N-Arachidonoyl L-Serine, a Putative Endocannabinoid, Alters the Activation of N-Type Ca\textsuperscript{2+} Channels in Sympathetic Neurons

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**METHODS**

**Cell isolation and electrophysiology**

Superior cervical ganglion (SCG) neurons from male Wistar rats (150–300 g) were enzymatically dissociated and placed in short-term (<24 h) culture as described previously (Ikeda 2004). For some experiments, the neurons were incubated overnight in tissue culture media containing 500 ng/ml Bordetella pertussis toxin (PTX; List Biological Laboratories, Campbell, CA) as previously described (Guo and Ikeda 2004). Rats were killed by decapitation after anesthesia with CO2 as approved by the Institutional Animal Care and Use Committee. Neurons were voltage-clamped using the whole cell patch-clamp technique as described previously (Guo and Ikeda 2004). \( \text{I}_{\text{Ca}^-} \) tail currents were filtered at 10 kHz prior to digitization at 50 kHz. Series resistance was electronically compensated \( \approx 80\% \). Experiments were carried out at room temperature (22–26°C).

**Solutions and chemicals**

The external recording solution contained (in mM) 140 methanesulphonic acid, 145 tetraethylammonium hydroxide, 10 HEPES, 10 glucose, and 10 CaCl\textsubscript{2} and 0.0003 tetrodotoxin (Alomone Labs, Jerusalem, Israel), pH 7.4 with TEA-OH. For tail current experiments, the CaCl\textsubscript{2} was reduced to 5 mM. The pipette solution contained (in mM) 120 N-methyl-D-glucamine, 20 tetraethylammonium hydroxide, 11 EGTA, 10 HEPES, 10 sucrose, 10 HCl, 1 CaCl\textsubscript{2}, 4 MgATP, 0.3 Na\textsubscript{2}GTP, and 14 Tris creatine phosphate, pH 7.2 with methanesulphonic acid. The osmolalities of the bath and pipette solutions were adjusted with sucrose to 325 and 300 mOsmol/kg, respectively. N-arachidonoyl-L-alanine (ARA-A), N-arachidonoyl-L-glycine (ARA-G), N-arachidonoyl-L-Serine, and N-arachidonoyl-dopamine (ARA-DA) were purchased from Cayman Chemical (Ann Arbor, MI) as ethanol stock solutions (25–140 mM) and dissolved directly into the recording solution on the day of the experiment. Ethanol was <0.1% in all solutions and at this concentration produced no discernable effect on \( \text{I}_{\text{Ca}^-} \). Drugs were applied by positioning the outlet tube (200 \( \mu \text{m} \) ID) of a custom-designed gravity-fed microperfusion system \( \sim 100 \mu \text{m} \) from the cell body.

**Data analysis and statistics**

Nonlinear least-squares curve fitting was performed using a Marquardt-Levenberg algorithm from Igor Pro version 6.02A (WaveMetrics, Lake Oswego, OR). Statistical comparisons, as indicated in the text, were determined with Prism 4 version 4.0c (GraphPad Software, San Diego, CA). \( P < 0.05 \) was considered significant. Summary data are presented as means \( \pm \) SE.

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RESULTS

We tested the effect of ARA-A, ARA-G (Huang et al. 2001), ARA-S, and ARA-DA (Huang et al. 2002) application on Ca\(^{2+}\) currents (\(I_{Ca}\)) recorded from dissociated adult rat sympathetic neurons using whole cell patch clamp. Under these recording conditions, \(I_{Ca}\) arises primarily from \(\omega\)-conotoxin GVIA-sensitive N-type Ca\(^{2+}\) channels (Ikeda 1991). Currents were evoked with a 25-ms test pulse to −10 mV from a holding potential of −80 mV. Application of ARA-A (10 \(\mu\)M) or ARA-S (10 \(\mu\)M) produced a rapid and reversible enhancement of \(I_{Ca}\) amplitude (Fig. 1A) without overt modification of \(I_{Ca}\) kinetics (Fig. 1B). Conversely, application of ARA-DA (10 \(\mu\)M) to the same neuron produced minimal effects. A second series of sequential drug applications produced similar effects indicating that the minuscule ARA-DA response did not result from tachyphylaxis. ARA-G (10 \(\mu\)M) was tested in a separate set of experiments and produced results similar to those observed following ARA-S application (Fig. 1C). Comparison of the average change in \(I_{Ca}\) amplitude (Fig. 1C) revealed that ARA-S produced the largest increase (132 ± 14%, \(n = 14\)) and thus further experiments focused on this compound.

The effect of ARA-S at different potentials was examined by evoking \(I_{Ca}\) with 70-ms test pulses over the range −60 to +80 mV from a holding potential of −80 mV in the presence or absence of 10 \(\mu\)M ARA-S (Fig. 1D). Under control conditions, \(I_{Ca}\) became apparent around −30 mV, reached a maximum amplitude near +10 mV, and thereafter declined asymptotically toward baseline. In the presence of ARA-S, \(I_{Ca}\) was augmented several-fold at negative test potentials with lesser increases at more depolarized potentials. At very depolarized

![Figure 1](http://jn.physiology.org/)

**Figure 1.** Voltage-dependent enhancement of Ca\(^{2+}\) currents (\(I_{Ca}\)) by N-arachidonoyl t-serine and related compounds. Data are from whole cell patch-clamp recordings of rat sympathetic neurons obtained at room temperature (22–26°C). A: time course of \(I_{Ca}\) amplitude during exposure to 10 \(\mu\)M of N-arachidonoyl t-alanine (ARA-A, ■), N-arachidonoyl t-serine (ARA-S, □), or N-arachidonoyl dopamine (ARA-DA, ▪). B: \(I_{Ca}\) traces for the time course shown in A were evoked with 25-ms test pulses to +10 mV from a holding potential of −80 mV. Superimposed traces in the absence or presence of drug as indicated. C: mean ± SE increase in \(I_{Ca}\) amplitude produced by application of ARA-A, ARA-S, N-arachidonoyl l-glycine (ARA-G), and ARA-DA. With the exception of ARA-S vs. ARA-G, all means differ significantly (\(P < 0.001\)) from each other as determined from 1-way ANOVA followed by Newman-Keuls post hoc test. D: effect of ARA-S (10 \(\mu\)M) on \(I_{Ca}\) at different test potentials. \(I_{Ca}\) was evoked from a holding potential of −80 mV with 70-ms pulses to the indicated potential in the absence or presence (●) of ARA-S. E: current-voltage (I-V) curves were obtained in the absence (○) or presence of ARA-S (10 \(\mu\)M, ●) and then normalized to the \(I_{Ca}\) amplitude at +10 mV in the absence of ARA-S. The data represent the means ± SE for 10 neurons. F: concentration-response curve for ARA-S. Three concentrations of ARA-S were applied to each neuron (0.1, 1.0, and 10 or 0.3, 3.0, and 30 \(\mu\)M ARA-S). \(I_{Ca}\) was allowed to recover between applications.
test potentials (> +40 mV), $I_{Ca}$ amplitude was minimally affected, suggesting that a shift in Ca$^{2+}$ channel activation (Fig. 1E) rather than an increase in the number of available channels was responsible for the action of ARA-S. A concentration-response curve for ARA-S is illustrated in Fig. 1F. Three concentrations of ARA-S (0.1, 1.0, and 10 or 0.3, 3.0, and 30 µM) were applied to neurons allowing time to recover between each concentration. Potentiation of $I_{Ca}$ amplitude was evident starting at 3 µM and increased monotonically without evidence of saturation ≤30 µM, the highest concentration examined.

To investigate shifts in channel activation, Ca$^{2+}$ channel tail current amplitudes were determined in the absence or presence of ARA-S (10 µM). Tail currents result from the deactivation of channels on return to a hyperpolarized potential following a sojourn at a step potential that produces channel activation (Fig. 2A). Because tail currents are measured at a constant potential (here −40 mV), driving force, which is nonlinear for large ionic gradients such as Ca$^{2+}$, remains constant and thus $I_{Ca}$ amplitude can be equated with conductance. To facilitate accurate tail current measurement, analog filter bandwidth (−3 dB) and digital sampling rate were increased to 10 and 50 kHz, respectively. External [Ca$^{2+}$] was decreased to 5 mM, which served to decrease tail current amplitude and hence the effects of residual uncompensated series resistance. Under these conditions, tail current decays were well fit (following a 100-μs delay to allow uncompensated capacitive transients to settle) by a single-exponential function (Fig. 2B, —) with $\tau$ of ~0.5 ms. Activation curves were plotted as tail current amplitude, normalized to maximum amplitude (e.g., +80-mV step potential) in the absence of drug, versus step potential (Fig. 2C) and fit (—) with a two-component modified Boltzmann equation

$$I_{tail} = \frac{I_1}{1 + \exp[(V_{n} - V_{step})/k_1]} + \frac{I_2}{1 + \exp[(V_{n} - V_{step})/k_2]}$$

where $I_{tail}$ is the normalized tail current amplitude and $V_{step}$ is the step potential. $I_n$, $V_{n}$, and $k_n$ are the fractional amplitude, half activation potential, and slope factor, respectively for each component. Under control conditions, Ca$^{2+}$ channel activation curves from adult rat SCG neurons are composed of two components—a result of tonic G protein modulation (Ikeda 1991). In the presence of ARA-S, the activation was shifted toward more hyperpolarized potentials, retained a two-component profile, and achieved a similar maximum amplitude.

![Figure 2](http://jn.physiology.org/10.1152/jn.00689.2008/)

**Fig. 2.** ARA-S shifts $I_{Ca}$ activation curves toward hyperpolarized potentials. A: tail current voltage protocol (bottom) and representative traces, recorded at a step potential of −10 mV, obtained in the absence or presence of ARA-S (10 µM). External [Ca$^{2+}$] was decreased to 5 mM to reduce current amplitude thereby reducing series resistance error. B: tail current decay illustrated on an expanded time scale (same traces as in A). The initial 100 ms following the step pulse have been blanked. —, nonlinear least-square fits to single-exponential function. C: activation curves composed of mean ± SE (n = 5) normalized tail current amplitudes vs. step potential. Tail current amplitudes were determined from the exponential fit of the tail current decay phase. Amplitudes obtained in the absence (○) or presence (●) of ARA-S were normalized to the maximum amplitude in the absence of drug. —, the best fit of a 2-component Boltzmann equation (see text) to the data using nonlinear least-squares regression. D: voltage dependence of ARA-S effects illustrated by plotting the ratio of $I_{Ca}$ amplitude in the presence of ARA-S to the control condition for step (○) and tail currents (●) vs. step potential. E: table of mean ± SE Boltzmann equation parameters. A paired $t$-test was used for statistical comparison between the control and drug condition. ***, $P < 0.001$; *, $P < 0.05$. J Neurophysiol • VOL 100 • AUGUST 2008 • www.jn.org
Analysis of the activation parameters (Fig. 2E) revealed significant decreases in $V_{h1}$, $V_{h2}$, and $k_3$ but no change in the fractional contribution of each component. The hyperpolarizing shift in the activation curve account for the voltage-dependent $I_{Ca}$ increases as shown in Fig. 2D. Both the tail and step $I_{Ca}$ were affected to a much greater extent by ARA-S at hyperpolarized voltages. The coincidence of the step and tail $I_{Ca}$ data in Fig. 2D provide evidence for the fidelity of the tail current recordings.

Next, we asked whether ARA-S affects $I_{Ca}$ modulation by G-protein-coupled receptors. In SCG neurons, norepinephrine (NE) acts by binding to $\alpha_2$-adrenergic receptors (Schofield 1990) thereby liberating $G\beta\gamma$ from the heterotrimeric G protein complex resulting in voltage-dependent modulation (Bean 1989; Ikeda 1996). This form of $I_{Ca}$ modulation is detected using a double-pulse voltage protocol (Elmslie et al. 1990) (Fig. 3B) in which two identical test pulses to $+10$ mV are separated by a depolarizing conditioning pulse to $+80$ mV. Facilitation, defined as the ratio of postpulse to prepulse $I_{Ca}$ amplitude, provides a convenient index of $G\beta\gamma$-mediated modulation (Fig. 3A). Increases in facilitation result from relief of $G\beta\gamma$ inhibition produced by the conditioning pulse. Application of NE produced $I_{Ca}$ inhibition with a coincidental increase in facilitation that was similar in the presence of ARA-S. The mean inhibition of $I_{Ca}$, basal (i.e., in the absence of agonist) facilitation, and NE-induced facilitation were significantly different in the presence of ARA-S (Fig. 3C). However, the magnitude of the changes were small and possibly arose from effects of ARA-S on minor components of $I_{Ca}$ that do not arise from N-type Ca$^{2+}$ channels. Thus ARA-S does not appear to greatly influence either receptor-mediated or tonic modulation of N-type Ca$^{2+}$ channels by $G\beta\gamma$. The effects of ARA-S (10 mM) on neurons treated overnight with 500 ng/ml PTX were also examined. $I_{Ca}$ potentiation following ARA-S application was not significantly altered by pretreatment with PTX ($210 \pm 4$, $n = 3$ vs. $290 \pm 31\%$, $n = 9$ for control and PTX-treated, respectively). Conversely, NE-mediated $I_{Ca}$ inhibition was decreased ($13 \pm 6$, $n = 3$ vs. $52 \pm 3\%$, $n = 9$, for PTX and control, respectively) following PTX treatment providing evidence for toxin effectiveness. Thus activation of PTX-sensitive G proteins, namely $G_{i/o}$-containing heterotrimers, were not essential for $I_{Ca}$ increases resulting from ARA-S application.

**DISCUSSION**

Our data show that ARA-S produces a rapid and reversible augmentation of $I_{Ca}$ in sympathetic neurons that is voltage dependent and results from a hyperpolarizing shift in the activation curve. At depolarized potentials, the same maximal conductance is attained in the presence of ARA-S arguing against a recruitment of covert channels or a change in the maximum probability of opening. ARA-S did not interfere with or contribute to G protein modulation of N-type Ca$^{2+}$ channels. We thus conclude that $I_{Ca}$ enhancement by ARA-S occurs independently of G-protein-coupled receptors and possibly results from direct interaction with the CaV2.2 channel or alteration of plasma membrane surface potential.

There are two main implications of these findings. First, the ARA-S concentration (10 mM) used here falls well within the concentration range used to probe potential physiological roles of ARA-S and related endocannabinoids (Milman et al. 2006). Given the well-established role played by N-type Ca$^{2+}$ channels in providing Ca$^{2+}$ for synaptic transmission combined with the nonlinear relationship between [Ca$^{2+}$] and transmitter release (Xu et al. 2007), one can easily envision changes in synaptic transmission produced by applying ARA-S. Thus receptor-independent effects, such as the one demonstrated here, will need to be considered before meaningful interpretations of ARA-S action are entertained. Although agents such as Bordetella pertussis toxin are useful for separating receptor-dependent (at least in terms of GPCRs using $G_{i/o}$-containing heterotrimers) from receptor-independent effects, this ability...
degrades as the complexity of the system increases (e.g., in vivo experiments). Second, it is possible that direct actions of ARA-S on ion channels underlie physiological processes. For example, a related compound, anandamide, is proposed to influence physiological function by binding to CB1, CB2, and subsequently activating downstream signaling cascades or directly activating TRPV channels without the aid of signaling intermediates (Smart et al. 2000; van der Stelt and Di Marzo 2005; van der Stelt et al. 2005). Although endocannabinoids and related lipid compounds (Bradshaw and Walker 2005) often have direct effects on ion channel function (Oz 2006), the effect of ARA-S on N-type Ca2+ channels is somewhat unique. Closely related compounds such as anandamide, 2-AG (Guo and Ikeda 2004) and ARA-DA (Fig. 1C) either have no effect or produced inhibition at similar concentrations, whereas both ARA-A and ARA-G were capable of augmenting ICa to varying degrees (Fig. 1C). From this series of compounds, the presence of a carboxylic acid group was common to the substances that enhanced ICa amplitude. We could not find literature values for the pKz of ARA-S or related lipoamino acids thus the charge status of the carboxylic acid group at physiological pH is unclear. Given the pKz of the carboxylic acid group in the free amino acids (2.1–2.4), it seems likely that ARA-S, ARA-G, and ARA-A are negatively charged at pH 7.4. Thus a possible explanation for the effects of ARA-S and related lipoamino acids is alteration of the membrane surface potential (Hille 2001) following incorporation of negative charges into the outer leaflet of the plasma membrane. The net result would be a negative shift in channel activation as seen following ARA-S application. It should be noted, however, that enhancement of L-type ICa in ventricular myocytes by long chain fatty acids occurred without shifting activation or inactivation along the voltage axis (Huang et al. 1992) thus arguing against this explanation as a universal mechanism for Ca2+ channel modulation by negatively charged lipophilic agents.

The actions of ARA-S are somewhat reminiscent of the effects of arachidonic acid on N-type Ca2+ channels in SCG neurons (Liu and Rittenhouse 2003; Liu et al. 2001). Although ARA-S, a conjugate of arachidonic acid and serine, likely breaks down to arachidonic acid, ARA-S lacks a Ca2+ channel inhibitory component characteristic of arachidonic acid effects. Therefore, it is unlikely that arachidonic acid mediates the effects of ARA-S although we cannot conclusively rule out this possibility. The stimulatory effects of ARA-S on N-type Ca2+ channels are strikingly similar to those observed for the fatty acid analogs palmitoyl coenzyme A and arachidonoyl coenzyme A (Barrett et al. 2001). Thus the lipid moiety in these compounds may share a common mechanism for Ca2+ channel stimulation that is conferred by the charge on the head group. At present, many details relevant to the biology of ARA-S, including synthetic and degradative pathways, partition efficiency, and sites of action have not been investigated. This void in our knowledge limits speculation as even fundamental facts, such as the relevant physiological concentration of ARA-S, remain unknown.

REFERENCES


G R A N T S

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