Sphingosine Inhibits Voltage-operated Calcium Channels in GH4C1 Cells*

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In the present study we investigated the mechanism of inhibitory action of sphingosine (SP) on voltage-activated calcium channels (VOCCs) in pituitary GH4C1 cells. Using the patch-clamp technique in the whole-cell mode, we show that SP inhibits Ba2+ currents (I_{Ba}) when 0.1 mM BAPTA is included in the patch pipette. However, when the BAPTA concentration was raised to 1–10 mM, SP was without a significant effect. The effect of SP was apparently not mediated via a kinase, as it was not inhibited by staurosporine. By using the double-pulse protocol (to release possible functional inhibition of the VOCCs by G proteins), we observed that G proteins apparently evoked very little functional inhibition of the VOCCs. Furthermore, including GDP\textbeta\textgammaS (guanyl-5'-yl thiophosphate) in the patch pipette did not alter the inhibitory effect of SP on the Ba2+ current, suggesting that SP did not modulate the VOCCs via a G protein-dependent pathway. Single-channel experiments with SP in the pipette, and experiments with excised outside-out patches, suggested that SP directly inhibited VOCCs. The main mechanism of action was a dose-dependent prolongation of the closed time of the channels. The results thus show that SP is a potent inhibitor of VOCCs in GH4C1 cells, and that calcium may be a cofactor in this inhibition.

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§ The abbreviations used are: SP, sphingosine; PKC, protein kinase C; VOCC, voltage-activated calcium channel; f1, ohm(s); GDP\textbeta\textgammaS, guanyl-5'-yl thiophosphate; GTP\textgammaS, guanosine 5'-3-O-(thio)triphosphate; HBSS, Hapes-buffered salt solution.

Sphingosine (SP) and related sphingolipids are considered potent endogenous inhibitors of protein kinase C (PKC) (1), as well as activators of proliferation (2–4). Recently, it has been shown that SPs stimulate the release of sequestered calcium in many cell types. A role for sphingosine and sphingosine 1-phosphate as possible second messengers has been postulated (9, 10).

An interesting observation made recently is that sphingosines regulate the gating of a novel type of calcium channel located in membranes of intracellular calcium stores in endothelial cells (11). Furthermore, sphingosines inhibit calcium entry through voltage-activated calcium channels (VOCCs) in cardiac cells (12), and capacitative calcium entry in Jurkat T cells (13). An inhibitory effect of sphingosines on depolarization-evoked calcium entry has also been observed in synaptosomes (14). In brain microsomes, the sphingosine derivative sphingosine phosphorylcholine stimulated calcium release via the ryanodine receptor (15), whereas sphingosine blocked activation of the ryanodine receptor in cardiac cells (16). Another interesting observation is that SP inhibits sustained, VOCC-dependent calcium gradients in hippocampal neurons after stimulation with either glutamate or N-methyl-d-aspartate (17). This SP-evoked inhibition was attributed to inhibition of PKC, but in the light of recent observations, it may be the result of SP-evoked blockade of VOCCs. Thus, SPs appear to be potent regulators of calcium signaling. However, the mechanisms involved are not yet known.

In pituitary cells, changes in intracellular free calcium ([Ca2+]i) are of crucial importance for the regulation of hormone synthesis and secretion (18, 19). The increase in [Ca2+]i may be the result of agonist-evoked activation of phospholipase C and the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate, and the concomitant release of sequestered calcium (20–22). Agonist-evoked activation of phospholipase C also results in the activation of VOCCs (23–26). In pituitary cells, the L- and the T-type VOCCs have been identified (27, 28). In particular, L-type VOCCs are considered important for the stimulation of hormone synthesis and secretion (23, 24, 29–30), but also for the steady-state regulation of [Ca2+]i (31).

Stimulating GH4C1 pituitary cells with diacylglycerols activates sphingomyelinase, resulting in the production of sphingosine and ceramides (32, 33). In a recent study, we observed that SPs potently inhibited calcium entry via VOCCs in GH4C1 pituitary cells (34). This effect was independent of an action of PKC. In GH4C1 cells, PKC is assumed to participate in the regulation of VOCCs (20, 23, 35, 36) (see also Refs. 37 and 38), and thus both PKC and SPs may be important modulators of VOCCs in these cells. In the present study, we have investigated the mechanisms of action of SP on VOCCs in GH4C1 cells. Using whole-cell, single-channel, and outside-out patch-clamp methods, we show that SP inhibits the gating of VOCCs in GH4C1 cells. This mechanism may, in part, be dependent on intracellular calcium.

EXPERIMENTAL PROCEDURES

Materials—Fura 2-AM was obtained from Molecular Probes (Eugene, OR). Sphingosine, staurosporine, GTP\textgammaS, GDP\textbeta\textgammaS, and tetrodotoxin were acquired from Sigma. Ham’s F-10 nutrient mixture was from Life Technologies, Inc., and serum was from Biological Industries (Israel). All other reagents were of analytical grade.

Culture dishes were obtained from Falcon Plastics (Oxnard, CA). [3H]PN 200-110 (81 Ci/mmol) was from Amersham (Little Chalfont, Buckinghamshire, UK).

Cell Culture—Clonal rat pituitary GH4C1 cells were generously given by Dr. Armen H. Tashjian, Jr. (Harvard University, Boston, MA). The cells were grown in monolayer culture in Ham’s F-10 nutrient mixture with 15% (v/v) horse serum and 2.5% fetal bovine serum (Ham’s F-10+.
A1
control currents.

A2
10 μM sphingosine

B1
1.0
0.5
0.0
-0.5
-1.0

B2
0.0
0.5
1.0

FIG. 1. The inhibitory effect of SP on calcium channel currents in GH4C1 cells. Representative traces of Ba^{2+} currents obtained by depolarizing the cells from −70 mV to the potentials indicated. A1, control currents. A2, currents obtained after the application of 10 μM SP. B, current-voltage relationship of the Ba^{2+} current. Panel shows control currents (●) and currents obtained after the application of 10 μM SP (○). The data shown are from the same cell. In this experiment, the patch pipette contained 0.1 mM BAPTA.

medium in a water-saturated atmosphere of 5% CO₂ and 95% air at 37 °C, as described previously (39, 40). Before an experiment, the cells from a single donor culture were harvested with 0.02% EDTA solution (5 mg/ml) for 10 min in a high potassium HBSS-III buffer (containing (in mM): KCl, 140; MgCl₂, 1.13; glucose, 10; HEPES, 10 (pH 7.2), adjusted with KOH) to abolish the membrane potential. In these experiments, the pipettes were filled with HBSS-II lacking BAPTA but containing 4 mM CaCl₂ and 10 μM EGTA to give a free calcium concentration of 100 nM. This resting calcium concentration was chosen because of the apparent calcium-dependent effects of sphingosine observed in the whole-cell and cell-

The external solution used for the excised outside-out patch recordings was HBSS-II. The internal pipette solution in these experiments was HBSS-II lacking BAPTA but containing 4 mM CaCl₂ and 10 mM EGTA to give a free calcium concentration of 100 nM. This resting calcium concentration was chosen because of the apparent calcium-dependent effects of sphingosine observed in the whole-cell and cell-

This normalization of the results, where the steady state current before application of test compound was considered 100%, was made to make a comparison between different measurements feasible.

The cell-attached single-channel recordings, the cells were bathed in a high potassium HBSS-III buffer (containing (in mM): KCl, 140; MgCl₂, 1.13; glucose, 10; HEPES, 10 (pH 7.2), adjusted with KOH) to abolish the membrane potential. In these experiments, the pipettes were filled with HBSS-IV buffer (containing (in mM): BaCl₂, 60; TEA-Cl, 10; HEPES, 10 (pH 7.2), adjusted with TEA-OH), and had a resistance of 15–20 MΩ. Leak and capacitive currents in the single-channel experiments were subtracted and compensated for in all recordings. Data analysis was made using the pCLAMP6 program (Axon Instruments).

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FIG. 2. BAPTA attenuates the inhibitory effect of SP on VOCCs in whole-cell recordings. Time course of the run-down of normalized individual currents (I/Iₜₘₐₓ) measured at the end of a 200-ms depolarizing pulse, where Iₜₘₐₓ is the maximal current measured immediately prior to addition of test compound, and I the current measured at time points after addition of test compound. The cells were held at −70 mV and then step depolarizations to +10 mV were made. In these experiments, the patch pipette contained 0.1 mM (A1 and B1) or 1 mM (A2 and B2) BAPTA. Control cells (A1 and A2), and cells perfused with 10 μM SP (B1 and B2). The crossed lines indicate the calculated time where the current was decreased by 50% (Iₜₘₐₓ see Equation 1 under “Experimental Procedures”). In A1 and B1, the data shown are from 5 separate cells, and in A2 and B2 from 7 separate cells each.

\[
\frac{I_{\text{max}}}{b} = \frac{1}{1 + \left(\frac{t}{T_{\text{50}}}\right)} + d \quad \text{(Eq. 1)}
\]

The current signals were filtered at 2.3 kHz, sampled at 2 kHz, and stored on an Atari Mega/Ste computer. The current signals were filtered at 2.3 kHz, sampled at 2 kHz, and stored on an Atari Mega/Ste computer. The cells were held at −70 mV with test pulses to −20 mV and +10 mV made throughout the experiment to avoid additional rundown of the channels. For the calculations, we used data derived from pulses to ±10 mV. A stock solution of sphingosine (10 mM) was made in ethanol. The final concentration of the solvent did not exceed 0.2%. This concentration of the solvent did not have any effects on VOCCs in GH4C1 cells.

To calculate the half-inactivation time (Tₜₘₐₓ) of the inward outward current, a logistic equation was used, where \( t \) is the time from rupturing the cell membrane, \( b \) is the slope, and \( d \) is the residual current after full inactivation (Equation 1).

\[
\frac{I_{\text{max}}}{I_{\text{max}}} = \frac{1}{1 + \left(\frac{t}{T_{\text{50}}}\right)} + d \quad \text{(Eq. 1)}
\]
attached experiments. Thick-walled glass pipettes were used, which had a resistance of 9.5–11.5 MΩ. The outside-out patches were obtained using standard techniques (41). Patches with a resistance less than 5 GΩ were rejected from the experiments. Usually, the patches had a resistance higher than 20 GΩ. The excised patches were clamped at −250 mV to obtain more stable patches than those obtained at −270 mV, with test pulses to −210 mV and −110 mV. The current signals were sampled at 5 kHz, and filtered at 1 kHz using an Axoclamp-1A amplifier and Clampex software (Axon Instruments). After the sampling, no additional digital filtering was used. Data analysis was made using pClamp 6 software and Microcal Origin software.

RESULTS

Effects of Sphingosine on VOCCs in GH4C1 Cells—In a recent study, we showed that sphingosine derivatives inhibit the activation of VOCCs in GH4C1 cells, but the mechanism remained unclear (34). Using the whole-cell mode of the patch-clamp technique, we now found that 10 μM SP potently suppressed the current amplitude without any profound shift in the current-voltage relationship, suggesting that there is no voltage dependence of the inhibition (Fig. 1). VOCCs are prone to inhibition via a calcium-dependent mechanism (see Ref. 42). To evaluate whether SP could modulate VOCCs via this mechanism, different concentrations of BAPTA were included in the pipette solution. With 0.1 mM BAPTA in the pipette solution, SP rapidly inhibited $I_{\text{Ba}}$ (Fig. 1). In control cells, the half-time value ($T_{50}$) for the rundown was 267 ± 42 s, whereas in the presence of 10 μM SP, $T_{50}$ was 143 ± 8 s ($p < 0.05$). However, when the pipette solution contained 1 mM or 10 mM BAPTA, the rundown in the presence of SP proceeded at a rate equal to that observed in control cells (Fig. 2). This result suggests that the inhibitory effect of SP on the VOCCs is mediated via a calcium-dependent mechanism.

In some studies, BAPTA has been shown to act as a kinase inhibitor (43). To test whether the effect of SP was mediated via activation of protein kinases, the cells were treated with 200 nM staurosporine. Following a 15-min pretreatment of the cells with staurosporine, SP still (in the continuous presence of staurosporine) inhibited $I_{\text{Ba}}$ in a manner similar to that observed in control cells (in these experiments, the $T_{50}$ value was 149 ± 36 s, $n = 3$; $p < 0.05$).

Fig. 3. G proteins do not modify $I_{\text{Ba}}$ in GH4C1 cells. A, double-pulse protocol to release functional inhibition of VOCCs by G proteins. A1, pulse protocol. The cell was held at −70 mV and then depolarized to +10 mV. The cell was then depolarized first to +100 mV and then to +10 mV. The interval between the pulses in the double-pulse experiment was 1 ms. A2, current traces obtained using the protocol depicted in A1. The patch pipette contained 0.1 mM BAPTA. A3, an experiment identical to that shown in A2, except that the pipette also contained 300 μM GTPγS. B, the cell was held at −70 mV and then step depolarizations to +10 mV were made as described in Fig. 2. The patch pipette contained 0.1 mM BAPTA and 2 mM GDPγS. When a stable current was obtained, the cell was perfused with 10 μM SP. In this experiment, the $T_{50}$ was 118 s.

Fig. 4. Effect of SP as seen in single-channel recordings in GH4C1 cells. The experiments were performed using the cell-attached patch-clamp configuration. A1, consecutive sweeps from recordings in a control cell. A2, calculated open probability ($P_{\text{open}}$) of a single L-type Ca$	ext{^{2+}}$ channel during a 200-ms depolarization to +10 mV for the cell shown in A1. The consecutive sweeps shown in A1 are depicted by a solid bar. B, consecutive sweeps (B1) and calculated $P_{\text{open}}$ (B2) from a recording with 3 μM sphingosine in the pipette. The $P_{\text{open}}$ in consecutive sweeps was calculated using the routine provided by the pClamp 6 software. The results in A and B are from separate cells.

Sphingosine and VOCC
It is well known that G proteins may have a constitutive inhibitory effect on VOCCs, and that agonist-evoked inhibition of VOCCs may be mediated via a G protein-dependent mechanism (see Ref. 44). However, in GH4C1 cells, G proteins seem to have a very modest effect on the VOCCs, as evaluated using the double-pulse protocol (to release the possible functional inhibition of Ca\(^{2+}\) channels by G protein (45); Fig. 3). In these experiments, we could not detect any difference in \(I_{\text{Ba}}\). Addition of 2 mM GDP/BS (to inhibit G protein activation) to the pipette solution had no observable effects on the inhibitory action of SP on the calcium channel current. In these experiments, the \(IT_{50}\) value was 87 ± 14 s (n = 4; \(p < 0.05\), Fig. 3). Taken together, the above results exclude G proteins as the mediators of the observed SP-evoked inhibition of the VOCCs.

**Effects of SP on Single Ca\(^{2+}\) Channels in GH4C1 Cells**—To test whether SP has a direct effect on the VOCCs, single-channel analyses using the cell-attached mode of the patch clamp technique were performed with SP in the pipette solution. SP rapidly inhibited the Ba\(^{2+}\) current (Fig. 4), suggesting a direct effect of SP on the VOCCs. An analysis of the kinetic characteristics of the single channels showed that SP inhibited the VOCCs mainly by increasing the closed time of the channels (Fig. 5 and Table I). For the control cells, two conductance states were found. Using 3 \(\mu\)M and higher concentrations of SP, only one conductance amplitude could usually be detected. The values of the single-channel dwell times and amplitudes are summarized in Table I. When the pipette solution contained 10 \(\mu\)M SP, no openings of the VOCCs were observed in 7 out of 9 cells.

**Action of Sphingosine on Excised Outside-out Patches**—To exclude an effect of cytosolic factors, we tested the action of sphingosine on excised outside-out patches. In these experiments, the free Ca\(^{2+}\) concentration was buffered to 100 nM. This Ca\(^{2+}\) concentration is below the resting intracellular Ca\(^{2+}\) concentration, which in our GH4C1 cells was 204 ± 15 nM (mean ± S.E., n = 6) as determined with Fura 2. After depolarization of the patch membrane to −10 mV, frequent openings of one or, rarely, two Ca\(^{2+}\) channels were observed (Fig. 6). We observed a substantial run-down of the channels during the first 5 min of the recording. Thus, sphingosine was usually applied within 1 min of the recordings. The calculated dwell times of the channels and their amplitudes (Table II) were quite similar to those obtained in the cell-attached recordings, suggesting that the functional properties of Ca\(^{2+}\) channels were well preserved in the outside-out recordings. Interestingly, in the outside-out configuration, we observed a second long-lasting open state of the Ca\(^{2+}\) channels, which was not observed in cell-attached recordings (Table II and Fig. 6). Presently, we do not have an explanation for this observation.

Application of 10 \(\mu\)M sphingosine led to a dramatic decrease in the open probability of the channels (Fig. 7). In 7 out of 8 cells, a complete inhibition of the channel activity was observed within 1 min after application of sphingosine.

**DISCUSSION**

In the present study, we have investigated the effect of SP on VOCCs in GH4C1 cells. Our results suggest that SP inhibits

### Table I

**Effect of sphingosine on the amplitude and kinetics of single VOCCs**

Sphingosine was added to the electrode filling solution at the concentrations indicated. When the pipettes contained ≥3 \(\mu\)M SP, it proved difficult to measure two current amplitudes. When 10 \(\mu\)M SP was tested no openings were observed in 7 out of 9 cells tested. The results obtained in the two other cells were impossible to analyze. The values given are the mean ± SE, if not indicated differently.

| Concentration (n) | Amplitude | Closed time | Open time |
|-------------------|-----------|-------------|-----------|
| \(\mu\)M          | \(A_1\) | \(A_2\) | \(\tau_1\) | \(\tau_2\) | \(pA\) | \(\text{ms}\) | \(\text{ms}\) | \(\text{ms}\) |
| 0 (9)             | 0.54 ± 0.03 | 0.68 ± 0.04 | 0.57 ± 0.07 | 9.8 ± 2.2 | 0.31 ± 0.03 |
| 1 (7)             | 0.51 ± 0.03 | 0.61 ± 0.03 | 0.87 ± 0.07 | 15.7 ± 3.9 | 0.23 ± 0.04 |
| 3 (4)             | 0.52 ± 0.04 | XXX\(^a\) | 0.51 ± 0.14 | 29.7 ± 11\(^c\) | 0.23 ± 0.04 |
| 5 (3)             | 0.53 ± 0.03 | 0.67\(^b\) | 0.50 ± 0.01 | >10\(^d\) | 0.22 ± 0.05 |

\(^a\) No currents were detected.
\(^b\) Only one amplitude was detected.
\(^c\) \(p < 0.05\).
\(^d\) When the electrode contained 5 \(\mu\)M SP, it was not possible to reliably measure the duration of \(\tau_2\) in all experiments.
the VOCC-mediated current by prolonging the closed state of the channels, apparently in a calcium-dependent manner. Our present study is the first to show such properties of SP.

An important kind of modulation of VOCCs occurs via G protein-mediated mechanisms. In some cell types, the effects of SP derivatives have been shown to be in part mediated by a G protein (8, 46, 47). Furthermore, several recent reports strongly suggest the existence of membrane receptors for SP derivatives, and this putative receptor appears to be coupled to a pertussis toxin-sensitive G protein (48–50). However, by using several manipulations known to affect G protein-dependent mechanisms (i.e. the double-pulse protocol to release the possible functional inhibition of Ca$^{2+}$ channels by G protein (45), and by including GDPβS in the pipette solution), we could not influence the action of SP. Thus, an effect of SP mediated via a G protein seems unlikely in the present work.

Sphingosine derivatives have been reported to inhibit the release of sequestered calcium in excitable cells (Refs. 16 and 51; but see also Ref. 15). We have been unable to detect an SP-evoked increase in [Ca$^{2+}$]i in intact GH4C1 cells using Fura 2 (34); thus, we think it unlikely that SP could inhibit the channels through the mobilization of intracellular Ca$^{2+}$. Nev-

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**FIG. 6.** Calcium currents in an excised outside-out patch from GH4C1 cells. A, the trace shows a 500 ms pulse to −10 mV from a holding potential of −50 mV. Single-channel openings to one main conductance and one subconductance state can be observed. Furthermore, the channels had two open state dwell time probabilities and two closed states (see Table II). B, open probability (P(open)) histogram of consecutive sweeps from the cell shown in A.

**TABLE II**

| Characterization of the amplitudes and kinetics of single VOCCs in outside-out patches |
|---|
| The values given are the mean ± S.E. of 10 patches from separate cells. |
| Amplitude | Closed time | Open time |
| A1 | A2 | τ1 | τ2 | τ1 | τ2 |
| pA | ms | ms | ms | ms |
| 0.82 ± 0.05 | 0.58 ± 0.04 | 1.09 ± 0.09 | 8.6 ± 1.6 | 0.38 ± 0.03 | 2.7 ± 0.8 |

**FIG. 7.** Effect of SP on an excised outside-out patch from GH4C1 cells. Upper panel, open probability (P(open)) of a single Ca$^{2+}$ channel calculated from a 500-ms pulse to −10 mV before and after application of 10 μM sphingosine. Bottom panels, the traces show original recordings used for calculating the open probabilities before (A) and after (B) application of sphingosine. The arrows in the upper panel mark the calculated open probabilities corresponding to respective traces. Similar results were obtained in 9 other patches from 9 other cells.

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eternal, our study showed that in the presence of strong intracellular Ca\(^{2+}\) buffering (achieved by including 1–10 mM BAPTA in the pipette solution), the effect of SP was abolished. This result suggests, but does not prove, that calcium is necessary for the SP-evoked inhibition of VOCCs. In control cells, the concentration of BAPTA in the pipette did not affect the \(I_{\text{T,20}}\) value of the run-down of the VOCCs. Another possibility is that BAPTA inhibited a kinase in our cells, as recent studies have indicated that BAPTA may inhibit tyrosine kinases (43). This explanation appears unlikely, as pretreatment of the cells with the potent kinase inhibitor staurosporine neither abolished nor potentiated the effect of SP.

A striking effect of SP was observed on the kinetics of single VOCCs. Using 5 \(\mu\)M and higher concentrations of SP, we found that the single channels were inhibited almost immediately after the gigaseal formation. Also in the excised outside-out experiments, the effect of SP on the open probability of the channels was almost immediate. These results suggest that SP inhibits the VOCCs directly (or possibly via a membrane-delimited action). We observed that SP significantly prolonged the closed time of the channels, without significant effects on either the amplitude, or the open time probability. These data suggest that SP is not an open-channel blocker. Similar results were found recently for the SP-evoked inhibition of single \(\kappa\)-channels in smooth muscle cells (52). In addition, Yasui and Palade (53) suggested that the effect of SP on VOCCs in ventricular myocytes could be due to an effect of SP on channel gating. However, no single-channel experiments were performed in their study.

In the present report, we did not evaluate which types of VOCCs were suppressed by SP. In a recent report, we have shown data suggesting that SPs apparently inhibited the \(L\)-type of VOCCs (34). Theoretically, the effect of SP could be mediated via binding to the dihydropyridine binding site in the VOCCs. However, preliminary binding experiments showed that SP did not inhibit the binding of the dihydropyridine antagonist \(^{3}H\)PN 200-110 to GH4C1 cells.\(^{2}\) Furthermore, considering that at least four different binding sites for antagonists to the VOCCs are known (54), we cannot exclude the possibility that SP could bind to some other known site than that of dihydropyridines.

In conclusion, we have shown that SP, possibly directly or via a membrane-delimited action, inhibits VOCCs in GH4C1 cells by increasing the closed time probability of the channels. The action of SP apparently requires free intracellular Ca\(^{2+}\). Although the relatively rapid run-down of the channels in the present study precluded an investigation on the reversibility of the SP-evoked inhibition, our previous study clearly showed an almost total recovery of calcium entry after washout of SP (34). Thus, the effects of SP are not the result of an irreversible blockade of the channels. The physiological significance of SP in the regulation of pituitary cell function is still unclear. However, in preliminary studies, we have been able to measure significant endogenous levels of SP in GH4C1 cells.\(^{3}\) Considering that the regulation of pituitary hormone synthesis and secretion is critically dependent on intracellular Ca\(^{2+}\) dynamics, SP may be an important regulator of pituitary cell function.

\(^{2}\) K. Karhánapää and K. Törnquist, unpublished observation.

\(^{3}\) K. Törnquist and H. Vuorela, unpublished results.