

Extra- and Intracellular Sphingosylphosphorylcholine Promote Spontaneous Transmitter Release from Frog Motor Nerve Endings

EUGEN BRAILOIU and NAE J. DUN

Department of Pharmacology, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee

Received December 16, 2002; accepted March 11, 2003

This article is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

Similar to phosphatidylinositol bisphosphate, sphingomyelin breakdown generates several lipids, including sphingosylphosphorylcholine (SPC), that are putative signaling molecules. The present study was undertaken to evaluate the involvement of SPC in transmitter release process. Intracellular recordings were made from isolated frog sciatic-sartorius nerve-muscle preparations, and the effects of SPC on neurosecretion in the form of miniature endplate potentials (MEPPs) were assessed. Extracellular application of SPC mixture (D,L-SPC) at 1, 10, and 25 μ M increased the MEPP frequency by 68, 96, and 127%, respectively. D-erythro-SPC (dissolved in dimethyl sulfoxide but not coupled to bovine serum albumin), but not L-threo-SPC, was active extracellularly; the former (at 10 μ M) increased the MEPP frequency by 143%. D-erythro-SPC treatment did not significantly change the median amplitude or frequency-distribution of MEPPs. Intracellular delivery via liposomes, in which 10, 100, or 1000 μ M SPC mixture was entrapped in liposomal

aqueous phase, induced a concentration-dependent increase in MEPP frequency of 45, 91, and 100%, respectively. D-erythro-SPC and L-threo-SPC at the concentration of 100 μ M increased the MEPP frequency by 117 and 67%, respectively, or 91 and 61%, respectively, when coupled to bovine serum albumin. Pretreatment with thapsigargin significantly reduced but did not abolish the effects of extracellular D-erythro-SPC (10 μ M) or liposomes containing 100 μ M D-erythro-SPC. Liposomes filled with 100 μ M D-myo-inositol 1,4,5-trisphosphate (IP₃) enhanced the MEPP frequency to the same magnitude as 100 μ M D-erythro-SPC entrapped in liposomes. However, administration of 100 μ M D-erythro-SPC and IP₃ entrapped in the same liposomes enhanced the MEPP frequency by 70%, which was less than that produced by these two compounds alone. The result provides the first electrophysiological evidence that SPC can modulate transmitter release by an extra- or intracellular action at the frog motor nerve ending.

Neurosecretion is a process critically dependent on a transient increase in intracellular Ca²⁺, which may be caused by an influx of Ca²⁺ from extracellular milieu and/or release from intracellular calcium stores (Petersen and Cancela, 1999). The major calcium stores in frog motor nerve terminals include smooth endoplasmic reticulum (SER) (Pezzati et al., 2001), mitochondria (Calupca et al., 2001), and secretory vesicles (Pezzati et al., 2001). Second messengers can activate or mobilize these stores. For example, injection of IP₃, cyclic ADP ribose (cADPR), and NAADP into the presynaptic site of *Aplysia californica* motoneurons enhances transmitter output (Chameau et al., 2001). Similarly, liposomal delivery of IP₃, cADPR, or NAADP into frog motor nerve terminals increases spontaneous transmitter release (Brailoiu and Miyamoto, 2000; Brailoiu et al., 2001). In the case of IP₃, a similar enhancement in transmitter release was reported at

the crayfish glutamatergic motor synapse (Dixon and Atwood, 1989).

Sphingomyelin is a major constituent of synaptic plasma membranes (Cotman et al., 1969), endoplasmic reticulum (Vale, 1980), and synaptic vesicles (Breckenridge et al., 1973; Deutsch and Kelly, 1981). Similar to phosphatidylinositol bisphosphate, sphingomyelin breakdown generates several active lipid messengers, such as sphingosine 1-phosphate (S1-P) (Le Stunff et al., 2002; Pyne and Pyne, 2002), sphingosylphosphorylcholine (SPC) (Meyer zu Heringdorf et al., 1997, 2002), and ceramide 1-phosphate (Le Stunff et al., 2002). These lipids can act as extracellular messengers to activate G-protein-coupled receptors (Kluk and Hla, 2002; Meyer zu Heringdorf et al., 2002; Xu, 2002) or as intracellular second messengers to release Ca²⁺ from internal stores (Ghosh et al., 1990; Meyer zu Heringdorf et al., 2002), including brain microsomes (Dettbarn et al., 1995; Furuya et al., 1996; Huang and Chueh, 1996).

This study was supported by grants NS18710 and NS39646 from the National Institutes of Health.

ABBREVIATIONS: SER, smooth endoplasmic reticulum; IP₃, D-myo-inositol 1,4,5-trisphosphate; cADPR, cyclic adenosine diphosphate-ribose; S1-P, sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; MEPP, miniature end-plate potential; HEK, human embryonic kidney; SCA_{MPER}, sphingolipid Ca²⁺-release-mediating protein of the endoplasmic reticulum.

Aside from the fact that sphingomyelin metabolism occurs in neurons including motoneurons (Irie and Hirabayashi, 1999), little is known regarding the possible involvement of sphingomyelin-derived messengers in neurosecretion in general and transmitter release in motoneurons in particular. In our previous study, we show that intracellularly but not extracellularly applied S1-P enhanced spontaneous transmitter release from frog motor nerve terminals, a process that is subject to receptor desensitization (Brailoiu et al., 2002). In view of a role for S1-P in modulating neurosecretion in motor nerve endings (Brailoiu et al., 2002), we were interested to know whether or not SPC might also affect transmitter release. If positive, does SPC act extracellularly or intracellularly or both?

Materials and Methods

SPC mixture, thapsigargin, IP₃, and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). *D-erythro*-SPC and *L-threo*-SPC were from Matreya (Pleasant Gap, PA). For extracellular administration, SPC mixture and thapsigargin (10^{-2} M and 10^{-3} M, respectively, stock solutions) were dissolved in dimethyl sulfoxide (DMSO). *D-erythro*-SPC and *L-threo*-SPC were dissolved in methanol. Before the experiments, methanol was removed under vacuum conditions, and the lipids were redissolved in DMSO to a 10^{-2} M stock solution (Betto et al., 1997). For other series of experiments, *D-erythro*-SPC or *L-threo*-SPC/methanol aliquots were dried under a low-pressure concentrator and dissolved in 1 mg/ml fatty acid-free bovine serum albumin (Meyer zu Heringdorf et al., 1996). For intracellular delivery, SPC mixture, *D-erythro*-SPC and *L-threo*-SPC were dissolved in methanol (10^{-2} M stock solution). In another set of experiments, *D-erythro*-SPC/BSA or *L-threo*-SPC/BSA complex was entrapped in liposomes.

For intracellular administration, reverse-phase evaporation vesicles (REV liposomes) (Szoka and Papahadjopoulos, 1978) were prepared from 60 mg/ml egg yolk phosphatidylcholine. Drugs were entrapped into liposomes as described previously (Brailoiu et al., 2002). Liposome batches were dialyzed (Sigma dialysis sacs) against control Ringer's solution [1/600 (v/v), 150 min] to remove nonincorporated agent, and the Ringer's solution was changed every 30 min. Liposome suspensions were administered by continuous perfusion (1.5 ml/min) after 1/20 (v/v) dilution in control Ringer's solution.

Frogs (*Rana pipiens*) were decapitated and rapidly double-pithed, and sciatic-sartorius nerve-muscle preparations were isolated. Every effort was made to use the minimum number of animals required for valid statistical analyses. Procedures were reviewed and approved by the University Committee for Animal Care. Muscles were mounted in a 3-ml Sylgard-lined Petri dish bath that was continuously perfused with Ringer's solution using a dual-chambered roller pump. The Ringer's solution contained 110 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 2.0 mM Tris, pH 7.2, and 5.6 mM glucose. Ca²⁺-free Ringer's solution contained 108 mM NaCl, 2.5 mM KCl, 1.8 mM MgCl₂, 2.5 mM EGTA, 2.0 mM Tris, pH 7.2, and 5.6 mM glucose.

MEPPs were recorded using conventional microelectrode (3 M KCl, 5–15 M Ω) techniques similar to those described previously (Brailoiu and Miyamoto, 2000). Selection of recordings was made from impalements that showed large MEPP size (>0.3 mV), good signal-to-noise ratio (baseline peak-to-peak noise <0.1 mV), and high and stable muscle resting membrane potential (>-80 mV, with <3 mV decline during the control period). Resting potentials ranged between -80 and -90 mV in different fibers. Data from muscle fibers that showed more than 10% drop in the resting membrane potential during an experiment were not used. Experiments were conducted at the ambient room temperature (21–22°C), and only one trial was carried out on each muscle. Preparations were equilibrated for at least 30 min before use. Signals were fed into a high-impedance

preamplifier (A-M Systems, Carlsberg, WA) and viewed on a R5103N oscilloscope (Tektronix, Beaverton, OR). Signal-to-noise ratio was increased with a band-pass filter (1 kHz) and boosted for interfacing with a data acquisition unit with 1 MHz digitization frequency (RC Electronics, Goleta, CA). MEPPs were recorded with a modified videocassette recorder (AM Vetter, Rebersburg, PA) for off-line analysis.

MEPP frequency represents the number of miniature endplate potentials per 60 s. MEPP amplitudes (100 samples for each time

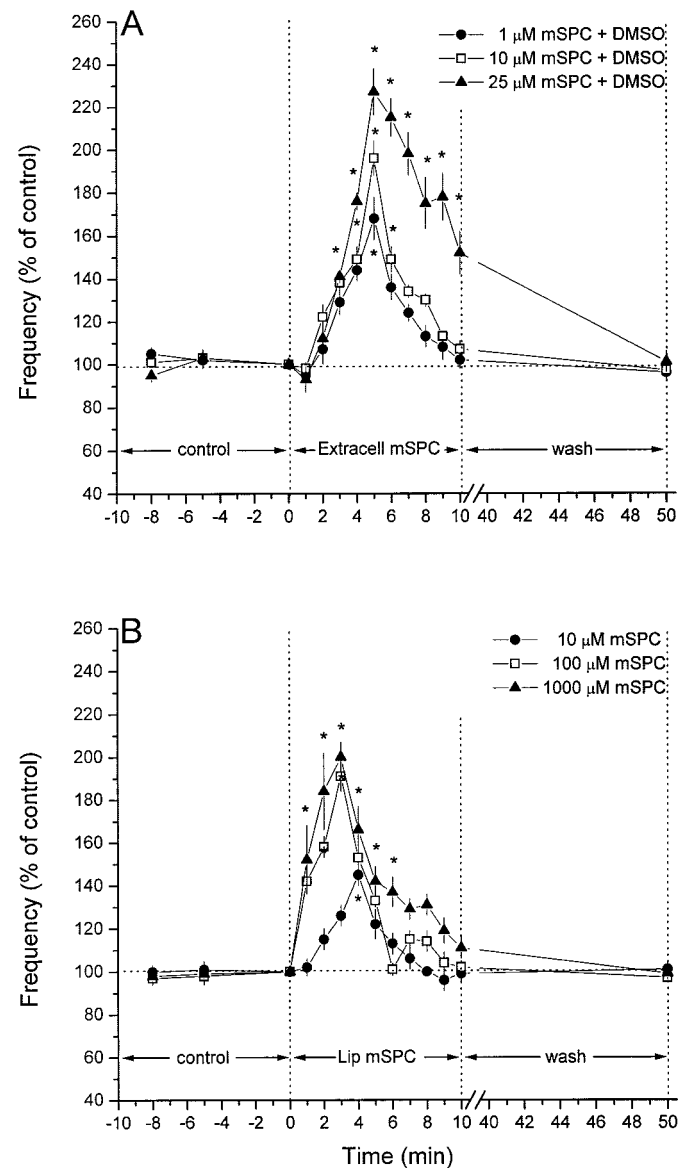


Fig. 1. Concentration-dependent increase of MEPP frequency by extracellular and intracellular administration of SPC mixture to the frog nerve-muscle preparations. **A**, percentage changes in MEPP frequency as a function of time. Effects of 1 (●), 10 (□), and 25 μM (▲) mixtures of SPC administered extracellularly. The peak effect occurs 5 min after SPC application. MEPP frequency 100% = 1.14 s^{-1} (1 μM SPC), 0.87 s^{-1} (10 μM SPC), and 1.26 s^{-1} (25 μM SPC). **B**, plots of effects of liposomes containing 10 (●), 100 (□), and 1000 μM (▲) mixture SPC on MEPP frequency are superimposed for comparison. The peak effect occurs at 3 min for 100 and 1000 μM SPC-filled liposomes, and 4 min for 10 μM. Note that the maximal effects seem faster than extracellular SPC administration (3 versus 5 min). MEPP frequency 100% = 1.34 s^{-1} (10 μM SPC), 1.22 s^{-1} (100 μM SPC), and 0.97 s^{-1} (1000 μM SPC). Each point represents the mean \pm S.E.M. from 6 experiments. *, $P < 0.05$, statistically significant different from control.

point) were measured using stored digitized data and a grid template on a flat screen monitor. To minimize the effects of junction-to-junction variation, data for each experiment were expressed as percentage of values at time 0, and results from six single experiments were averaged (plots show mean \pm S.E.M.). Analysis of statistical differences was made by comparing each point with points obtained in control Ringer's solution, with $p < 0.05$ indicating significant differences (Student's t test followed by analysis of variance and Bonferroni's test). Relative amplitude-frequency histograms were compared using the Kolmogorov-Smirnov test for significant difference ($p < 0.05$).

Occasionally, MEPPs of much larger amplitude, referred to herein as giant MEPPs, were recorded. Giant MEPPs are spontaneous potentials with amplitudes of more than twice that of the regular MEPPs and with a slower, smoother rising phase (Brailoiu et al., 2002).

Results

Extracellular Administration of SPC Mixture. Local application of 1, 10, or 25 μM SPC mixture induced a significant and concentration-dependent increase in MEPP frequency of 68, 96, and 127%, respectively (Fig. 1A), with no significant changes in the muscle resting potential and MEPP amplitude. The effect was relatively short-lasting, reaching its peak 5 min after application, and returned to baseline in the ensuing 5 min (Fig. 1A). After application of 25 μM SPC mixture, MEPP frequency returned to the control level 40 min after wash (Fig. 1A).

Discrimination between D-erythro-SPC and L-threo-SPC. Local administration of 10 μM D-erythro-SPC dissolved in DMSO (0.1% final concentration in the bath) induced an increase in MEPP frequency of 143% (Fig. 2A), with a peak effect at 5 to 6 min. This effect was stronger and more sustained than that observed with 10 μM SPC mixture. There was no significant change in median amplitude or frequency-amplitude distribution (data not shown). On the

other hand, administration of 10 μM D-erythro-SPC/BSA complex had no significant effect on the frequency (Fig. 2B) and amplitude of MEPPs or the muscle resting potential. At the concentration as high as 50 μM , D-erythro-SPC/BSA complex had no significant effect on the MEPP frequency or amplitude ($n = 3$; data not shown).

Local application of 10 μM L-threo-SPC dissolved in DMSO or L-threo-SPC/BSA complex had no effects on the MEPP frequency (Fig. 2, C and D), amplitude, or the muscle resting potential.

Intracellular Administration of SPC Mixture. Perfusion with liposomes containing 10, 100, or 1000 μM SPC mixture in aqueous phase induced a concentration-dependent increase in MEPP frequency of 45, 91, and 100%, respectively (Fig. 1B). The final concentration within the nerve terminal was estimated to be 100-fold lower than that in the liposome vesicle for molecules of the size of SPC (Brailoiu et al., 2001). The enhancing effect was phasic, with a peak after 3 min of perfusion. There was no significant change in MEPP amplitude or the muscle resting potential. The effect induced by SPC-filled liposomes had a faster onset compared with that obtained by extracellular SPC application (3 min versus 5 min).

Discrimination between D-erythro-SPC and L-threo-SPC. Perfusion with liposomes containing 1 mg/ml BSA dissolved in 140 mM KCl significantly decreased the MEPP frequency by 20% (Fig. 3, B and D). Administration of liposomes containing 100 μM D-erythro-SPC increased the MEPP frequency by 117% (Fig. 3A), the effects were more sustained than that of the SPC mixture. Again, there was no significant change in the median MEPP amplitude. However, the relative frequency-histogram indicates an increase in the number of giant MEPPs (data not shown). In contrast with extracellular application of D-erythro-SPC/BSA complex, perfusion of liposomes containing 100 μM D-erythro-SPC/

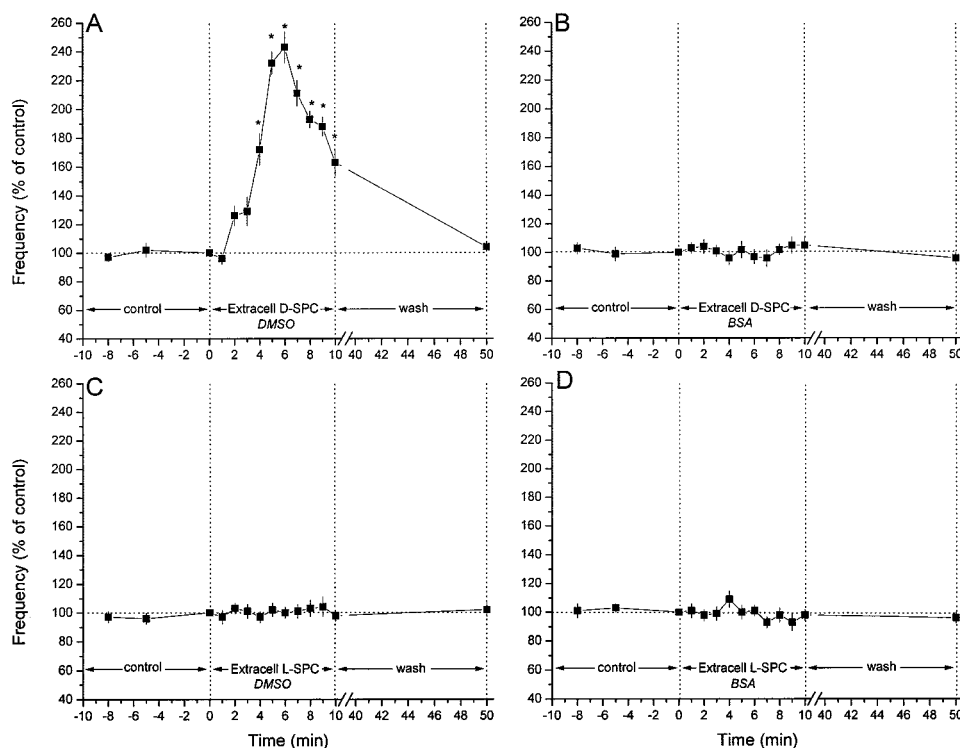


Fig. 2. Effects of extracellular administration of D- or L-SPC on MEPP frequency at the frog neuromuscular junction. A, effect of extracellular administration of 10 μM D-erythro-SPC dissolved in DMSO. The maximum effect occurs after 6 min and is significantly higher than that induced by 10 μM SPC mixture (143 versus 94%). MEPP frequency 100% = 0.92 s^{-1} . B, plot of effects of extracellular application of 10 μM D-erythro-SPC/BSA complex reveals no change in MEPP frequency. MEPP frequency 100% = 0.82 s^{-1} . C, extracellular administration of 10 μM L-threo-SPC dissolved in DMSO induced no statistically significant increase in MEPP frequency, indicating that the extracellular SPC binding site is stereospecific. MEPP frequency 100% = 1.47 s^{-1} . D, extracellular administration of 10 μM L-threo-SPC/BSA induced no statistically significant increase in MEPP frequency. MEPP frequency 100% = 1.26 s^{-1} . For all series, each point represents the mean \pm S.E.M. from six experiments. *, $P < 0.05$, statistically significant different from control.

BSA transiently enhanced the MEPP frequency by 91% (Fig. 3B); the MEPP amplitude or muscle resting potential was not changed by D-erythro-SPC/BSA.

Perfusion with 100 μM L-threo-SPC or L-threo-SPC/BSA entrapped into liposomes enhanced the MEPP frequency by 67 and 61% (Fig. 3, C and D). As shown above, these compounds when applied extracellularly had no significant effects on MEPP frequency.

Extra- and Intracellular D-erythro-SPC Induced Effect Partly Insensitive to Thapsigargin. In this series of experiments, we used thapsigargin to produce a functional deletion of SER in the nerve terminals. Thapsigargin inhibits Ca^{2+} -ATPase in SER (Takemura et al., 1989), thereby abolishing Ca^{2+} release in response to IP_3 or cADPR (Berridge, 1993; Lee, 2001). Muscles were incubated in Ca^{2+} -free Ringer's solution plus 2.5 mM EGTA and 1 μM thapsigargin for 30 min, followed by restoration of muscles to normal Ca^{2+} -containing Ringer's solution and 1 μM thapsigargin for another 30 min.

After thapsigargin treatment, extracellular administration of 10 μM D-erythro-SPC dissolved in DMSO enhanced the transmitter release by only 74%, which is significantly

smaller compared with the effect on nontreated preparations (Fig. 4A). Perfusion of liposomes containing 100 μM D-erythro-SPC on preparations pretreated with thapsigargin still induced a phasic increase in MEPP frequency of 76% (Fig. 4B). This effect was smaller, but statistically significant, compared with that observed on untreated muscles.

Effects of Liposomes Containing D-erythro-SPC in Ca^{2+} -Free and after Depletion of Intracellular Ca^{2+} Stores. After depletion of thapsigargin-sensitive Ca^{2+} stores (procedure described above), muscles were perfused 30 min with Ca^{2+} -free Ringer's solution, which totally abolished neurotransmitter release. Administration of liposomes containing 100 μM D-erythro-SPC had no effect ($n = 3$; data not shown).

Interferences between IP_3 and D-erythro-SPC. Administration of 100 μM IP_3 -filled liposomes induced an increase in MEPP frequency of 105% (Fig. 5), without affecting the muscle resting potential. Perfusion of liposomes containing both 100 μM IP_3 and D-erythro-SPC enhanced the MEP frequency by only 70% (Fig. 5), a value that is smaller, but statistically significant, compared with that caused by IP_3 or D-erythro-SPC alone. This increase was followed by a de-

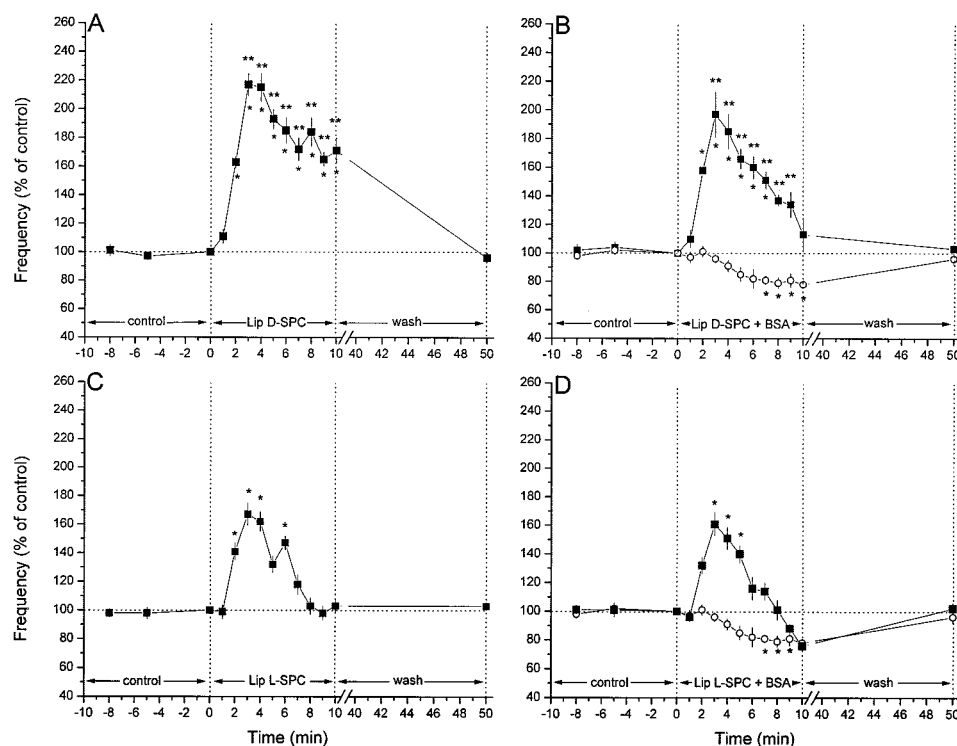


Fig. 3. Effects of liposomal delivery of D- or L-SPC on MEPP frequency at the frog neuromuscular junction. A, perfusion of liposomes containing 100 μM D-erythro-SPC induced a statistically significant increase in MEPP frequency. The peak effect occurs faster than extracellular D-erythro-SPC (3 versus 6 min), and the magnitude is not significantly different from that induced by 100 μM mixture SPC entrapped liposomes (117 versus 91%). MEPP frequency 100% = 0.77 s^{-1} ; $n = 6$. **, $P < 0.05$, statistically significant difference compared with the effect induced by perfusion of liposomes containing 100 μM L-threo-SPC. B, plots of effects of liposomes containing 100 μM D-erythro-SPC/BSA complex (■) versus 1 mg/ml BSA entrapped liposomes (○). In contrast with extracellular application, intracellular delivery of D-erythro-SPC/BSA complex enhances the MEPP frequency. The maximal effect (97% increase) appears after 3 min of perfusion. Furthermore, the vehicle control (liposomes containing BSA) induced a decrease in MEPP frequency. MEPP frequency 100% = 1.12 s^{-1} for liposomes containing 100 μM D-erythro-SPC/BSA and 1.44 s^{-1} for liposomes containing only BSA. C, perfusion of liposomes containing 100 μM L-threo-SPC increased MEPP frequency by 67%, which is statistically significant. Similar to D-erythro-SPC filled liposomes, the maximal effect occurs after 3 min of perfusion. However, note that the effect is significantly smaller than that induced by D-erythro-SPC (67 versus 117%) and that the MEPP frequency returns to control level before wash (e.g., at time = 8 min); $n = 6$. D, plots of effects of liposomes containing 100 μM L-threo-SPC/BSA complex (■) versus 1 mg/ml BSA entrapped liposomes (○). Intracellular delivery of L-threo-SPC/BSA complex enhances the MEPP frequency by 61%. Note that the MEPP frequency returns to control after 8 min and drops 20% below the control level at 10 min (same level induced by liposomes containing BSA). MEPP frequency 100% = 0.96 s^{-1} for liposomes containing 10^{-4} M L-threo-SPC/BSA and 1.44 s^{-1} for liposomes containing only BSA; $n = 6$. For all series, each point represents the mean \pm S.E.M. *, $P < 0.05$, statistically significant different from control.

crease (30%) below the control level in MEPP frequency after 10 min of liposome perfusion ($n = 6$).

Discussion

The major observation of this study is that extra- or intracellular application of SPC enhances spontaneous transmitter release from frog motor nerve-muscle junctions. Extracel-

lular application of SPC mixture caused a concentration-dependent increase in spontaneous transmitter release, as assessed by MEPP frequency, without affecting the median MEPP amplitude. This suggests the existence of plasmalemmal SPC receptors and/or diffusion of this lipid into the nerve terminal. When DMSO was used as a vehicle, discrimination between the D- and L-isoforms reveals that only D-erythro-SPC, the naturally occurring form, is active in enhancing transmitter release. Similar to the SPC mixture, the median amplitude as well as the frequency-amplitude distribution is not changed by D-erythro-SPC. This stereo-selective response was also observed in HEK-293 cells (Meyer zu Heringdorf et al., 1998). Contrary to HEK-293 cells, in which D-erythro-SPC/BSA is active (Meyer zu Heringdorf et al., 1998), the D-erythro-SPC/BSA complex is found to have no significant effect on MEPP frequency at the frog nerve-muscle junction. One possible explanation is that the D-erythro-SPC/BSA complex is too large to diffuse into the intact synaptic cleft. This is in contrast with cell suspension, where the molecule is likely to be interacting directly with receptors exposed from the surface of plasmalemmal membrane. This suggests that D-erythro-SPC might act via plasmalemmal receptors and/or by diffusing into cytoplasm. Because both the D- and L-isoforms act intracellularly, it is possible that extracellularly applied D-erythro-SPC may diffuse into cytoplasm and act on intracellular receptors. Because a stereospecific transporter for lipids has yet to be documented, our observation that the D- but not the L-isoform is effective when applied extracellularly argues against the idea that SPC, irrespective of the isoform, acts only intracellularly. As

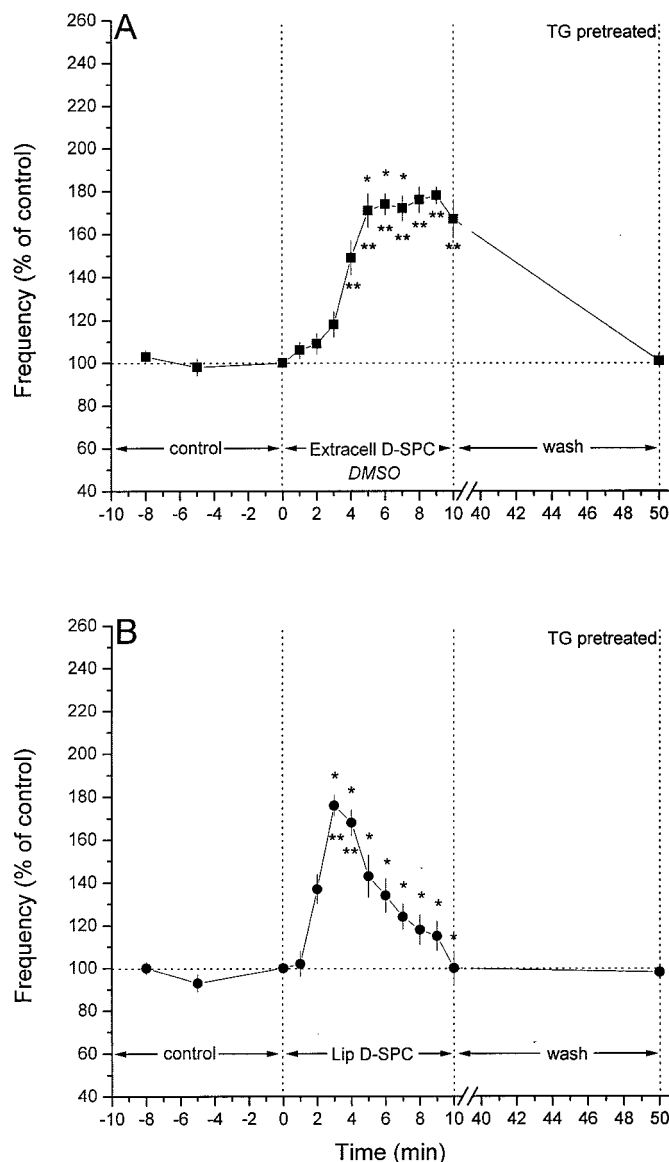


Fig. 4. Effects of extracellular administration of D-erythro-SPC or liposomal delivery of D-erythro-SPC on MEPP frequency in nerve-muscle preparations pretreated with thapsigargin. A, effect of extracellular administration of 10 μM D-erythro-SPC dissolved in DMSO on preparations pretreated with thapsigargin (TG). MEPP frequency 100% = 1.72 s^{-1} . Each point represents the mean \pm S.E.M. from 6 experiments. *, $P < 0.05$, statistically significant difference compared with the effect of 10 μM D-erythro-SPC/DMSO on non-thapsigargin-treated preparations (see Fig. 2A). **, $P < 0.05$, statistically significant different from control. B, plots of the effects of liposomes containing 100 μM D-erythro-SPC on MEPP frequency in frog nerve-muscle preparations treated with thapsigargin. MEPP frequency 100% = 1.83 s^{-1} . Each point represents the mean \pm S.E.M. from six experiments. *, $P < 0.05$, statistically significant difference compared with the effect of 100 μM D-erythro-SPC-filled liposomes on non-thapsigargin-treated preparations (see Fig. 3A). **, $P < 0.05$, statistically significant different from control.

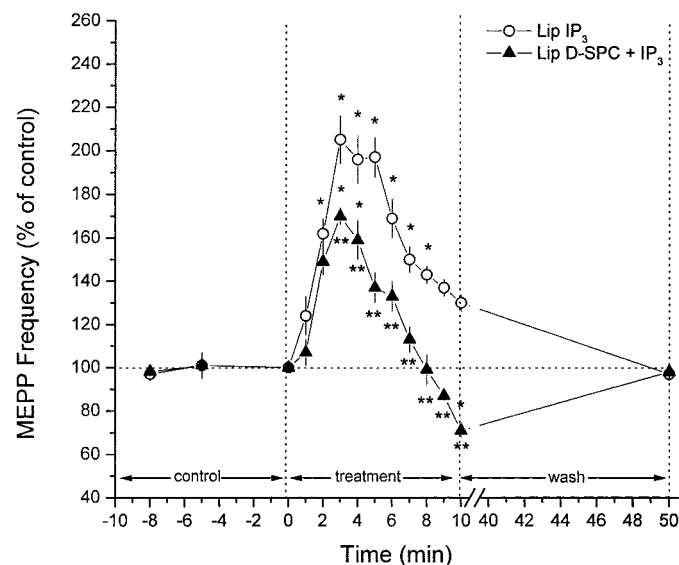


Fig. 5. Effect of liposomes containing 100 μM IP_3 (○) and 100 μM D-erythro-SPC plus 100 μM IP_3 (▲) on MEPP frequency. IP_3 increases the MEPP frequency to about the same degree as D-erythro-SPC (compare with Fig. 3A) (no statistically significant differences between these two treatments). However, when administered together, the MEPP frequency increase is significantly smaller compared with D-erythro-SPC or IP_3 alone. Moreover, the MEPP frequency drops below the control level in preparations treated with liposomes containing D-erythro-SPC and IP_3 ($n = 6$) MEPP frequency 100% = 1.16 s^{-1} for 100 μM IP_3 -filled liposomes and 1.33 s^{-1} for D-erythro-SPC + IP_3 (both 100 μM) entrapped liposomes. *, $P < 0.05$, statistically significant different from control; **, $P < 0.05$, statistically significant difference between the effects induced by liposomes containing both D-erythro-SPC + IP_3 versus D-erythro-SPC- or IP_3 -filled liposomes.

a corollary, *D-erythro*-SPC probably acts via a stereospecific plasmalemmal receptor situated at the presynaptic site. Similar to other models (for review, see Meyer zu Heringdorf et al., 2002), activation of these receptors induces a response partly by mobilizing Ca^{2+} from thapsigargin-sensitive Ca^{2+} pool.

SPC acts intracellularly to release Ca^{2+} from internal stores in different types of permeabilized cell (Ghosh et al., 1990, 1994; Yule et al., 1993; Kindman et al., 1994), via a nonstereospecific receptor (Meyer zu Heringdorf et al., 1998). SPC is also able to elicit a rapid Ca^{2+} release from cerebral and cerebellar microsomes (Dettbarn et al., 1995; Furuya et al., 1996; Huang and Chueh, 1996). In the frog nerve-muscle preparations, perfusion of liposomes containing the SPC mixture induced a concentration-dependent enhancement in MEPP frequency, without affecting the median amplitude. The onset of MEPP frequency increase seems to be faster (3 versus 5 min) in the case of intracellular application compared with that caused by extracellular application. Similar to HEK-293 cells (Meyer zu Heringdorf et al., 1998), both *D-erythro*-SPC and *L-threo*-SPC administered intracellularly enhance the spontaneous transmitter release. In contrast to HEK-293 cells, in which these compounds elicit a release of Ca^{2+} with the same magnitude and time course (Meyer zu Heringdorf et al., 1998), *D-erythro*-SPC is more potent than *L-threo*-SPC in our preparations, where comparison was made with one single concentration. It should also be noted that both *D-erythro*-SPC/BSA and *L-threo*-SPC/BSA complex administered intracellularly are active in enhancing the MEPP frequency, suggesting that SPC is coupled to albumin. This is in contrast to extracellular application, in which only the *D-erythro*-SPC is active. The presence of internal Ca^{2+} stores in frog motor nerve terminals (Pezzati et al., 2001), together with the ability of SPC to release Ca^{2+} from brain microsomes (Dettbarn et al., 1995; Furuya et al., 1996; Huang and Chueh, 1996) suggest that SPC enhances transmitter release by mobilizing Ca^{2+} from internal stores.

Unlike other models, in which where SPC was ineffective in eliciting an effect after depletion of intracellular Ca^{2+} stores (Yule et al., 1993; Meyer zu Heringdorf et al., 1998), the effect of SPC is only partly inhibited by thapsigargin pretreatment. Our observation that administration of IP_3 or cADPR induced no facilitatory effect on MEPP frequency at frog motor nerve terminals after thapsigargin pretreatment (Brailoiu et al., 2001) indicates that SPC may act on thapsigargin-insensitive Ca^{2+} stores represented in nerve terminals by synaptic vesicles (Brailoiu et al., 2001) or cause Ca^{2+} sensitization (Shirao et al., 2002). It is less likely for SPC to release Ca^{2+} from mitochondrial stores because among several lipid metabolites, only arachidonic acid in lower concentrations has been shown to be effective (Huang and Chueh, 1996). Moreover, SPC at high concentration (100 μM) damages the mitochondrial functions (Strasberg and Callahan, 1988), resulting in a decrease of MEPP frequency. Because the intracellular SPC-induced effect is abolished in the absence of extra- and intracellular Ca^{2+} , the possibility that the thapsigargin-insensitive effect is caused by a heretofore-unknown Ca^{2+} sensitive intracellular pathway activated by SPC cannot be excluded.

Future studies are needed to determine whether or not SPC acts on 'sphingolipid Ca^{2+} -release-mediating protein of the endoplasmic reticulum' (SCAMPER), which is proposed to

be an endoplasmic reticulum Ca^{2+} channel (Mao et al., 1996). Alternatively, there is some evidence that SPC may bind to SCAMPER and function as a modulatory protein that opens the ryanodine Ca^{2+} channel (Betto et al., 1997; Schnurbus et al., 2002) in a manner similar to that proposed for cADPR. In the later case, cADPR binds to FK-506 binding- or 100-kDa protein and activates ryanodine receptors (for review, see Lee, 2001).

The observation that the increase in MEPP frequency occurs rapidly, starting in the first minute after liposome perfusion, suggests that SPC may activate the 'recycling vesicular pool' rather than the 'storage' pool. Although the median amplitude of MEPPs is not changed by SPC, a frequency-amplitude histogram indicates an increase in giant MEPPs, similar to that observed in the presence of S1-P (Brailoiu et al., 2002). This suggests that sphingomyelin breakdown may be one of the intracellular pathways generating giant MEPPs. More importantly, the finding that an increase in the number of giant MEPPs is associated with intracellular application of SPC or S1-P (Brailoiu et al., 2002), but not extracellular application of SPC, provides additional evidence that extracellular SPC activates a different intracellular pathway(s) than sphingomyelin breakdown.

In other cells, the maximal Ca^{2+} release induced by SPC was in the same range as that caused by IP_3 (Yule et al., 1993; Meyer zu Heringdorf et al., 1998, 2002). Similar effects were observed in our model. Unexpectedly, the concomitant administration of SPC and IP_3 resulted in a significantly smaller increase in MEPP frequency than these two compounds alone. This may be explained by the alteration of the complex temporal and spatial interaction in Ca^{2+} signaling (Petersen and Cancela, 1999), which plays a critical role in exocytosis described by some investigators (Hirose et al., 1999). Concomitant application of IP_3 and SPC may inhibit Ca^{2+} -induced Ca^{2+} release mechanism, leading to an attenuated release of transmitters.

The estimated and expected concentration of S1-P and SPC in the blood is reported to be 250 nM and 15 μM , respectively, with an approximate ratio of 2:1 (Liliom et al., 2001). Our observation that extracellular SPC in micromolar concentrations activates motoneuron secretion raises the possibility that circulating SPC may function as a physiological regulator of acetylcholine release at the neuromuscular junction.

In conclusion, our study shows that extra- as well as intracellular SPC can enhance spontaneous transmitter release at the frog neuromuscular junction, partly by activating internal Ca^{2+} stores. The extracellular effect is stereospecific, in that only the *D-erythro*-SPC is active, whereas both isoforms are active intracellularly.

References

- Berridge MJ (1993) Inositol trisphosphate and calcium signalling. *Nature (Lond)* **361**:315–325.
- Betto R, Teresi A, Turcato F, Salvati G, Sabbadini RA, Krown K, Glembocki CC, Kindman LA, Dettbarn C, Pereon Y, et al. (1997) Sphingosylphosphocholine modulates the ryanodine receptor/calcium-release channel of cardiac sarcoplasmic reticulum membranes. *Biochem J* **322**:327–333.
- Brailoiu E, Cooper RL, and Dun NJ (2002) Sphingosine 1-Phosphate enhances spontaneous transmitter release at the frog neuromuscular junction. *Br J Pharmacol* **136**:1093–1097.
- Brailoiu E and Miyamoto MD (2000) Inositol phosphates and cyclic adenosine diphosphate-ribose increase quantal transmitter release at frog nerve terminals: possible involvement of smooth endoplasmic reticulum. *Neurosci* **95**:927–931.
- Brailoiu E, Miyamoto MD, and Dun NJ (2001) Nicotinic acid adenine dinucleotide phosphate enhances quantal neurosecretion at frog neuromuscular junction: possible action on synaptic vesicles in the releasable pool. *Mol Pharmacol* **60**:718–724.

- Breckenridge WC, Morgan IG, Zanetta JP, and Vincendon G (1973) Adult rat brain synaptic vesicles. II. Lipid composition. *Biochim Biophys Acta* **320**:681–686.
- Calupca MA, Prior C, Merriam LA, Hendricks GM, and Parsons RL (2001) Presynaptic function is altered in snake K^+ -depolarized motor nerve terminals containing compromised mitochondria. *J Physiol (Lond)* **532**:217–227.
- Chameau P, van de Vrede Y, Fossier P, and Baux G (2001) Ryanodine-, IP₃- and NAADP-dependent calcium stores control acetylcholine release. *Pflueg Arch Eur J Physiol* **443**:289–296.
- Cotman C, Blank ML, Moehl A, and Snyder F (1969) Lipid composition of synaptic plasma membranes isolated from rat brain by zonal centrifugation. *Biochemistry* **8**:4606–4612.
- Dettbarn C, Betto R, Salviati G, Sabbadini R, and Palade P (1995) Involvement of ryanodine receptors in sphingosylphosphorylcholine-induced calcium release from brain microsomes. *Brain Res* **669**:79–85.
- Deutsch JW and Kelly RB (1981) Lipids of synaptic vesicles: relevance to the mechanism of membrane fusion. *Biochemistry* **20**:378–385.
- Dixon D and Atwood HL (1989) Conjoint action of phosphatidylinositol and adenylate cyclase systems in serotonin-induced facilitation at the crayfish neuromuscular junction. *J Neurophysiol* **62**:1251–1259.
- Furuya S, Kurono S, and Hirabayashi Y (1996) Lysosphingomyelin-elicited Ca^{2+} mobilization from rat brain microsomes. *J Lipid Mediat Cell Signal* **14**:303–311.
- Ghosh TK, Bian J, and Gill DL (1990) Intracellular calcium release mediated by sphingosine derivatives generated in cells. *Science (Wash DC)* **248**:1653–1656.
- Ghosh TK, Bian J, and Gill DL (1994) Sphingosine 1-phosphate generated in the endoplasmic reticulum membrane activates release of stored calcium. *J Biol Chem* **269**:22628–22635.
- Hirose K, Kadowaki S, Tanabe M, Takeshima H, and Iino M (1999) Spatiotemporal dynamics of inositol 1, 4, 5-trisphosphate that underlies complex Ca^{2+} mobilization patterns. *Science (Wash DC)* **284**:1527–1530.
- Huang WC and Chueh SH (1996) Calcium mobilization from the intracellular mitochondrial and nonmitochondrial stores of the rat cerebellum. *Brain Res* **718**:151–158.
- Irie F and Hirabayashi Y (1999) Ceramide prevents motoneuronal cell death through inhibition of oxidative signal. *Neurosci Res* **35**:135–144.
- Kindman LA, Kim S, McDonald TV, and Gardner P (1994) Characterization of a novel intracellular sphingolipid-gated Ca^{2+} -permeable channel from rat basophilic leukemia cells. *J Biol Chem* **269**:13088–13091.
- Kluk MJ and Hla T (2002) Signaling of sphingosine-1-phosphate via the SIP/EDG-family of G-protein-coupled receptors. *Biochim Biophys Acta* **1582**:72–80.
- Le Stunff H, Peterson C, Liu H, Milstien S, and Spiegel S (2002) Sphingosine-1-phosphate and lipid phosphohydrolases. *Biochim Biophys Acta* **1582**:8–17.
- Lee HC (2001) Physiological functions of cyclic ADP-ribose and NAADP as calcium messengers. *Annu Rev Pharmacol Toxicol* **41**:317–345.
- Lilom G, Sun G, Bunemann M, Virag T, Nusser N, Baker DL, Wang DA, Fabian MJ, Brandts B, Bender K, et al. (2001) Sphingosylphosphocholine is a naturally occurring lipid mediator in blood plasma: a possible role in regulating cardiac function via sphingolipid receptors. *Biochem J* **355**:189–197.
- Mao C, Kim SH, Almenoff JS, Rudner XL, Kearney DM, and Kindman LA (1996) Molecular cloning and characterization of ScaMPER, a sphingolipid Ca^{2+} release-mediating protein from endoplasmic reticulum. *Proc Natl Acad Sci USA* **93**:1993–1996.
- Meyer zu Heringdorf D, Himmel HM, Jakobs KH (2002) Sphingosylphosphorylcholine-biological functions and mechanisms of action. *Biochim Biophys Acta* **1582**:178–189.
- Meyer zu Heringdorf D, Niederdraing N, Neumann E, Frode R, Lass H, Van Koppen CJ and Jakobs KH (1998) Discrimination between plasma membrane and intracellular target sites of sphingosylphosphorylcholine. *Eur J Pharmacol* **354**:113–122.
- Meyer zu Heringdorf D, van Koppen CJ and Jakobs KH (1997) Molecular diversity of sphingolipid signalling. *FEBS Lett* **410**:34–38.
- Meyer zu Heringdorf D, van Koppen CJ, Windorfer B, Himmel HM and Jakobs KH (1996) Calcium signalling by G protein-coupled sphingolipid receptors in bovine aortic endothelial cells. *Naunyn-Schmiedeberg's Arch Pharmacol* **354**:397–403.
- Petersen OH and Cancela JM (1999) New Ca^{2+} -releasing messengers: are they important in the nervous system? *Trends Neurosci* **22**:488–495.
- Pezzati R, Meldolesi J, and Grohovaz F (2001) Ultra rapid calcium events in electrically stimulated frog nerve terminals. *Biochem Biophys Res Commun* **285**:724–727.
- Pyne S and Pyne NJ (2002) Sphingosine 1-phosphate signalling and termination at lipid phosphate receptors. *Biochim Biophys Acta* **1582**:121–131.
- Schnurbus R, de Pietri Tonelli D, Grohovaz F, and Zacchetti D (2002) Re-evaluation of primary structure, topology and localization of Scamper, a putative intracellular Ca^{2+} channel activated by sphingosylphosphocholine. *Biochem J* **362**:183–189.
- Shirao S, Kashiwagi S, Sato M, Miwa S, Nakao F, Kurokawa T, Todoroki-Ikeda N, Mogami K, Mizukami Y, Kuriyama S, et al. (2002) Sphingosylphosphorylcholine is a novel messenger for Rho-kinase-mediated Ca^{2+} sensitization in the bovine cerebral artery: unimportant role for protein kinase C. *Circ Res* **91**:112–119.
- Strasberg PM and Callahan JW (1988) Lysosphingolipids and mitochondrial function. II. Deleterious effects of sphingosylphosphorylcholine. *Biochem Cell Biol* **66**:1322–1332.
- Szoka F Jr and Papahadjopoulos D (1978) Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc Natl Acad Sci USA* **75**:4194–4198.
- Takemura H, Hughes AR, Thastrup O and Putney JW Jr (1989) Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. Evidence that an intracellular calcium pool and not inositol phosphate regulates calcium fluxes at the plasma membrane. *J Biol Chem* **264**:12266–12271.
- Vale MG (1980) Molecular arrangement of phosphatidylcholine and sphingomyelin in sarcoplasmic reticulum membranes. *Arch Biochem Biophys* **204**:148–152.
- Xu Y (2002) Sphingosylphosphorylcholine and lysophosphatidylcholine: G protein-coupled receptors and receptor-mediated signal transduction. *Biochim Biophys Acta* **1582**:81–88.
- Yule DI, Wu D, Essington TE, Shayman JA, and Williams JA (1993) Sphingosine metabolism induces Ca^{2+} oscillations in rat pancreatic acinar cells. *J Biol Chem* **268**:12353–12358.

Address correspondence to: Nae J. Dun, Department of Pharmacology, James H. Quillen College of Medicine, East Tennessee State University, PO Box 70577, Johnson City, TN 37614-1708. E-mail: dunnae@etsu.edu