Modulation of Neuronal Voltage-gated Calcium Channels by Farnesol

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The modulation of presynaptic voltage-dependent calcium channels by classical second messenger molecules such as protein kinase C and G protein βγ subunits is well established and considered a key factor for the regulation of neurotransmitter release. However, little is known of other endogenous mechanisms that control the activity of these channels. Here, we demonstrate a unique modulation of N-type calcium channels by farnesol, a dephosphorylated intermediate of the mammalian mevalonate pathway. At micromolar concentrations, farnesol acts as a relatively non-discriminatory rapid open channel blocker of all types of high voltage-activated calcium channels, with a mild specificity for L-type channels. However, at 250 nM, farnesol induces an N-type channel-specific hyperpolarizing shift in channel availability that results in ~50% inhibition at a typical neuronal resting potential. Additional experiments demonstrated the presence of farnesol in the brain (rodents and humans) at physiologically relevant concentrations (100–800 pmol/g (wet weight)). Altogether, our results indicate that farnesol is a selective, high affinity inhibitor of N-type Ca²⁺ channels and raise the possibility that endogenous farnesol and the mevalonate pathway are implicated in neurotransmitter release through regulation of presynaptic voltage-gated Ca²⁺ channels.

Calcium entry into the cytosol is a crucial mediator of a range of cellular responses, including cell proliferation and neurotransmitter release (1, 2). Internal calcium levels are precisely regulated through differential expression and modulation of multiple types of voltage-dependent calcium channels (3–6). These channels are key pharmacological targets, and the identification of novel means of regulating calcium channel activity remains of critical importance for the treatment of a variety of neurological disorders, including migraines, pain, and ischemia (7, 8).

Molecular cloning has identified genes encoding at least nine different neuronal calcium channel α₁ subunits (termed α₁A through α₁D). Functional expression studies have shown that α₁A encodes P- and Q-type calcium channels (9, 10); α₁B defines an α-conotoxin GVIA-sensitive N-type channel (11, 12); α₁C, α₁D, and α₁P are L-type calcium channels (13–16); α₁E, α₁H, and α₁I are members of the family of T-type calcium channels (17–19); and α₁K is a unique calcium channel with properties common to both high and low threshold calcium channels (20, 21). The activities of voltage-dependent calcium channels are extensively modulated by cytoplasmic messenger molecules. Although the short-term modulation of these channels by protein kinases (22, 23, 24) and G protein βγ subunits (25–31) has been well documented, little is known about mechanisms that mediate their long-term regulation.

Farnesol is an isoprenoid intermediate of the mevalonate pathway, produced by dephosphorylation of farnesyl pyrophosphate (Fig. 1) (32, 33). This pathway plays a central role in cell growth and differentiation; controls the production of ubiquitin and cholesterol; and provides the substrates to G protein prenylation reactions in a number of tissues, including brain (32, 33). Farnesol was recently shown to induce a low affinity inhibition of L-type calcium currents in vascular smooth muscle (37), thus raising the possibility that neuronal voltage-gated Ca²⁺ channels might also be regulated by farnesol. To test this hypothesis, we examined the effects of exogenous farnesol on neuronal voltage-dependent calcium channels exogenously expressed in human embryonic kidney (HEK) cells. We observed that at submicromolar concentrations, farnesol mediated an N-type channel-selective hyperpolarizing shift in steady-state inactivation that resulted in a selective inhibition of N-type calcium channels at a typical neuronal resting potential of ~70 mV. To further establish the physiological relevance of our findings in HEK cells, we assessed the presence of farnesol in the brain. Using mass spectroscopy, we were able to detect farnesol in human and rodent brain tissue specimens at concentrations similar to those inducing selective inhibition of the N-type Ca²⁺ channels in HEK cells.

Overall, our data indicate that farnesol is a high affinity inhibitor of N-type calcium channels. The data suggest a novel mechanism for the precise regulation of brain Ca²⁺ homeostasis and neurotransmitter release implicating the mevalonate pathway and brain farnesol production.

MATERIALS AND METHODS

Transient Transfection of HEK Cells—Human embryonic kidney (HEK) 293 cells were grown in standard Dulbecco’s modified Eagle’s...
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medium supplemented with 10% fetal bovine serum and 0.4 mg/ml neomycin. The cells were grown to 85% confluence, split with trypsin/EDTA, and plated on glass coverslips at 10% confluence 12 h prior to transfection. Immediately prior to transfection, the medium was replaced, and the cells were transiently transfected with cDNAs encoding calcium channel α₁, β₃, and α₁b subunits (at a 1:1:1 molar ratio) using a standard calcium phosphate protocol. After 12 h, the medium was replaced with fresh Dulbecco’s modified Eagle’s medium, and the cells were allowed to recover for 12 h. Subsequently, the cells were incubated at 25 °C in 5% CO₂ for 1–2 days prior to recording. Human embryonic kidney cells stably expressing N-type α₁b channels were maintained and plated for electrophysiological recordings as described previously (29).

**Patch Clamp Recordings**—Immediately prior to recording, individual coverslips were transferred to a 3-cm culture dish containing recording solution composed of either 20 mM BaCl₂, 1 mM MgCl₂, 10 mM HEPES, 40 mM tetraethylammonium chloride, 10 mM glucose, and 87.5 mM CsCl (pH 7.2). Whole cell patch clamp recordings were performed using an Axopatch 200B amplifier (Axon Instruments, Inc., Foster City, CA) linked to a personal computer equipped with pCLAMP Version 6.0. Patch pipettes (Sutter borosilicate glass, BP150-86-15) were pulled using a Sutter P-87 microelectrode puller, fire-polished using a Narashige Microforge, and showed typical resistances of 2–4 MΩ. The internal pipette solution contained 105 mM CsCl, 25 mM tetraethylammonium chloride, 1 mM CaCl₂, 11 mM EGTA, and 10 mM HEPES (pH 7.2).

All-trans-farnesol (trans-trans-5,7,11-trimethyl-2,6,10-dodecatrien-1-ol; Sigma) was prepared as a 50 mM stock in 100% ethanol, diluted into the recording solution at the appropriate final concentrations, and perfused directly onto the cell using a home-built gravity-driven microperfusion system. At the applicable concentrations, ethanol by itself had no effect on calcium channel activity. Data were filtered at 1 kHz and recorded directly onto the hard drive of the computer. Data were analyzed using Clampfit (Axon Instruments, Inc.). All curve fitting was carried out with SigmaPlot Version 4.0 (Jandel Scientific). Steady-state inactivation curves were fitted with the Boltzmann equation: Ipeak (normalized) = 1/(1 + exp((V - V½)/k25.65)), where V and V½ are the conditioning and half-inactivation potentials, respectively, and k is a slope factor. Unless stated otherwise, all error bars are S.E. values; numbers in parentheses displayed on the figures reflect numbers of experiments; and *p values given reflect Student’s t tests.

**Brain Farnesol Analysis**—Brain specimens (~2 g) were homogenized for 1.5 min with 15 parts (w/v) methanol/ethanol/water mixture (2:5:2.5:95, v/v) using a Polytron homogenizer (Brinkmann Instruments) set at 2. One-half of each homogenate was added, when appropriate, to 2-cis,6-trans-farnesol used as an internal standard (~<0.2% all-trans-farnesol) or all-trans-farnesol (Fluka Chemical Corp., Ronkonkoma, NY). After centrifugation, the supernatant was applied to a 6-ml Oasis™ cartridge (Waters Associates, Milford, MA). The cartridge was washed with a 5% (v/v) methanol/water mixture, and farnesol was eluted with pure methanol. A mixture of 100 μl of N,O-bis-(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane (Pierce) was used to derivatize the lipids recovered in the eluate. A 2.5-μl aliquot was then injected using splitless injection into a Hewlett-Packard 5890 gas chromatograph equipped with a Hewlett-Packard 5970 mass-selective detector operated in the selected-ion monitoring mode, monitoring ions at m/z 107, 155, and 143 at a dwell time of 100 ms. After an initial hold of 1.5 min at 100 °C, the gas chromatograph oven was programmed at 20 °C/min to 300 °C. Under these conditions, all-trans-farnesol eluted at 8.8 min (8.6 min for cis,trans-farnesol). The area of ion at m/z 107 was used for quantitation, whereas ions at m/z 135 and at m/z 143 were used as qualifying ions by ratioing them to ion at m/z 107. Runs were terminated at 20 min.

**RESULTS**

**Farnesol Mediates a Low Affinity Block of All Types of High Voltage-activated Brain Calcium Channels**—We have previously shown that farnesol mediates a low (micromolar) affinity inhibition of native smooth muscle L-type calcium channels (37). To examine whether this inhibition was selective for L-type channels, we exogenously applied farnesol to four major types of expressed high voltage-activated neuronal calcium channels (α₁A, α₁B, α₁C, and α₁D). Fig. 2 depicts the effects of micromolar farnesol concentrations on N-type (α₁B + β₁B + α₂) calcium channels stably expressed in HEK 293 cells. As shown in Fig. 2A, at a holding potential of −100 mV, application of farnesol resulted in rapidly developing peak current inhibition of N-type calcium channels. This effect was dose-dependent (Fig. 2, A, C, and D) and completely reversible upon washout. There was little if any effect on the position of the peak of the current-voltage relation (Fig. 2B).

Fig. 2E compares the effects of 25 μM farnesol on peak current amplitude of four different types of high voltage-activated calcium channels. Under identical experimental conditions (coexpression with α₃ and β₁₀, holding potential of −100 mV, and 20 mM external barium), 25 μM farnesol inhibited α₁A, α₁B, α₁C, and α₁D peak current levels by 27 ± 5% (n = 8), 57 ± 7% (n = 10), 82 ± 3% (n = 10), and 36 ± 7% (n = 7), respectively. Thus, over the time course of a typical neuronal action potential, L-type channels were the most effectively inhibited channel isoform, followed by N-type channels (IC₅₀ values obtained from dose-response curves for peak current inhibition
Farnesol Is a Rapid Open Channel Blocker of Non-L-type Calcium Channels—Upon examination of the current waveform in the presence of farnesol, a dramatic, concentration-dependent, and reversible speeding of the time course of inactivation became apparent (Fig. 2C). A qualitatively similar behavior was also observed with transiently expressed α1E and α1A channels, albeit to a somewhat reduced degree (data not shown). In principle, this could be due to a drug-mediated promotion of inactivation or to a rapid open channel block that occurs immediately upon channel opening. If a farnesol-bound channel were to simply inactivate at a faster rate, then at a half-maximal concentration, one would expect 50% of the channels to inactivate rapidly, whereas the remaining portion would inactivate with the normal (control) rate. As evident from the raw data in Fig. 2C, no such biphasic response was observed. Instead, the time course of current decay accelerated with increasing farnesol concentrations and remained monophasic at all concentrations tested. When the time course of current decay was fitted monophasically, corrected for the control inactivation rate, and then plotted as a function of farnesol concentration (Fig. 3A), a linear relation was obtained, consistent with a mechanism by which farnesol rapidly binds to open channels immediately upon membrane depolarization. In this scenario, the slope of the regression line would reflect the association rate constant (4.8 ms$^{-1}$ μM$^{-1}$), and the intercept on the y axis would be equivalent to the dissociation rate constant (7.6 ms$^{-1}$), translating into a $K_d$ of 1.6 μM. A dose-response curve for current inhibition at the end of a 100-ms test pulse (i.e. close to equilibrium) yielded an IC$_{50}$ of 3.5 μM, which is consistent with the $K_d$ value obtained from Fig. 3A.

To determine whether a rapid open channel blocking mechanism could account for the observed reduction in peak current levels, we carried out a simple simulation (Fig. 3B) in which we assumed that a block can occur immediately upon channel opening (and thus, prior to reaching peak current amplitude). With a time constant for current activation of 1.8 ms and using our experimental measurements of the decay rate at various farnesol concentrations, we were able to reproduce the qualitative features of peak current inhibition observed experimentally. Our simulation can also account for the apparent discrepancy between the $K_d$ value obtained from the kinetic analysis (1.6 μM) and the IC$_{50}$ value obtained from Fig. 2D (21 μM) since at the time of peak, the open channel block would not yet be fully developed.

Unlike α1A, α1B, and α1E channels, α1C (L-type) channels underwent little change in “inactivation” kinetics in the presence of farnesol (data not shown), but nonetheless exhibited substantial inhibition. This observation suggests that the block of the L-type channels is almost fully developed prior to channel opening (resting block). This is in stark contrast with the inhibition of non-L-type channels, which develops during the course of the test depolarization. Thus, when comparing the IC$_{50}$ values for peak current inhibition (as in Fig. 2E), it is important to consider that unlike in the case of L-type channels, the IC$_{50}$ for peak current inhibition of non-L-type channels likely underestimates the true farnesol affinity (see above).
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The development and reversal of the peak current block were exclusively governed by a bimolecular interaction between the channel and farnesol, then the recovery rate constant obtained from the washout kinetics should be equivalent to that predicted from the concentration dependence of the time constant of block development (i.e. the block already occurs during the initial rising phase of the current). The simulated traces were generated using the following equation: \( I = (1 - \exp(-t \tau_d)) \exp(-t \tau_f) \), where \( t \) is the duration of the test depolarization, \( \tau_d \) is the time constant for activation (set to 1.8 ms), and \( \tau_f \) is the time constant for current decay. The values used for \( \tau_f \) reflect mean time constants for current decay determined experimentally at 0, 1, 5, 10, or 25 \( \mu \)M farnesol. Note that according to this simulation, increasing farnesol concentrations mediate a progressive decrease in the predicted peak current amplitude, suggesting that an open channel block can account for the bulk of peak current inhibition observed in our experiments. C, kinetic analysis of the time course of development of the peak current block. Here, the inverse of the time constant for the development of the peak current block (as determined from exponential fits to data such as those depicted in Fig. 2A) is plotted as a function of farnesol concentration. Whereas the data are adequately described by a linear relation, the unblocking rate constant predicted from the linear regression differs dramatically from that determined from washout of the drug, indicating that the kinetics of development of the peak current block are not limited by a simple drug channel interaction, but may require a diffusion-limited step.

Open Channel Block by Farnesol May Require Partition into the Lipid Phase—The development and reversal of the peak current block of N-type channels occur at a time scale of ~1 min (Fig. 2A); and yet, the rate constants for the N-type channel block and unblock are predicted to occur in the low millisecond range (Fig. 3A). This suggests the involvement of one or more rate-limiting steps independent of the actual farnesol channel interactions. If the development and recovery of the farnesol peak current block were exclusively governed by a bimolecular interaction between the channel and farnesol, then the recovery rate constant obtained from the washout kinetics should be equivalent to that predicted from the concentration dependence of the time constant of block development (i.e. the block already occurs during the initial rising phase of the current). The simulated traces were generated using the following equation: \( I = (1 - \exp(-t \tau_d)) \exp(-t \tau_f) \), where \( t \) is the duration of the test depolarization, \( \tau_d \) is the time constant for activation (set to 1.8 ms), and \( \tau_f \) is the time constant for current decay. The values used for \( \tau_f \) reflect mean time constants for current decay determined experimentally at 0, 1, 5, 10, or 25 \( \mu \)M farnesol. Note that according to this simulation, increasing farnesol concentrations mediate a progressive decrease in the predicted peak current amplitude, suggesting that an open channel block can account for the bulk of peak current inhibition observed in our experiments. C, kinetic analysis of the time course of development of the peak current block. Here, the inverse of the time constant for the development of the peak current block (as determined from exponential fits to data such as those depicted in Fig. 2A) is plotted as a function of farnesol concentration. Whereas the data are adequately described by a linear relation, the unblocking rate constant predicted from the linear regression differs dramatically from that determined from washout of the drug, indicating that the kinetics of development of the peak current block are not limited by a simple drug channel interaction, but may require a diffusion-limited step.

Many compounds affecting voltage-dependent ion channels exhibit varying affinities for different kinetic states, thus raising the possibility that the shifts in inactivation properties might occur at farnesol concentrations well below those required for an open channel block. To investigate this possibility, HEK cells expressing N-type calcium channels were held at a more depolarized potential (~70 mV), and the concentration
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of external permeant ion was reduced from 20 to 5 mM to more closely approach the surface potentials experienced by the channels in their native environment. Initially, we applied 2 μM farnesol and observed a >50% inhibition of peak current levels (data not shown). We then further reduced the farnesol levels to 250 and 100 nM and examined the effects on channel activity. As shown in Fig. 5A, at the more depolarized holding potential of −70 mV, application of 100 nM farnesol resulted in a slowly developing, yet substantial reduction in peak current amplitude, which could only be partially reversed upon wash-out. Fig. 5B depicts the effects of 100 nM farnesol on the position of the steady-state inactivation curve of α1B channels for five paired experiments. Compared with Fig. 4, the half-inactivation potential was shifted 13 mV more negative due to the less effective surface charge screening occurring in 5 mM barium. Upon application of 100 nM farnesol, the half-inactivation potential was shifted further to −58.3 ± 1.3 to −71.2 ± 3.2 mV (p = 0.004), resulting in an ~45% inhibition of N-type currents at a holding potential of −70 mV, consistent with the data in Fig. 5A. Fig. 5C depicts current records obtained from α1B channels in the absence and presence of 250 nM farnesol at a holding potential of −70 mV. Similar to Fig. 5A, farnesol reduced peak current amplitude in a partially reversible manner. The peak current reduction was accompanied by only a mild increase in the apparent rate of inactivation, which is indicative of a relative lack of the pronounced open channel block observed at higher farnesol concentrations (i.e. Fig. 2C). There was no detectable effect of submicromolar farnesol on current activation properties. Fig. 5D compares the effect of 250 nM farnesol (Vₜₜ = −70 mV) on the peak current levels of four types of high voltage-activated calcium channels. As shown, the effect of 250 nM farnesol on peak current amplitude was most pronounced for α1B channels (43 ± 4% inhibition, n = 6), whereas only a minor (~<10%) reduction in the peak current levels of α1A (P/Q-type) and α1E (R-type) channels was observed. There was no effect on α1C (L-type) channels. Hence, our data show that farnesol is a high affinity inhibitor of N-type calcium channels and constitute the first description of selective blockade of these channels by a small organic molecule.

Farnesol Is Naturally Occurring in Brain Tissue—One of the enzymes required for the production of farnesol (farnesyl-pyro-phosphate synthase) is known to be expressed in the brain (38), thus suggesting that farnesol could be present endogenously in neuronal tissue. To investigate this possibility, a farnesol assay using gas chromatography and mass spectrometry was developed and applied to the detection of farnesol in human and rodent brain lipid extracts. Mouse brains (male BXD mice, weighing ~25 g) were collected and flash-frozen in liquid nitrogen immediately after sacrifice. The specimens were then pooled (n = six animals/pool) and processed as described under “Materials and Methods.” As illustrated in Fig. 6, authentic all-trans-farnesol was found in these brain extracts. Identification was established by comparison with pure all-trans-farnesol using two criteria: retention time (8.7–8.8 min) and ratios of two qualifying ions (ions at m/z 135 and m/z 143) to ion at m/z 107 (0.90–0.93 and 0.68–0.76 for ions at m/z 135 and at m/z 143, respectively) (Fig. 6A and B). In contrast, there was no detectable cis,trans-farnesol (Fig. 6B), thus allowing the use of cis,trans-farnesol as an internal standard (Fig. 6C). Farnesol concentrations were estimated to be 417 and 373 pmol/g ( wet weight) in these pools. Rat brains (male Harlan Sprague-Dawley, 12–16 weeks of age; n = two to three brains/pool) were also analyzed. Authentic all-trans-farnesol was found in all samples (average concentration = 590 pmol/g (wet weight), range of 180–745). Finally, brain necropesy specimens (frontal cortex) from four individuals, a 42-year-old male who died in a motor
FIG. 6. Gas chromatograph-mass spectrometry analysis of farnesol in brain samples. Lipids were extracted as described under "Materials and Methods." A, electron impact mass spectrum of the trimethylsilyl ether of all-trans-farnesol. In this experiment, a methanol/ethanol/water solution containing 100 ng of all-trans-farnesol was applied to an OASIS cartridge. The eluate was derivatized and analyzed by gas chromatography-mass spectrometry (scan run mode). The insert shows the ion chromatograms for three selected ions during the scan run (retention time = 8.73 min). Despite their relatively low abundance, these ions were free of interferences and were therefore chosen for the assay. The area of ion at m/z 107 was used for quantitation, whereas ions at m/z 135 and 143 were used as qualifying ions (the ratios to ion at m/z 107 were 0.92 and 0.69 for ions at m/z 135 and 143, respectively). B, typical selected-ion monitoring chromatogram (107, 135, and 143 atomic mass units (amu)) of derivatized lipids from native neuronal tissue (mouse brain; ~1.2 g (wet weight)). The presence of authentic all-trans-farnesol (FOH) was established based on the retention time (8.78 min) of the three selected ions and the ratios of the qualifying ions to ion 107 (0.92 for ion at m/z 135 and 0.72 for ion at m/z 143). No cis,trans-farnesol was present. Similar results were obtained with rat and human brain homogenates (data not shown). C, duplicate sample added to 250 ng of cis,trans-farnesol as an internal standard (retention time = 8.58 min). In this experiment, the brain farnesol concentration was estimated at 417 pmol/g (wet weight) using the area of ion at m/z 107.
vehicle accident, a 55-year-old male who died from a ruptured berry aneurysm, a 70-year-old female with Huntington’s disease, and a 46-year-old male with coronary artery disease (specimens collected within 24 h post mortem), were obtained from the Oregon Brain Bank and analyzed. Authentic all-trans-farnesol was detected in all samples at concentrations estimated at 248, 290, 180, and 110 pmol/g (wet weight) for the four specimens, respectively. Under our experimental conditions, none of the untreated rat or human brain extracts had detectable levels of cis,trans-farnesol.

These results demonstrate that farnesol is a naturally occurring substance in the brain and provide indirect evidence for a complete metabolic pathway supporting farnesol production and degradation in the central nervous system. More important, our results suggest that brain farnesol levels may be sufficiently high to mediate substantial inhibition of N-type calcium channels by reducing the availability of the channel for opening.

**DISCUSSION**

Farnesol has previously been shown to mediate a low affinity inhibition of native smooth muscle L-type calcium channels (37). Here, we present several novel aspects of the actions of farnesol on voltage-dependent calcium channels from brain tissue. First, at hyperpolarized membrane potentials, farnesol induces a relatively nonselective peak current inhibition of transiently expressed high voltage-activated calcium channels. Second, we have presented evidence that farnesol mediates a selective, high affinity inhibition of inactivated N-type channels, making this compound the first small organic high affinity blocker with selectivity for a non-L-type calcium channel. Finally, we show that these selective effects on N-type channels occur at physiological farnesol concentrations. Thus, farnesol may constitute the first endogenous high affinity ligand to be identified for any type of voltage-dependent calcium channel.

At hyperpolarized holding potentials (~100 mV), micromolar concentrations of farnesol mediated a dramatic speeding of the rate of current decay of N-type channels and, to a somewhat lesser extent, α1A and α1E channels. Our kinetic analysis is consistent with a mechanism in which farnesol rapidly blocks the channel immediately subsequent to opening. The open channel-blocking site is probably not accessible directly from the external aqueous phase since development and reversal of the blocking effects required minutes despite the rapid association and dissociation kinetics suggested by our kinetic analysis. Hence, farnesol must likely partition into the membrane phase and perhaps into the cytoplasm before it is capable of producing an open channel block. Consistent with such a mechanism, the presence of farnesol did not interfere with the development of or recovery from ω-conotoxin GVIA block (data not shown), further supporting the notion that farnesol does not directly block the pore from the extracellular side of the channel. Instead, the rapid open channel block by farnesol might occur by physical occlusion of the pore from the cytoplasmic side; however, substantiation of such a hypothesis will require further investigation.

Whereas the farnesol-induced reduction in peak current amplitude could be accounted for by assuming that all of the inhibition was due to an open channel block, the peak current inhibition of L-type channels appeared to be mediated by a distinct mechanism. We observed little speeding of the rate of current decay (i.e. open channel block); and yet, L-type α1C channels underwent the largest degree of peak current inhibition. These considerations suggest that the L-type channel block by farnesol may differ from that of non-L-type channels via its state dependence. Whether the site(s) of farnesol action encompasses one of the previously identified antagonist inter-

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