampal slice is blocked by nordihydroguaiaretic acid. *Neurosci. Lett.* **88**:81–85 (1988).
13. Axelrod, J., and T. D. Reisine. Stress hormones: their interaction and regulation. *Science* (*Washington D. C.*) **224**:452–459 (1984).
14. Matteson, D. R., and C. M. Armstrong. Properties of two types of calcium channels in clonal pituitary cells. *J. Gen. Physiol.* **87**:161–182 (1986).
15. Armstrong, D., and R. Eckert. Voltage activated calcium channels that must be phosphorylated to respond to membrane depolarization. *Proc. Natl. Acad. Sci. USA* **84**:2518–2522 (1985).
16. Korn, S. J., and R. Horn. Influence of sodium-calcium exchange on calcium current rundown and the duration of calcium-dependent chloride currents in pituitary cells, studied with whole cell and perforated patch recording. *J. Gen. Physiol.* **94**:789–812 (1989).
17. Hess, P., J. B. Lansman, and R. W. Tsien. Different modes of Ca channel gating behavior favoured by dihydropyridine Ca agonists and antagonists. *Nature* (*Lond.*) **311**:538–544 (1984).
18. Hoel, P. G. *Introduction of Mathematical Statistics*, Ed. 4. John Wiley, New York (1971).
19. van den Bosch, H. Intracellular phospholipases A. *Biochim. Biophys. Acta* **604**:191–246 (1980).
20. Rouzer, C. A., and B. Samuelsson. Reversible, calcium-dependent membrane association of human leukocyte 5-lipoxygenase. *Proc. Natl. Acad. Sci. USA* **84**:7393–7397 (1987).
21. Lee, K. S., and R. W. Tsien. Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. *Nature* (*Lond.*) **302**:790–794 (1983).
22. Courtney, K. R. Mechanism of frequency-dependent inhibition of sodium currents in frog myelinated nerve by the lidocaine derivative GEA 968. *J. Pharmacol. Exp. Ther.* **195**:225–236 (1975).
23. Rouzer, C. A., and S. Kargman. Translocation of 5-lipoxygenase to the membrane in human leukocytes challenged with ionophore A23187. *J. Biol. Chem.* **263**:10980–10988 (1988).
24. Rouzer, C. A., A. W. Ford-Hutchinson, H. E. Morton, and J. W. Gillard. MK886, a potent and specific leukotriene biosynthesis inhibitor blocks and reverses the membrane association of 5-lipoxygenase in ionophore-challenged leukocytes. *J. Biol. Chem.* **265**:1436–1442 (1990).
25. Egan, R. W., and P. H. Gale. Comparative biochemistry of lipoxygenase inhibitors, in *Prostaglandins, Leukotrienes and Lipoxins* (J. M. Bailey, ed.). Plenum Press, New York, 593–607 (1985).
26. Baader, W. J., A. Hatzelmann, and V. Ullrich. The suppression of granulocyte functions by lipophilic antioxidants. *Biochem. Pharmacol.* **37**:1089–1098 (1988).
27. Volterra, A., and S. A. Siegelbaum. Antagonistic modulation of S-K^+ channel activity by cyclic AMP and arachidonic acid metabolites: role for two G proteins, in *Arachidonic Acid Metabolism in the Nervous System* (A. I. Barkai and N. G. Bazan, eds.). New York Academy of Sciences, New York, 219–236 (1989).
28. Bug, W., L. Role, S. A. Siegelbaum, and L. Simmons. Arachidonic acid inhibits calcium current in chick sympathetic neurons. *Soc. Neurosci. Abstr.* **15**:177 (1989).
29. Cohen, C. J., T. Bale, and M. D. Leibowitz. Calcium agonist and antagonist effects of fatty acids in guinea pig atrial myocytes. *Biophys. J.* **57**:307A (1990).
30. Hagiwara, S., and L. Byerly. Calcium channel. *Annu. Rev. Neurosci.* **4**:69–125 (1981).
31. Latorre, R. The large calcium-activated potassium channel, in *Ion Channel Reconstitution* (C. Miller, ed.). Plenum Press, New York, 431–467 (1986).
32. Bean, B. P. Multiple types of calcium channels in heart muscle and neurons: modulation by drugs and neurotransmitters, in *Calcium Channels: Structure and Function* (D. W. Wray, R. I. Norman, and P. Hess, eds.). New York Academy of Sciences, New York, 334–345 (1989).

[1] S. J. Korn and A. Bolden. Influence of inhibitors of arachidonic acid metabolism on ACTH secretion from AtT-20 cells. Manuscript in preparation.

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