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Mobilization of intracellular calcium by intracellular flash photolysis of caged dihydrosphingosine in cultured neonatal rat sensory neurones*°

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The ability of dihydrosphingosine to release Ca^{2+} from intracellular stores in neurones was investigated by combining the whole cell patch clamp technique with intracellular flash photolysis of caged, *N*-(2-nitrobenzyl)dihydrosphingosine. The caged dihydrosphingosine (100 μM) was applied to the intracellular environment *via* the CsCl-based patch pipette solution which also contained 0.3% dimethylformamide and 2 mM dithiothreitol. Cultured dorsal root ganglion neurones from neonatal rats were voltage clamped at −90 mV and inward whole cell Ca^{2+}-activated currents were recorded in response to intracellular photorelease of dihydrosphingosine. Intracellular photorelease of dihydrosphingosine (about 5 μM) was achieved using a Xenon flash lamp. Inward Ca^{2+}-activated currents were evoked in 50 out of 57 neurones, the mean delay to current activation following photolysis was 82 ± 13 s. The responses were variable

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Abbreviations: HS, horse serum; G_{M1}, G_{M3}, G_{T1b}, G_{D1b}, glycosphingolipid gangliosides; DRG, dorsal root ganglion; NMDG, *N*-methyl-D-glucamine.

with neurones showing transient, oscillating or sustained inward currents. High voltage-activated Ca^{2+} currents evoked by 100 ms voltage step commands to 0 mV were not attenuated by photorelease of dihydrosphingosine. Controls showed that alone a flash from the Xenon lamp did not activate currents, and that the unphotolysed caged dihydrosphingosine, and intracellular photolysis of 2-(2-nitrobenzylamino) propanediol also did not evoke responses. The dihydrosphingosine current had a reversal potential of -11 ± 3 mV ($n = 11$), and was carried by two distinct Cl^- and cation currents which were reduced by 85% and about 20% following replacement of monovalent cations with *N*-methyl-D-glucamine or application of the Cl^- channel blocker niflumic acid (10 μM) respectively. The responses to photoreleased dihydrosphingosine were inhibited by intracellular application of 20 mM EGTA, 10 μM ryanodine or extracellular application of 10 μM dantrolene, but persisted when Ca^{2+} free saline was applied to the extracellular environment. Intracellular application of uncaged dihydrosphingosine evoked responses which were attenuated by photolysis of the caged Ca^{2+} chelator Diazo-2. Experiments also suggested that extracellular application of dihydrosphingosine can activate membrane conductances. We conclude that dihydrosphingosine directly or indirectly mobilises Ca^{2+} from ryanodine-sensitive intracellular stores in cultured sensory neurones.

Sphingolipids appear to play a number of diverse roles in cell signalling, acting as intercellular as well as intracellular messengers (Hakomori & Igarashi, 1995; Spiegel & Milstein, 1995). Previous studies have shown that sphingolipids can modulate, in a variety of ways, mechanisms which regulate intracellular Ca^{2+} concentration. Firstly, sphingolipids can directly or indirectly, *via* other signalling pathways, mobilize Ca^{2+} from intracellular stores. Application of sphingosine-1-phosphate released Ca^{2+} from intracellular stores and activated Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes (Durieux *et al.*, 1993). Similarly, Ca^{2+} responses have been seen in permeabilized cultured smooth muscle cells following generation of sphingosine-1-phosphate which may directly interact with Ca^{2+} release channels (Ghosh *et al.*, 1994) or may exert its effect by activation of phospholipase D and generation of phosphatidic acid. The glycosphingolipid gangliosides, G_{M1} and G_{M3}, have also been found to liberate Ca^{2+} from stores and activate platelets (Yatomi *et al.*, 1996). Additionally, both sphingosine and sphingosine-1-phosphate differentially modulate Ca^{2+} signals in transformed oligodendrocytes. Sphingosine and inhibition of sphingosine-1-phosphate formation promoted oscillatory type Ca^{2+} responses while sphingosine-1-phosphate generated non-oscillatory type Ca^{2+} responses (Fatatis & Miller, 1996). Secondly, sphingosine inhibited Ca^{2+} uptake into intracellular stores by regulation of Ca^{2+}-ATPase in pancreatic acinar cells and thus by attenuating Ca^{2+} homeostatic mechanisms prolonged events triggered by an increase in intracellular Ca^{2+} (Pandol *et al.*, 1994). Thirdly, Ca^{2+} entry through voltage-activated Ca^{2+} channels has been found to decrease as a result of dihydrosphingosine mediated inhibition of protein kinase C activity (Bartschat & Rhodes, 1995). Decreased re-phosphorylation and enhanced protein phosphatase activity can both promote Ca^{2+} channel inactivation and this may account for modulation of voltage activated Ca^{2+} channel function. Early studies showed that G_{T1b}, G_{D1b} and G_{M1} reduced excitotoxic neuronal damage in cultures suggesting that glycosphingolipids may have a clinical neuroprotective use in neurological disorders. This action may be due to gangliosides preventing Ca^{2+}-dependent protein kinase C translocation to the cell membrane after glutamate receptor activation (Favaron *et al.*, 1988).

The aim of this study was to investigate Ca^{2+} homeostatic mechanisms and entry pathways as possible sites of action for dihydrosphingosine using cultured dorsal root ganglion (DRG) neurones as a model system. Cultured DRG neurones express a variety of receptors

and ion channels including ryanodine sensitive Ca^{2+} release channels, a variety of high voltage-activated Ca^{2+} channels, and Ca^{2+}-activated nonselective cation and Cl^- channels. Whole cell Ca^{2+}-activated currents can be used as a physiological index of a rise in intracellular Ca^{2+} close to the cell membrane evoked by photorelease of Ca^{2+} from DM-nitrophen or release of Ca^{2+} from intracellular stores (Currie *et al.*, 1992; 1995; Scott *et al.*, 1995). Cultured DRG neurones have proved useful in studying the regulation of high voltage-activated Ca^{2+} channels following G-protein activation (Dolphin *et al.*, 1988), and the mobilization of Ca^{2+} from intracellular stores by novel agents including cytosolic sperm factor and cyclic ADP ribose (Currie *et al.*, 1992).

Intracellular flash photolysis of caged compounds combined with whole cell patch clamp recording is a powerful approach for rapidly delivering molecules or ions of interest to the intracellular environment and recording subsequent changes in electrophysiological properties of cells (Gurney, 1994). Caged dihydrosphingosine, used in this study, is a photolabile and biologically inert compound which can be introduced *via* the patch pipette solution into an intracellular environment using the whole cell recording technique. Once inside a cell it can be rapidly photocleaved in a controlled fashion by a flash of intense near UV light, to liberate free dihydrosphingosine and a by-product, 2-nitrosobenzaldehyde. Responses to an intracellular concentration jump in dihydrosphingosine can then be investigated.

MATERIALS AND METHODS

Cell culture. One to three-day old Wistar rats were decapitated, the spinal column was dissected free and the dorsal root ganglia removed and washed in phosphate buffered saline (PBS, pH 7.4). The DRG neurones were then dissociated enzymatically in Ham's nutrient mixture F14 with 10% horse serum (F14/HS) containing 0.125% collagenase for 13 min and then in PBS with 0.25% trypsin for 6 min at 37°C, and mechanically by trituration in F14/HS (900 μl) containing DNase (100 μl, 160 kunitz · ml^{-1}). The suspension of DRG cells was diluted to 5 ml with F14/HS and preplated for 3 h. The dead cells and clumps of cells were removed by gentle washing and discarded. More vigorous washing removed the DRG neurones leaving the fibroblasts and glia stuck to the pre-plate. The DRG neurone rich suspension was then plated on polyornithine-laminin coated coverslips and bathed in 1.5 ml culture medium F14/HS containing nerve growth factor (20 ng/ml), penicillin (5000 IU/ml), streptomycin (5000 μg/ml) and $NaHCO_3$ (14 mM). The cultured DRG neurones were maintained at 37°C in 100% humidity and gassed with 95% air and 5% CO_2 and the cultures were re-fed with fresh culture medium every 5 to 7 days.

Synthesis of caged, N-(2-nitrobenzyl)dihydrosphingosine. *N*-(2-Nitrobenzyl)-DL-*erythro*-dihydrosphingosine, the only dihydrosphingosine preparation used in this work, was prepared through the N-alkylation of DL-*erythro*-dihydrosphingosine with 2-nitrobenzyl bromide to give an 83% yield following chromatography on a silica gel column. The proposed structure was supported by mass spectroscopy (MS) and proton magnetic resonance spectroscopy (^{1}H-NMR). Caged propanediol, 2-(2-nitrobenzylamino)propanediol, was synthesised using the same approach (Zehavi, 1997).

Whole cell recording and flash photolysis. The whole cell variant of the patch clamp technique (Hamill *et al.*, 1981) was used to investigate the electrophysiological effects of both the intracellular photorelease and extracellular application of dihydrosphingosine on DRG neurones which had been in culture for 2 days to 3 weeks. Experiments were carried out at room temperature (21–23°C). For voltage clamp experiments, borosilicate glass patch pipettes (3–8 MΩ) were filled with CsCl-

based patch pipettes solution containing (in mM): 140 CsCl, 0.1 $CaCl_2$, 1.1 or 20 EGTA, 2 $MgCl_2$, 2 ATP, 10 Hepes. In some current clamp experiments, KCl-based patch pipette solution was used containing (in mM): 140 KCl, 0.1 $CaCl_2$, 1.1 or 20 EGTA, 2 $MgCl_2$, 2 ATP, 10 Hepes. The pH and osmolarity of these patch pipette solutions were corrected to 7.2 and 310–320 mOsm $\cdot$ l^{-1} with Tris and sucrose. Caged, *N*-(2-nitrobenzyl)dihydrosphingosine (100 μM) or 2-(2-nitrobenzylamino)propanediol were included in the CsCl-based patch pipette solution with 0.3% dimethylformamide and 2 mM dithiothreitol and experiments were also carried out using patch pipette solutions containing 10 μM ryanodine, 10 μM dantrolene, 5–10 μM dihydrosphingosine with 0.4% dimethylformamide and 2.5 mM Diazo-2, a caged Ca^{2+} chelator (Adams *et al.*, 1989) (Molecular Probes Inc.). The extracellular bathing solution for voltage clamp experiments contained (in mM): 130 choline chloride, 2 $CaCl_2$, 3 KCl, 0.6 $MgCl_2$, 1 $NaHCO_3$, 10 Hepes, 5 glucose, 25 tetraethylammonium chloride, 0.0025 tetrodotoxin (Sigma). For some experiments, modified solutions were used in which either no Ca^{2+} was added to the extracellular bathing medium (nominally Ca^{2+}-free solution) or in which all monovalent cations were replaced by a large impermeant cation *N*-methyl-D-glucamine (NMDG, pH adjustment with HCl). The recording solutions used in the voltage clamp experiments were designed to inhibit Na^+ and K^+ currents and isolate voltage-activated Ca^{2+} currents and Ca^{2+}-activated nonselective cation and Cl^- currents. For the current clamp experiments, a NaCl-based extracellular bathing solution was used containing (in mM): 130 NaCl, 2 $CaCl_2$, 3 KCl, 0.6 $MgCl_2$, 1 $NaHCO_3$, 10 Hepes, 5 glucose. The pH and osmolarity of these extracellular bathing solutions were corrected to 7.4 and 320 mOsm $\cdot$ l^{-1} with NaOH and sucrose. NMDG-based extracellular bathing medium, 10 μM niflumic acid, or 10 μM dihydrosphingosine in 0.4% dimethylformamide were applied to the extracellular environment by low pressure ejection (about 7 kPa) from a blunt pipette positioned 50–100 μm from the neurone under study. An Axoclamp-2A (Axon Instruments Inc.) amplifier, operated at a sampling rate of 15–25 kHz in discontinuous single electrode voltage clamp mode, was used to record Ca^{2+}-activated Cl^- currents and Ca^{2+}-activated cation currents evoked in response to intracellular photolysis of caged dihydrosphingosine. Experiments were also conducted to determine whether dihydrosphingosine attenuated high voltage-activated Ca^{2+} currents activated from a holding potential of -90 mV by 100 ms depolarizing voltage step commands to 0 mV. The same amplifier was also used in bridge mode to record changes in input resistance when dihydrosphingosine was applied to the extracellular environment. Neurones were held at -75 mV by constant current injection and the input resistance was measured from the changes in the electrotonic potentials evoked by -100 pA current commands of 100–300 ms.

Intracellular photolysis of caged dihydrosphingosine, 2-(2-nitrobenzylamino)propanediol or diazo-2 was carried out after at least 5 min equilibration in the whole cell recording configuration which allowed diffusion of the constituents of the patch pipette solution into the neurone. Photolysis was achieved using an XF-10 Xenon flash lamp (Hi-Tech Scientific) with a UG11 bandpass filter. A 1 ms, 200 V flash of intense near UV light had a power output of about 8 mJ mm^{-2} and gave about 5% photolysis in our system. The mean quantum yield was 0.32 (n = 6) for the photolysis of 2-(2-nitrobenzylamino)propanediol, measured using HPLC reverse phase chromatography; monitored at λ_{254}, and a mobile phase of 40% methanol containing 100 mM potassium phosphate and 4 mM potassium acetate at pH 3.7. The estimated percentage photolysis of caged dihydrosphingosine and Diazo-2 was also about 5%.

Data analysis. Data were captured and stored on digital audio tape using a Biologic

digital tape recorder (DTR 1200) and on a Gould 2200S pen recorder. Analysis of data was performed off-line on a Tandon computer using Cambridge Electronic Design voltage clamp analysis software (version 5.5). All voltage-activated Ca^{2+} currents had scaled linear leakage and capacitance currents subtracted to obtain values for the net inward current. Data are given as mean ± standard error of the mean (S.E.M.) values and statistical significance was determined using a paired or independent Student's *t* test as appropriate.

RESULTS

Actions of dihydrosphingosine on high voltage-activated calcium currents

Given previous findings on the modulation of voltage-activated Ca^{2+} currents by sphingolipids (Bartschat & Rhodes, 1995), we started this study by investigating the actions of intracellular photoreleased dihydrosphingosine on high voltage-activated Ca^{2+} currents in DRG neurones held at −90 mV and depolarized to 0 mV by 100 ms voltage step commands. Control experiments showed that up to 5 flashes from the Xenon flash lamp and 0.3% dimethylformamide and 2 mM dithiothreitol in the patch pipette solution had no significant effect on high voltage-activated Ca^{2+} currents activated every 30 s for 8 min (n = 17). After 8 min equilibration in the whole cell recording configuration, intracellular photorelease of 5–15 μM dihydrosphingosine did not attenuate high voltage-activated Ca^{2+} currents activated every minute for 8 min (Fig. 1A). The mean net Ca^{2+} current amplitudes measured at the peak and at the end of the 100 ms voltage step command were −0.80 ± 0.09 nA and −0.57 ± 0.07 nA, respectively, under control conditions and −0.80 ± 0.09 nA and −0.55 ± 0.09 nA (n = 5; NS) 8 min after intracellular photolysis of caged dihydrosphingosine. Additionally, intracellular flash photolysis of caged dihydrosphingosine during (at 30 ms) a 100 ms depolarizing voltage step command had no significant effect on high voltage-activated Ca^{2+} currents (Fig. 1B). During these experiments on high voltage-activated Ca^{2+} currents, it was apparent that intracellular photorelease of dihydrosphingosine, after a delay, did activate inward currents which were usually transient in nature (Fig. 1C).

Intracellular photorelease of dihydrosphingosine activates calcium-dependent currents

Intracellular flash photolysis of caged dihydrosphingosine, after a delay, activated inward currents from a holding potential of −90 mV in 50 out of the 57 cultured DRG neurones studied. These inward currents were variable in nature ranging from rapid transient events and oscillatory currents to more sustained currents (Fig. 2A). The mean delay to activation after intracellular photorelease of 5–15 μM dihydrosphingosine was 82 ± 13 s and the mean peak amplitude of these inward currents was −1.22 ± 0.08 nA (n = 50). No inward currents were evoked by flashes of intense near UV light in the absence of caged dihydrosphingosine (n = 3). As additional controls the caged dihydrosphingosine was left unphotolysed in neurones for 15 min (n = 7) and up to 20 μM of the biologically inactive compound propanediol was photoreleased inside the neurones (n = 7); no inward currents were activated by either of these protocols (Fig. 2B, C). The photolysis of 2-(2-nitrobenzylamino)propanediol and caged dihydrosphingosine yield equimolar concentrations of the by-product 2-nitrosobenzaldehyde which in the presence of dithiothreitol was inactive. The negative results of these controls indicated that the inward currents were evoked as a result of intracellular photorelease of dihydrosphingosine.

The ionic nature of the inward currents activated by dihydrosphingosine were determined by reversal potential estimation, removal of extracellular Ca^{2+} from the bathing

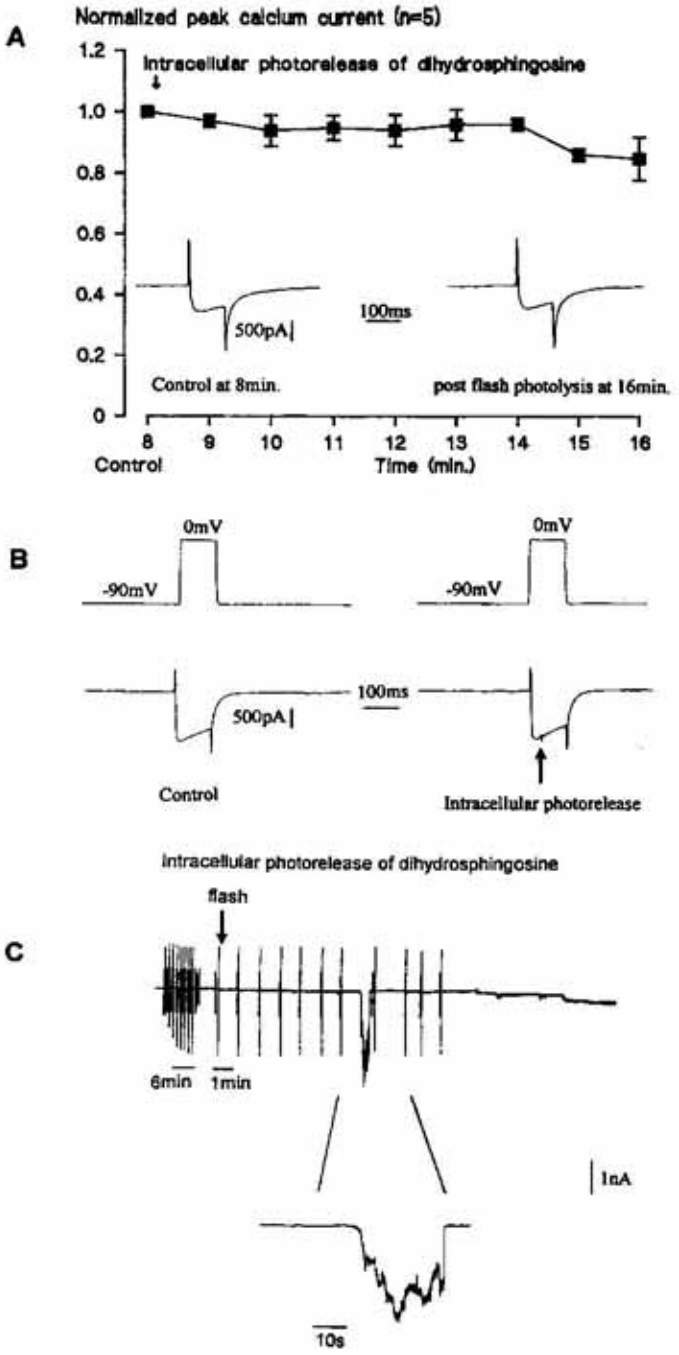

Figure 1. Intracellular photolysis of caged dihydrosphingosine on high voltage-activated Ca^{2+} currents.

A. Intracellular photorelease of dihydrosphingosine had no significant effect on mean normalized peak Ca^{2+} current amplitude over an 8 min period. Data are shown with standard errors for 5 experiments and inset traces show currents activated at 0 mV under control conditions and 16 min in the experiment, 8 min after photolysis. B. Records of a control Ca^{2+} current and a Ca^{2+} current activated during photolysis of caged dihydrosphingosine. C. Expanded record showing equilibration over 6 min with the Ca^{2+} current increasing as a result of intracellular Cs^{+} block of K^{+} conductances and stability of the Ca^{2+} current after intracellular photolysis of dihydrosphingosine. Inset shows an expanded record of the dihydrosphingosine evoked current. The holding potential for all cells was -90 mV.

solution, replacing monovalent cations in the extracellular bathing solution with the large impermeant cation NMDG and by extracellular application of the Cl^{-} channel blocker ni-

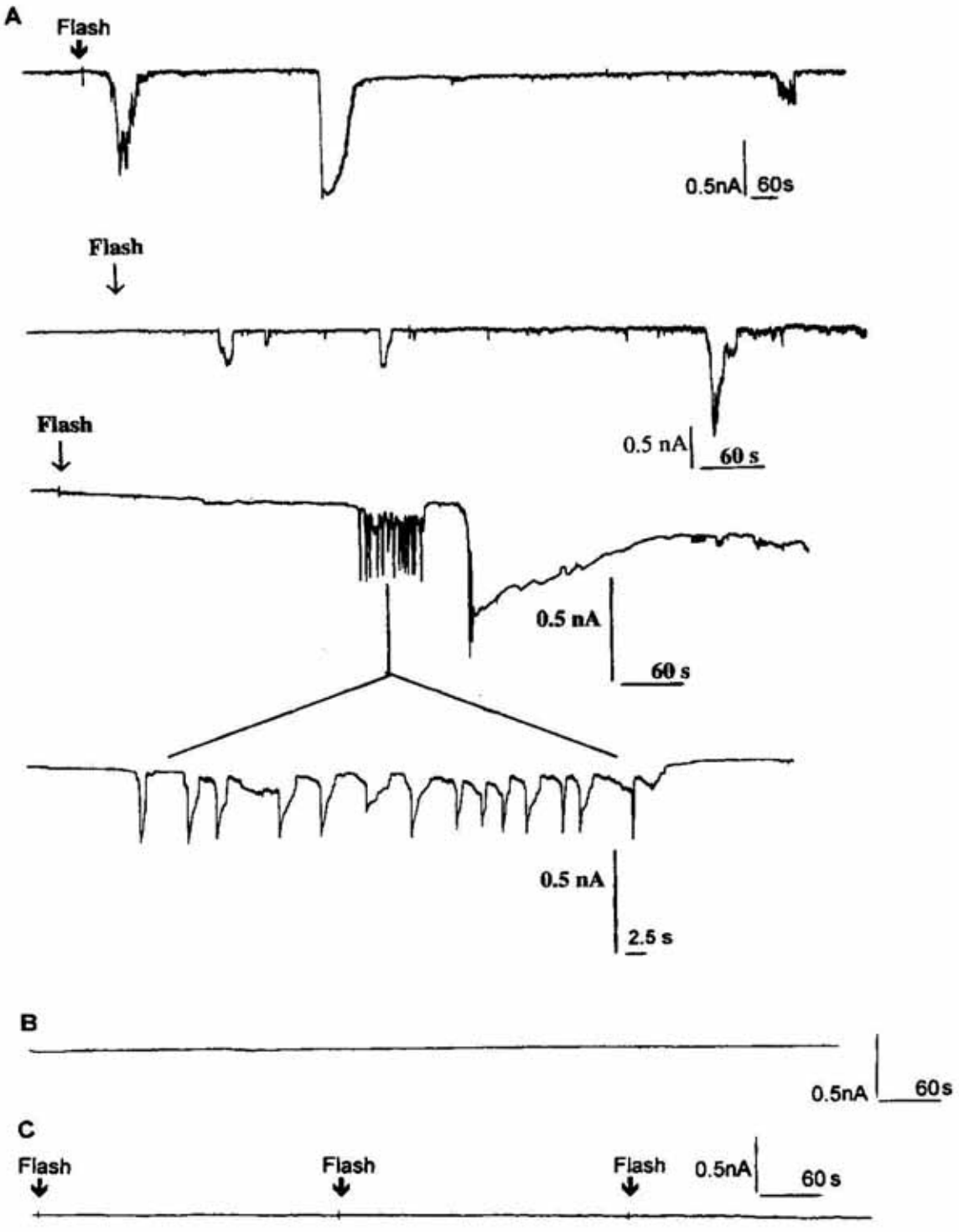

Figure 2. Inward currents activated by intracellular photorelease of caged dihydrosphingosine.

A. Inward currents evoked following photorelease of 5 μM dihydrosphingosine recorded from three different neurones. An expanded record shows the oscillatory nature of some of the responses. B. Control record showing no responses to caged dihydrosphingosine when no intracellular photolysis was carried out. C. Record showing no effect of intracellular photolysis of 2-(2-nitrobenzylamino)propanediol. The holding potential for all cells was -90 mV.

current-voltage relationship which could not be extended to potentials positive to -50 mV because of contamination by voltage-activated currents. The mean estimated reversal potential was -11 ± 3 mV (n = 11, Fig. 3) which is close to the predicted equilibrium potential for Cl^- and nonselective cation conductances under our recording conditions. The inward currents activated by intracellular photorelease of dihydrosphingosine were still evoked when Ca^{2+}-free extracellular bathing solution was applied, and had a mean peak amplitude of -1.19 ± 0.17 nA (n = 7, Fig. 4A). A major component of the dihydrosphingosine-activated inward current was carried by monovalent cations, perfusion with NMDG-containing extracellular bathing solution reversibly reduced the inward current at -90 mV by 85 ± 5% (n = 9, Fig. 4B). The mean peak current values with standard choline-based and NMDG-based bathing solutions were -0.99 ± 0.21 nA and -0.17 ± 0.07 nA respectively (n = 9, $P < 0.001$). Part of the current, about 20% (n = 2), was inhibited by extracellular pressure ejection of 10 μM niflumic acid suggesting an efflux of Cl^- contributed to the inward current at -90 mV (Fig. 4C). Two ionic elements contributing to the inward current were confirmed when the current was completely abolished by combined extracellular application of 10 μM niflumic acid made up in NMDG-containing extracellular bathing solution (n = 3, Fig. 4D).

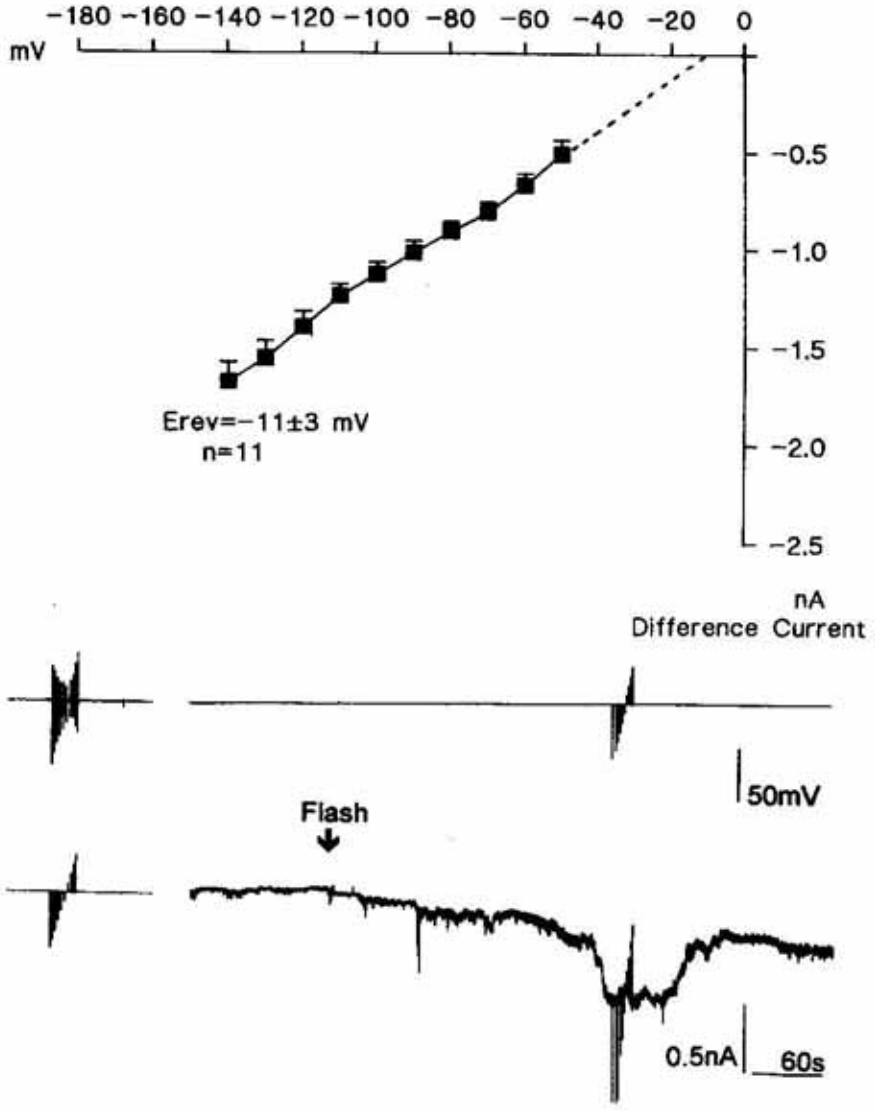

Figure 3. Estimated reversal potential (Erev) of difference current evoked by dihydrosphingosine.

Graph giving the difference currents obtained by subtracting the leak currents generated under control conditions from the currents at the same voltages activated during an inward current evoked by intracellular photorelease of dihydrosphingosine. Extrapolation of the difference current-voltage relationship gave the estimated reversal potential. Records show examples of the voltage step commands, the resultant control leak currents and currents evoked during a response to dihydrosphingosine.

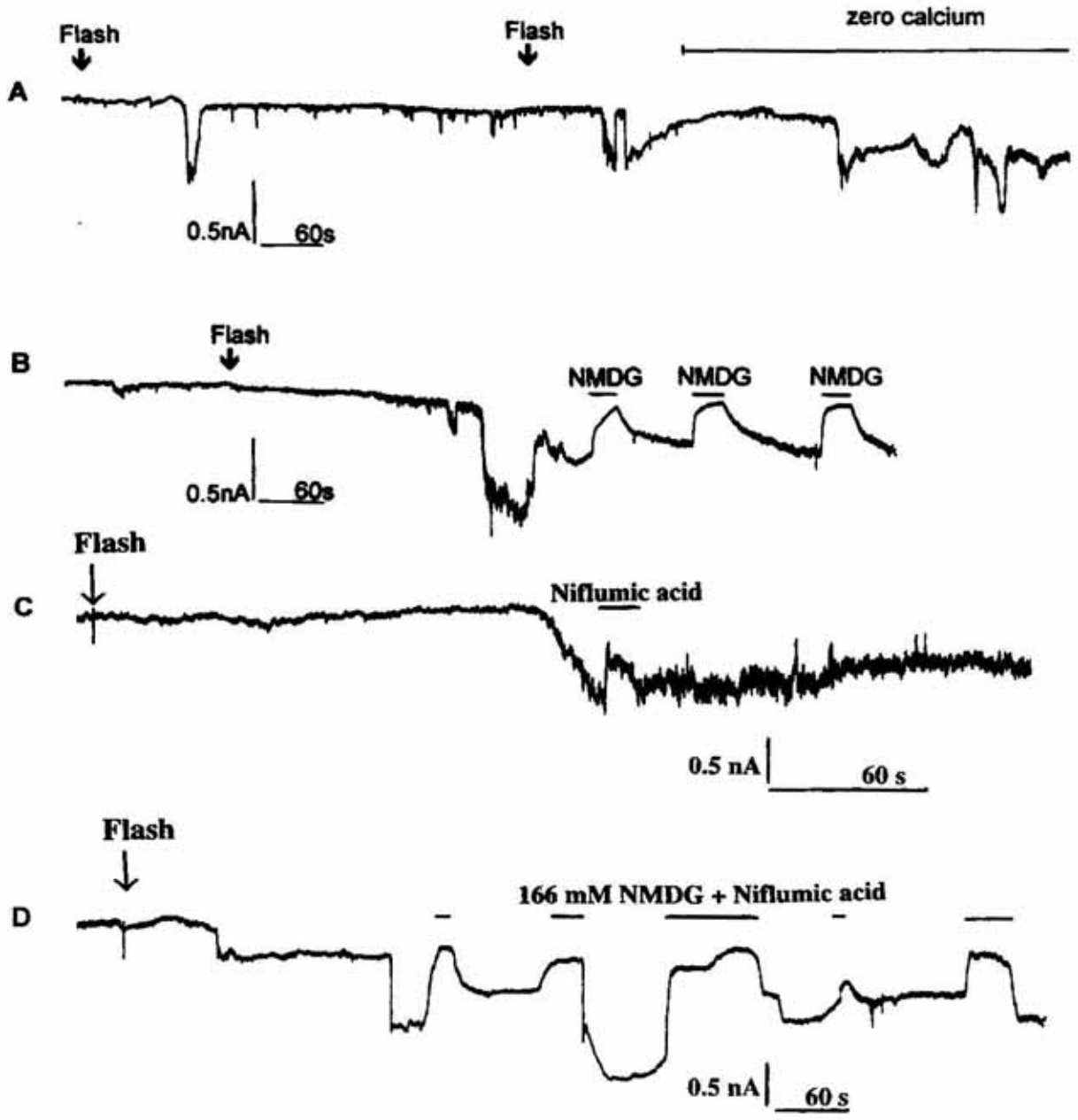

Figure 4. Intracellular photorelease of dihydrosphingosine evokes Ca^{2+}-activated currents.

A. Responses to intracellular photorelease of dihydrosphingosine persisted during perfusion of Ca^{2+} free-saline (zero calcium). B. Record showing that *N*-methyl-D-glucamine (NMDG)-based extracellular bathing solution attenuated inward currents evoked by intracellular photorelease of dihydrosphingosine. C. Record showing that extracellular application of 10 μM niflumic acid attenuated inward currents evoked by intracellular photorelease of dihydrosphingosine. D. Record showing that NMDG-based extracellular bathing solution containing 10 μM niflumic acid reversibly abolished inward currents evoked by intracellular photorelease of dihydrosphingosine. All neurones were held at -90 mV.

Intracellular photorelease of dihydrosphingosine activated currents similar to those produced by direct intracellular application of Ca^{2+} and by agents which mobilize Ca^{2+} from intracellular stores, such as caffeine, cytosolic sperm factor, and cyclic ADP-ribose (Currie *et al.*, 1992). The next series of experiments were designed to determine whether intracellular photolysis of caged dihydrosphingosine activated Ca^{2+}-dependent currents as a result

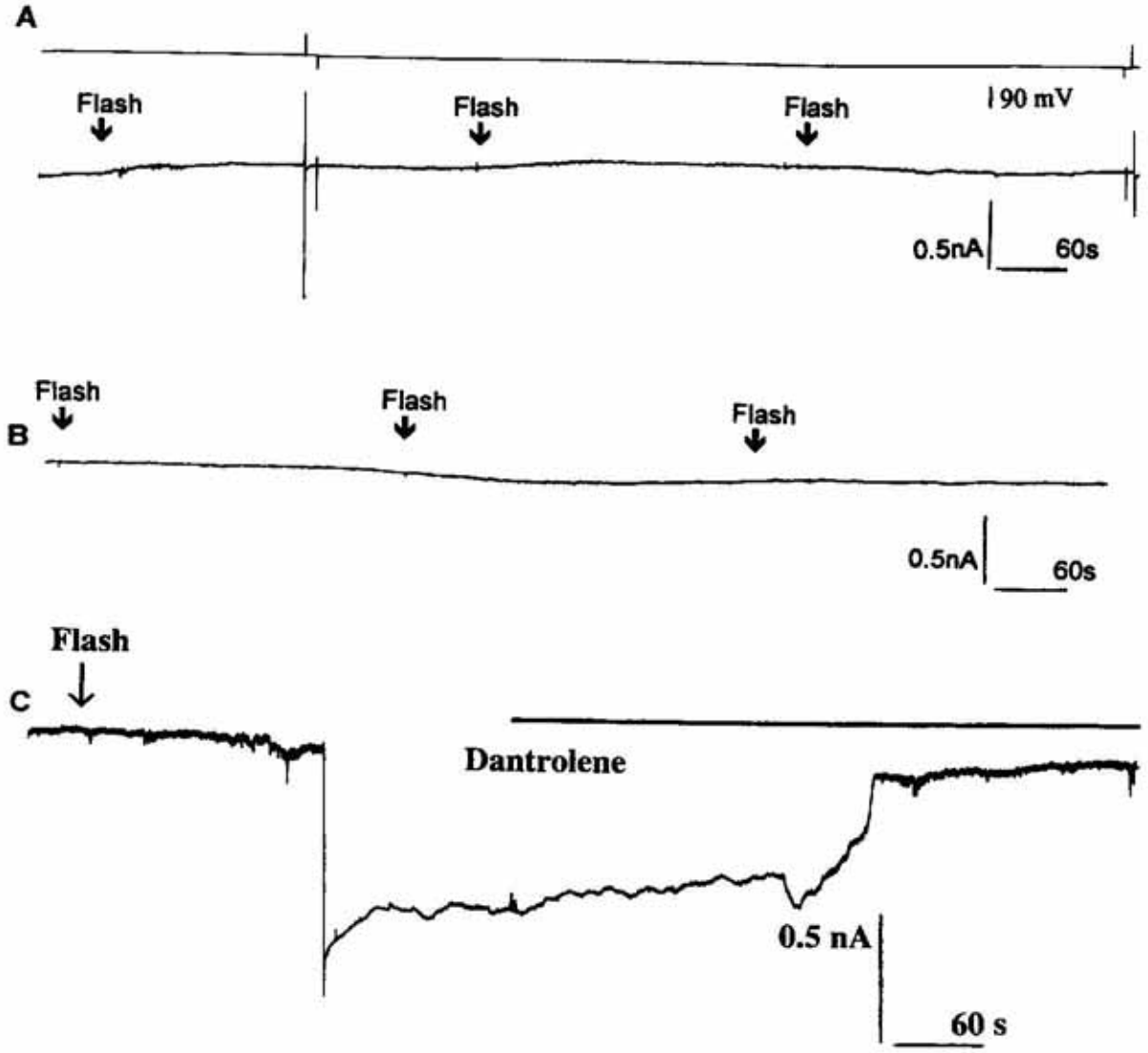

Figure 5. Intracellular photorelease of dihydrosphingosine evokes Ca^{2+}-activated currents by releasing Ca^{2+} from intracellular stores.

Records show that the inward currents evoked by dihydrosphingosine were abolished by: A, intracellular application of 10 μM ryanodine. The deflections are voltage-activated whole cell currents evoked to check that the neurone was still being recorded from using the whole cell recording technique. B, intracellular application of CsCl-based patch pipette solution containing a high concentration of EGTA (20 mM), and C, extracellular application of 10 μM dantrolene. All neurones were held at -90mV.

of Ca^{2+} released from intracellular stores. Inclusion of 10 μM ryanodine, the plant alkaloid and Ca^{2+} release channel blocker, in the patch pipette solution prevented activation of inward currents by photorelease of dihydrosphingosine (n = 12, Fig. 5A). Responses were also prevented when CsCl-based patch pipette solution containing a high concentration of the Ca^{2+} chelator EGTA (20 mM instead of 1.1 mM) was used (n = 7, Fig. 5B). Additionally, extracellular application of 10 μM dantrolene, which is an antihyperthermia drug and has been shown to prevent caffeine-induced release of Ca^{2+} from stores, gradually

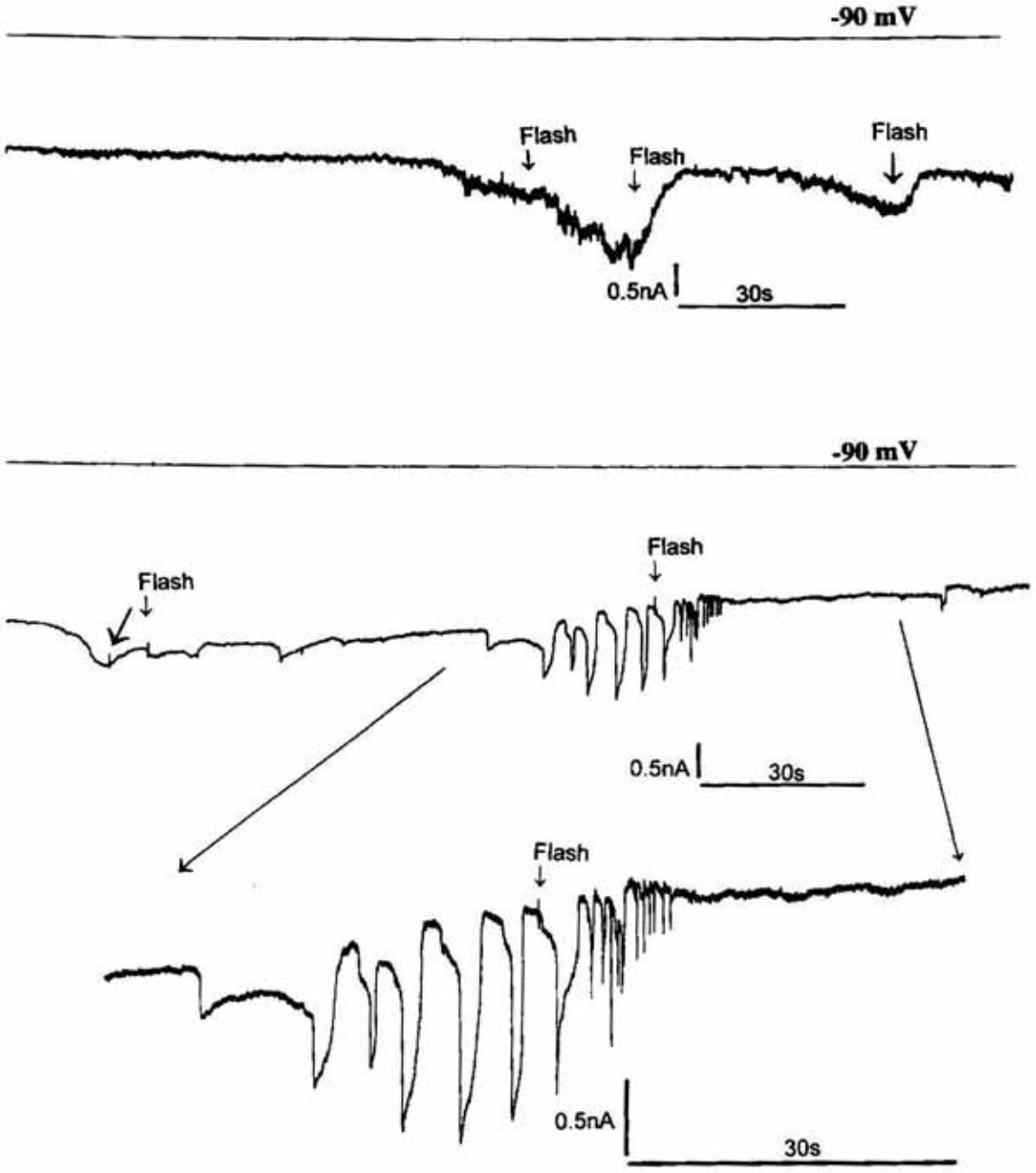

Figure 6. Intracellular photolysis of the caged Ca^{2+} chelator diazo-2 attenuates dihydrosphingosine activated inward currents.

Records from two different cultured DRG neurones showing attenuation of inward currents activated by dihydrosphingosine following photolysis of Diazo-2. The neurones were held at −90 mV.

inhibited the Ca^{2+}-activated inward current and completely prevented further activation of inward currents by dihydrosphingosine (n = 3, Fig. 5C). Extracellular application of 10 μM dantrolene reduced the peak current evoked by intracellular photorelease of dihydrosphingosine by 78 ± 3%, from −0.88 ± 0.11 nA to −0.19 ± 0.03 nA ($P < 0.0005$, n = 8). These results indicate that Ca^{2+}-activated nonselective cation and Cl^- currents were evoked by intracellular photorelease of dihydrosphingosine as a result of release of Ca^{2+} from an intracellular store which was sensitive to both ryanodine and dantrolene.

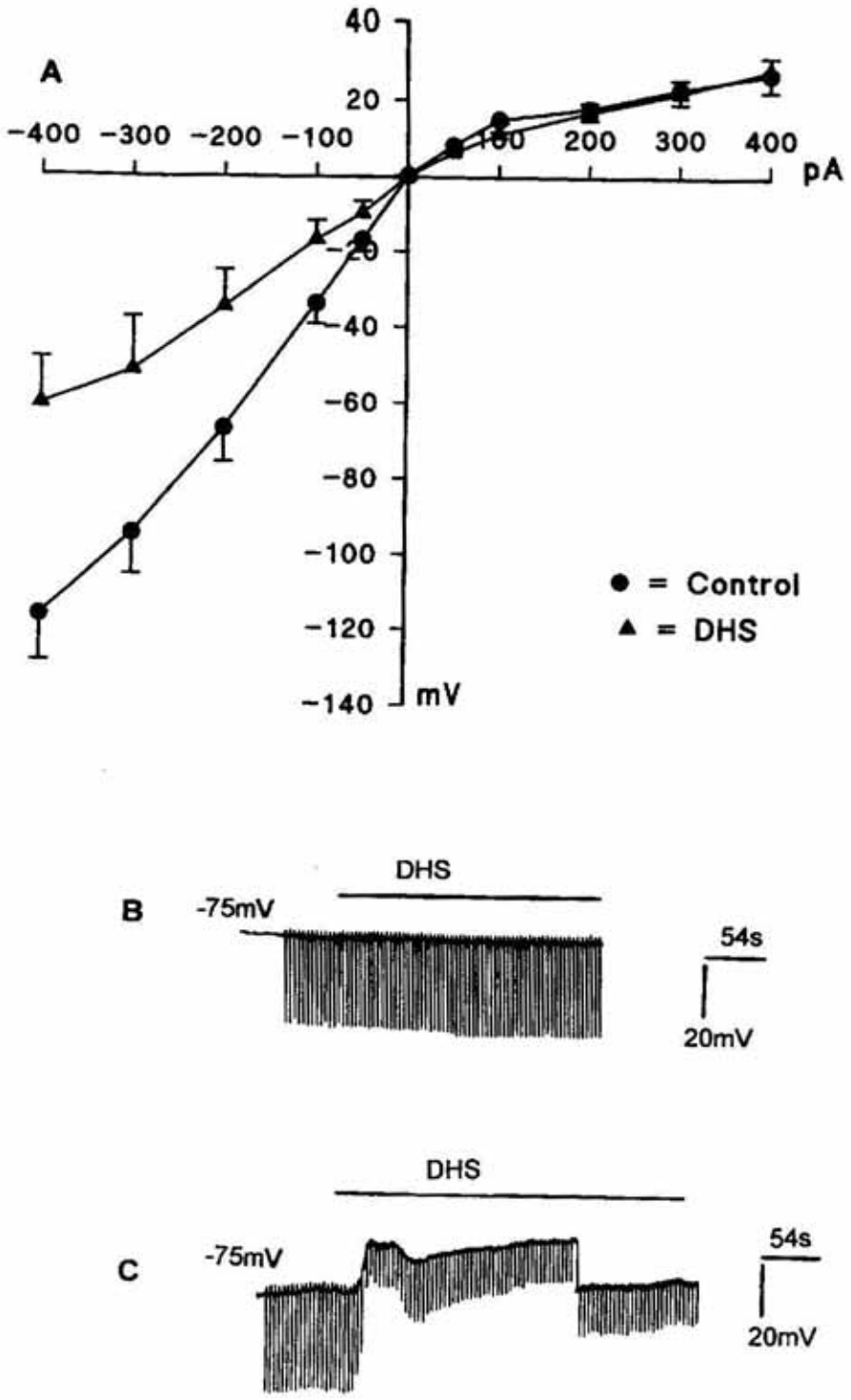

Figure 7. Extracellular application of dihydrosphingosine evokes a variety of conductances.

A. Current-voltage relationships under control conditions (● mean ± S.E.M., n = 5) and during 3–5 min application of 10 μM dihydrosphingosine (▲ mean ± S.E.M., n = 5), showing the increase in conductance evoked by dihydrosphingosine. The measurements were all made from neurones held at −75 mV. B. Records from neurones patch clamped with pipettes filled with KCl-based patch pipette solution containing 20 mM EGTA and 10 μM dantrolene. One cell showed no significant response to application of 10 μM dihydrosphingosine, (DHS), C. in contrast a second cell depolarised and showed a reduction in input resistance; note that the change in membrane potential was corrected by constant current injection. The downward deflections show electrotonic potentials evoked by 100 pA hyperpolarizing current step commands and constant current was applied to hold the neurones at −75mV.

Experiments were also undertaken to determine whether including 5 μM uncaged dihydrosphingosine in the patch pipette solution could activate inward currents when recording was done in the whole cell patch clamp configuration and whether these events were sensitive to intracellular photolysis of caged Ca^{2+} chelator, Diazo-2. One cultured DRG neurone out of 7 did not respond to intracellular application of dihydrosphingosine. The mean delay to activation (the time between entering the whole cell recording configuration and the development of the first event) was 328 ± 114 s and the mean peak current amplitude was −1.15 ± 0.19 nA (n = 6). Intracellular photolysis of Diazo-2 attenuated both events activated by dihydrosphingosine reducing the inward currents and oscillatory responses (n = 3, Fig. 6).

Extracellular application of dihydrosphingosine

The electrophysiological actions of uncaged dihydrosphingosine applied to the extracellular environment were assessed by measuring changes in electrotonic potentials evoked by 100 pA hyperpolarizing current step commands and determining the input resistance values of cultured DRG neurones current clamped at −75 mV. NaCl-Based bathing solution and KCl-based patch pipette solutions were used in this part of the study. Extracellular application of 10 μM dihydrosphingosine in NaCl-based extracellular bathing solution containing 0.4% dimethylformamide for 3 to 5 min evoked a mean decrease in input resistance from 338 ± 55 MΩ to 169 ± 49 MΩ (n = 5, $P < 0.01$). Dihydrosphingosine also caused transient depolarizations of the membrane potential which were corrected by constant hyperpolarizing current injection. The responses to extracellular application of dihydrosphingosine were seen in all cultured DRG neurones studied, and recovery of the input resistance values were seen about 5 min after removal of the perfusion pipette containing dihydrosphingosine. In two cases studied it was found that the input resistance recovered after dihydrosphingosine application to values greater than those obtained for controls, and in 1 case input resistance continued to increase over a 15 min recovery period. Dihydrosphingosine produced a reduction in electrotonic potentials over the hyperpolarizing range of the current-voltage relationship (Fig. 7A).

Experiments were then carried out to determine whether these responses to dihydrosphingosine applied to the extracellular environment involved intracellular events which resulted in release of Ca^{2+} from dantrolene-sensitive stores and subsequent activation of Ca^{2+}-activated ion channels. Cultured DRG neurones were recorded from using patch pipettes filled with KCl-based patch pipette solution with 20 mM EGTA and 10 μM dantrolene added. The inclusion of a high concentration of EGTA and dantrolene in the patch pipette solution had no significant effect on input resistance of the DRG neurones measured 5 min after entering the whole cell recording configuration. However, the resting membrane potential recorded from control neurones was significantly higher ($P < 0.005$) than the values measured from neurones with high intracellular EGTA and dantrolene. The mean resting membrane potentials and input resistance values were −67 ± 2 mV and 400 ± 49 MΩ (n = 8) and −52 ± 4 mV (n = 10) and 378 ± 61 MΩ (n = 9) for neurones recorded under standard conditions and recorded with high intracellular EGTA and 10 mM dantrolene, respectively. Extracellular application of dihydrosphingosine to neurones recorded with high EGTA and 10 μM dantrolene in the patch pipette solution only evoked a change in membrane potential and input resistance in 50% of neurones (4 out of 8 neurones, Fig. 7B, C). The data from responding and non-responding neurones was analysed separately. The non-responders had a mean input resistance of 343 ± 31 MΩ under control conditions and 388 ± 25 MΩ (n = 4, NS) during perfusion with

dihydrosphingosine. This modest and insignificant apparent increase in input resistance was also transiently observed in neurones recorded using the standard KCl-based patch pipette solution during the first few seconds of extracellular application of 10 μM dihydrosphingosine. The neurones with high intracellular EGTA and dantrolene which responded to extracellular application of dihydrosphingosine had an input resistance of 466 ± 124 MΩ under control conditions which was reduced to 236 ± 60 MΩ (n = 4, $P < 0.05$) during perfusion with dihydrosphingosine.

DISCUSSION

In this study we failed to observe modulation of high voltage-activated Ca^{2+} currents following intracellular photorelease of dihydrosphingosine from its caged precursor. This is in contrast to the effects previously seen with dihydrosphingosine on Ca^{2+} influx into K^+-stimulated hippocampal synaptosomes (Bartschat & Rhodes, 1995). This may relate to different methods being used, Ca^{2+}-sensitive fluorescent indicator compared with whole cell recording of Ca^{2+} currents other possible influencing factors include Ca^{2+} channel diversity and heterogeneous expression of Ca^{2+} channels in the different preparations. Dihydrosphingosine has been found to inhibit protein kinase C activity implicated in the phosphorylation of Ca^{2+} channels, in particular the N-type, and would therefore appear to be consistent with the modulation of N-type Ca^{2+} currents. However, clear universal roles for protein kinase C in neuronal Ca^{2+} channel modulation remains obscure due to some conflicting evidence (Dolphin, 1995).

The data presented in this study clearly supports a role for sphingolipids in the mobilization of Ca^{2+} from intracellular stores. Dihydrosphingosine raised intracellular Ca^{2+} sufficiently to activate Ca^{2+} -activated Cl^- channels and nonselective cation channels in the cell membrane of cultured DRG neurones (Scott *et al.*, 1995). This effect was sensitive to raising intracellular Ca^{2+} buffering (increasing EGTA or intracellular photolysis of Diazo-2), dantrolene and ryanodine but not reducing extracellular Ca^{2+}, suggesting the involvement of intracellular Ca^{2+} stores. The responses to dihydrosphingosine were variable in nature and included sustained, transient and oscillatory events, but were consistent with the types of activity seen with sphingosine and sphingosine-1-phosphate in oocytes (Durieux *et al.*, 1993) and transformed oligodendrocytes (Fatatis & Miller, 1996). Additionally, in cultured DRG neurones agents which can mobilize Ca^{2+} from stores, including caffeine, cyclic ADP-ribose, cGMP, the cytosolic sperm factor oscillin, and ryanodine, have evoked very variable Ca^{2+}-dependent inward currents with different rates of activation and durations (Currie *et al.*, 1992; Crawford *et al.*, 1997). Further experiments are underway to determine whether the effects of dihydrosphingosine involve a direct action or whether conversion to another sphingolipid or product is required. The intracellular photorelease of dihydrosphingosine has definite advantages over just including dihydrosphingosine in the patch pipette solution as there appears to be a delay of about 5 min produced as the sphingolipid diffuses into the neurone. Following intracellular photolysis of caged dihydrosphingosine, there is still a delay of about 1.5 min before the first response develops; this may be because of the hydrophobic nature of the caged precursor and the photolysis product, free dihydrosphingosine, and thus their distributions in DRG neurones. The delay may also relate to metabolic processing and the production of more active sphingolipids, possibly sphingosine or sphingosine-1-phosphate, or the stimulation of other intracellular signalling pathways. Some previous studies which have found that sphingolipids release Ca^{2+} from intracellular stores have implicated the phosphatidylinositol signalling pathway and inositol 1,4,5-trisphosphate-sensitive store as potential sites of action

(Ghosh *et al.*, 1994; Chao *et al.*, 1994). Two branches of evidence suggest that this may not be the case with the action of dihydrosphingosine in cultured DRG neurones. Firstly, the introduction of inositol 1,4,5-trisphosphate or analogues which are resistant to metabolic breakdown have failed to release enough intracellular Ca^{2+} at a location which allows activation of Ca^{2+}-dependent channels in the cell membrane (Currie *et al.*, 1992; 1995). Secondly, in this study intracellular application of ryanodine abolishes the responses to dihydrosphingosine and thus implicates the ryanodine-sensitive Ca^{2+} release channel.

Extracellular application of dihydrosphingosine activated increases in input conductance in cultured DRG neurones. These responses appeared to involve several distinct mechanisms and ion channels. In some cells high intracellular EGTA and dantrolene blocked the response suggesting an event dependent on the release of Ca^{2+} from intracellular stores, similar to responses seen with intracellular application and photolysis of caged dihydrosphingosine. However, in 50% of neurones a response was still initiated under recording conditions designed to prevent release of Ca^{2+} from stores and activation of Ca^{2+}-activated ion channels, suggesting a different type of conductance and mechanism of activation. This may involve an intracellular site of action, but possibly the activation of a cell membrane receptor and thus relate to a transmembrane signalling role for sphingolipids. Surface membrane receptors for glycosphingolipids, such as gangliosides, have been identified (Hakomori & Igarashi, 1995). However, details of the mechanism and type of conductance which might be activated by extracellular dihydrosphingosine remain to be identified. The possible cellular functions involving such sphingolipid mediated transmembrane signalling include cell recognition, the regulation of cell growth and proliferation and the control of apoptosis (Spiegel & Milstein, 1995; Hakomori & Igarashi, 1995).

In conclusion, mobilization of Ca^{2+} from intracellular stores is a key event, important in both the physiology and pathophysiology of neurones. Intracellular photorelease of dihydrosphingosine appears to either directly or indirectly mobilize Ca^{2+} from intracellular stores which are sensitive to ryanodine and dantrolene. It seems likely that sphingolipids, in addition to inositol phospholipids, cyclic ADP-ribose and other nucleotides, may modulate Ca^{2+}-induced Ca^{2+} release.

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