Somatostatin Inhibition of Hippocampal CA1 Pyramidal Neurons: Mediation by Arachidonic Acid and Its Metabolites

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We used electrophysiological methods in a slice preparation to study the mechanisms of somatostatin (SS) effects on hippocampal pyramidal neurons. SS hyperpolarizes hippocampal pyramidal neurons in part by augmenting the time- and voltage-dependent M-current (I_M), which has been shown to be reduced by muscarinic agonists. The SS effects are abolished by the phospholipase A₂ inhibitors 4-bromophenacyl bromide and quinacrine. Arachidonic acid (AA) mimics all the effects of SS on hippocampal pyramidal neurons. The effects of AA and SS on I_M are blocked by the lipoxygenase inhibitor nordihydroguaiaretic acid but not by the cyclooxygenase inhibitor indomethacin. Prostaglandins E₂, F_{2α}, and I₂ do not increase I_M . However, the specific 5-lipoxygenase inhibitors 5,6-methanoleukotriene A₄ methyl ester and 5,6-dehydroarachidonic acid both blocked the I_M -augmenting action of either SS or AA. Leukotriene C₄ (but not leukotriene B₄) increases I_M to the same extent as AA. I_M was not altered by the 12-lipoxygenase product 12-hydroperoxyeicosatetraenoic acid, and SS effects were not altered by the 12-lipoxygenase inhibitor baicalein. These data implicate 5-lipoxygenase metabolite(s) (probably leukotriene C₄) as a mediator for the I_M -augmenting effect of SS. In addition, when the I_M effect is blocked by lipoxygenase inhibitors, both SS and AA elicit another outward current that is not blocked by either lipoxygenase or cyclooxygenase inhibitors, suggesting a direct role of AA itself distinct from the I_M effect. SS did not alter significantly Ca²⁺-dependent action potentials or, in whole-cell recordings, inward currents likely to represent high-threshold Ca²⁺ currents. The combined results of these studies suggest that SS hyperpolarizes hippocampal neurons by two mechanisms, both mediated through the AA system. However, one mechanism (I_M) involves a metabolite of AA and is most effective at slightly depolarized potentials, whereas the other may involve AA itself and be more effective at membrane potentials near rest.

[Key words: hippocampus, somatostatin, arachidonic acid, phospholipase A₂, M-current, lipoxygenase, leukotrienes, voltage clamp]

Somatostatin (SS; also SS-14, SRIF) is reported to be the second or third most abundant neuropeptide in most mammalian brain regions (Crawley, 1985). In hippocampus, immunohistochemical studies have revealed many SS-containing interneurons and a profuse network of intrinsic and extrinsic SS-containing fibers that appear to project to pyramidal and granule neurons (see, e.g., Bakst et al., 1986; Joëls et al., 1990). In addition, behavioral and clinical findings suggest an important role for SS in cognitive functions (Epelbaum, 1986). In spite of the apparent importance of SS in brain function, little is known of the exact physiological mechanisms of SS action. Results of early electrophysiological studies seemed to support a role for SS as an excitatory transmitter (Dodd and Kelly, 1978). However, more recent *in vivo* and *in vitro* studies in our laboratory (Pittman and Siggins, 1981; Mancillas et al., 1986; Moore et al., 1988; Schweitzer et al., 1990) and elsewhere (Watson and Pittman, 1988) support the view that SS is predominantly an inhibitory transmitter in the hippocampus. The inhibition of spike discharge is accompanied by a hyperpolarization associated with an increase in conductance to K⁺ ions, at least in part via augmentation of the M-current (I_M) (Moore et al., 1988; Watson and Pittman, 1988; Schweitzer et al., 1990). SS also hyperpolarizes neurons in other brain areas by K⁺ mechanisms (Inoue et al., 1988; Jacquin et al., 1988; Twery and Gallagher, 1989; Wang et al., 1989). Whereas SS-28, thought to be the preprohormone for SS-14, can act like SS-14 in some brain regions (Siggins et al., 1982; Jacquin et al., 1988; Moore et al., 1988; Watson and Pittman, 1988), in others SS-28 elicits responses different from, or even opposite to (Wang et al., 1989), those evoked by SS-14.

The I_M is a time- and voltage-dependent K⁺ current that persists at slightly depolarized membrane potentials and is thought to clamp the membrane at potentials near rest (Brown and Adams, 1980; Adams et al., 1982). I_M is reduced by muscarinic cholinergic agonists, certain peptides (e.g., leutinizing hormone–releasing hormone, substance P, bradykinin; for review, see Brown, 1988), 5-HT, and the excitatory amino acid quisqualate (Charpak et al., 1990), and by this mechanism is responsible in part for the slow and late-slow EPSPs in sympathetic neurons (Adams and Brown, 1982). Hippocampal pyramidal neurons also exhibit an I_M , which may be responsible, at least in part, for the slow muscarinic EPSP evoked in hippocampal neurons by short stimulus trains (Halliwell and Adams, 1982; but see Madison et al., 1987). Recently, we reported that I_M in hippocampal neurons *in vitro* was not only reduced by muscarinic agonists but markedly augmented by SS, suggesting that two different receptors can reciprocally regulate one neuronal channel type (Moore et al., 1988). A similar reciprocal

Received June 9, 1992; revised Nov. 9, 1992; accepted Nov. 17, 1992.

We thank Nancy Callahan for typing the manuscript and Drs. F. E. Bloom and L. Chavez-Noriega for providing valuable criticism. This work was supported by grants from NIMH (MH 47680), NIAAA (AA 06420), and NATO.

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Figure 1. Schematic of AA metabolism. Phospholipid degradation by PLA_2 leads to AA formation. AA can then be transformed through different enzymatic pathways to an array of metabolites, such as PGs (prostaglandins), 5-HPETE and 12-HPETE (5- and 12-hydroperoxy-eicosatetraenoic acid), and LTs (leukotrienes A_4 , B_4 , C_4 , D_4 , and E_4). The different enzymes involved are PLA_2 (phospholipase A_2), COX (cyclooxygenase), and 5-LOX and 12-LOX (5- and 12-lipoxygenase). (The cytochrome P-450 system is not shown for clarity.) The inhibitors used are 4BPB (4-bromophenacyl bromide), Quin (quinacrine), NDGA (nordihydroguaiaretic acid), Indo (indomethacin), Bai (baicalein), MLTA (5,6-methanoleukotriene A_4 methylester), and DHAA (5,6-dehydroarachidonic acid) (for details on the drugs used and their specificity, see Blackwell and Flower, 1983; Higgs and Vane, 1983; Piomelli and Greengard, 1990).

I_M regulation, by ACh and isoproterenol, has been reported for smooth muscle cells (Sims et al., 1988).

The slow time course of SS-induced inhibitions and its ability to modulate GTP-binding proteins (North, 1989; Law et al., 1991) and the levels of second messengers (Schonbrunn, 1990) suggest involvement of some specific transduction systems in hippocampal SS effects. In sympathetic neurons, muscarinic reduction of I_M may be mediated through G-protein activation of phospholipase C (PLC), with subsequent generation of diacylglycerols and activation of protein kinase C (Brown et al., 1989). By contrast, in hippocampal CA1 pyramidal neurons muscarinic inactivation of I_M may not require protein kinase C activation (Malenka et al., 1986), but rather the inositol triphosphate side of the PLC cycle (Dutar and Nicoll, 1988). Another candidate for transduction is the cAMP system. Sims et al. (1988) recently showed that the augmentation of I_M by isoproterenol involves cAMP mediation in smooth muscle cells, and SS is known to modulate adenylate cyclase activity in brain (Chneiweiss et al., 1985; Wang et al., 1989).

The phospholipase A_2 (PLA_2) pathway (Fig. 1) is another source of mediators implicated in neuronal signal transduction (Piomelli et al., 1987a). This enzyme catalyzes the release of arachidonic acid (AA), yielding several eicosanoids via different metabolic pathways. Two major pathways for AA metabolism are (1) via cyclooxygenase (COX) to form prostanoids, and (2) via lipoxygenases (LOXs) to form hydroperoxyeicosatetraenoic acids (Needleman et al., 1986). Some 12-lipoxygenase (12-LOX)

products mediate or modulate the effects of the peptides FMRFamide and histamine on K^+ channels in *Aplysia* neurons (Piomelli et al., 1987a,b), and hyperpolarize hippocampal pyramidal neurons (Carlen et al., 1989; Pace-Asciak et al., 1990). Metabolites of the 5-lipoxygenase (5-LOX) pathway alter spontaneous extracellular firing rate in rat cerebellar Purkinje cells *in vivo* (Palmer et al., 1981) and are involved in the opening of a G-protein-gated muscarinic K^+ channel ($I_{K,ACh}$) in atrial cells (for reviews, see Piomelli and Greengard, 1990; Shimizu and Wolfe, 1990). AA itself and certain fatty acids also directly modulate K^+ and other conductances in peripheral tissues (reviewed by Ordway et al., 1991).

We recently reported data suggesting that an AA metabolite, perhaps a leukotriene, mediates the I_M -augmenting effects of SS in hippocampal pyramidal cells (Schweitzer et al., 1990). In the present studies, we report new data suggesting another hyperpolarizing action of SS possibly mediated by AA itself, and we confirm the involvement of 5-LOX metabolites in I_M augmentation. In addition, we now find little evidence for the effects of SS on other K^+ conductances or on large inward currents in rat hippocampal pyramidal neurons.

Materials and Methods

Slice preparation. We used standard intracellular and whole-cell patch-clamp recording techniques in the rat hippocampal slice, prepared as described previously (Pittman and Siggins, 1981; Moore et al., 1990). In brief, transverse hippocampal slices (taken from male Sprague-Dawley rats of 100–170 gm body weight) 350–400 μ m thick were cut on a brain slicer and incubated in gassed (95% O_2 , 5% CO_2) artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl, 130; KCl, 3.5; NaH_2PO_4 , 1.25; $MgSO_4 \cdot 7H_2O$, 1.5; $CaCl_2$, 2.0; $NaHCO_3$, 24; glucose, 10. Other ions and agents were added to this ACSF medium. After a short incubation period in an interface configuration, the slices were completely submerged and continuously superfused with warm (30–31°C) ACSF at a constant rate within the range of 2.0–4.0 ml/min. However, in five of nine cells studied with the leukotrienes, the flow rate was decreased to about 1–1.5 ml/min to ensure drug penetration into the slice, because their costliness dictated the use of small volumes. In this case, the slower flow rate was held constant throughout the recording period. The voltage-clamp studies were done at 30–31°C to slow down I_M kinetics; however, in a previous study, SS superfusion at 35–37°C elicited the same augmenting effect on I_M as at lower temperatures (Moore et al., 1988). In most studies, we used sharp glass micropipettes filled with KCl (3 M; tip resistances, 50–80 M Ω) to penetrate CA1 pyramidal neurons. Methods of superfusion, voltage-clamp recording, cell identification, drug administration, and data analysis were as described previously (Pittman and Siggins, 1981; Moore et al., 1988, 1990; Schweitzer et al., 1990).

Electrophysiological methods. Current- and voltage-clamp studies were performed with an Axoclamp preamplifier. In voltage-clamp mode, tetrodotoxin (0.5–1 μ M) was added to block synaptic transmission and Na^+ -dependent action potentials. In discontinuous single-electrode voltage-clamp mode, the switching frequency between current injection and voltage sampling was 3–4 kHz for intracellular recording. Electrode “settling time” and input capacitance neutralization at the headstage were monitored continuously on an oscilloscope (Finkel and Redman, 1985). Current and voltage records were filtered at 0.3 kHz and usually acquired by D/A sampling and acquisition software (pCLAMP, Axon Instruments). For I_M studies, we most often applied -5 , -10 , -15 , and -20 mV voltage commands in sequence three to five times for subsequent averaging. Current measures thus were derived from averages of three to five sweeps at each of the four voltage commands.

The various problems (e.g., space clamping) associated with voltage clamping of neurons with extended processes are discussed elsewhere (Halliwell and Adams, 1982; Johnston and Brown, 1983; Finkel and Redman, 1985). These problems may be less acute when dealing with relative changes following drug application (see Madison et al., 1987). Notwithstanding, we repeated some studies with SS in the presence of various pharmacological agents, to minimize the contribution of large non- K^+ conductances (e.g., Na^+ and Ca^{2+}) in remote dendrites (see

below). In some cells, we used whole-cell recording in a "blind" patch-slice configuration (Blanton et al., 1989) with the membrane potential held throughout at -60 to -70 mV except for short command steps.

M-current analysis. In hippocampal CA1 pyramidal neurons, I_M is best seen with holding potentials of -40 to -45 mV and hyperpolarizing command steps of 5 – 25 mV and durations of 700 – 1000 msec (Halliwell and Adams, 1982). Here I_M appears as a slow inward current "relaxation" following the instantaneous (ohmic) inward current drop (Fig. 2A). As the instantaneous current jump at command onset is larger than that at command offset, the relaxation represents the inactivation of an outward (K^+) current rather than the activation of an inward current. I_M amplitude was measured with software (CLAMPFIT, Axon Instruments) that fitted exponential curves (one exponent, r values = 0.95 – 0.99) to the I_M relaxation. We used the difference between the instantaneous peak current at command onset and the steady-state current just before command offset to quantify the magnitude of I_M (see Fig. 2). Tail (off-command) currents were not analyzed because of possible contamination with other currents. Usually, a current (capacitive) artifact of 5 – 20 msec duration was present at command onset (see also Halliwell and Adams, 1982). Therefore, for estimation of the instantaneous portion of I_M with hyperpolarizing commands, the current relaxation was fitted via two methods: (1) curve fitting to the peak of the initial (quasi-instantaneous) current (Fig. 2Aa) that usually fell within 5 – 20 msec of step onset after complete settling of the capacity transient, or (2) exponential fitting through the peak with extrapolation to command onset (Fig. 2Ab), based on the finite duration of the initial capacitive artifact and the exponential (monotonic) nature of the I_M relaxation. These methods gave essentially equivalent results with respect to relative effects of drugs, although the extrapolated I_M amplitude was obviously bigger (10 – 25% ; average 20%) than amplitude at peak (Fig. 2B). For statistical purposes the first method was used.

Q-current analysis. The Q-current (I_Q) is an anomalous or inward rectifier current carried by Na^+ and K^+ and seen in hippocampal pyramidal neurons (Halliwell and Adams, 1982). Like I_M , it produces a slow inward current relaxation with hyperpolarizing voltage steps, although only from hyperpolarized holding potentials (more negative than -65 mV) and via activation of an inward current. The amplitude of the relaxation increases as a function of the command hyperpolarization. I_Q and other forms of anomalous rectification were pharmacologically distinguished from I_M by their differential sensitivity to extracellular cesium (2 mM; to block I_Q) or the muscarinic agonist carbachol (CCh; 25 μ M; to block I_M).

Large inward conductances and whole-cell patch clamp. In studies designed to minimize the possibility that the current relaxations measured were artifacts of large, non- K^+ conductance changes (e.g., remotely in the dendrites), we injected a local anesthetic, Na^+ channel blocker QX-314 (25 – 100 mM in the recording pipette), while superfusing TTX (0.5 – 1 μ M) and $CdCl_2$ (50 – 300 μ M) to block Na^+ and Ca^{2+} currents. We verified the action of these agents in current-clamp mode by following the loss of current-evoked Na^+ - and Ca^{2+} -dependent action potentials.

To assess the effect of SS on Ca^{2+} currents in CA1 pyramidal neurons, we used whole-cell voltage clamp ("blind patch slice"; see Blanton et al., 1989) in continuous mode. The whole-cell patch pipette usually contained (in mM) Cs-gluconate, 130 ; HEPES, 10 ; $MgCl_2$, 2 ; Mg -ATP, 2 ; Na_2GTP , 1 ; tetraethylammonium (TEA), 2 ; BAPTA, 0.6 ; and leupeptin, 0.1 . Pipette tip resistance was 3 – 5 M Ω . To elicit high-threshold Ca^{2+} currents in the whole-cell configuration, we held the cells at around -60 mV and applied voltage steps from -52 to $+12$ mV (usually at $+8$ mV increments). TEA at 20 mM and TTX at 0.75 – 1 μ M were added to the extracellular ACSF to block K^+ channels and sodium spikes.

Drug administration. Drugs and peptides were made from a stock solution and added to the ACSF in known concentrations immediately before administration to the slice chamber. The usual drug protocol followed for agonist testing was to record currents during superfusion of ACSF alone ("control"), followed by switching of ACSF with drug and repeating these current measures after 5 – 10 min of drug, then followed by switching again to ACSF alone for 15 – 30 min with subsequent current measures ("washout"). The cell was depolarized to about -45 mV for I_M analysis at each of these three periods, but was held near resting potential between these periods to avoid the instabilities that occasionally develop with prolonged depolarization (see Halliwell and Adams, 1982). For tests of the enzyme inhibitors, because AA and SS did not affect all neurons (roughly 70%), the usual protocol involved first applying the agonist (AA or SS, with measurements as above), and then quickly adding the inhibitor to the agonist superfusate once a

positive agonist response was established. This protocol was feasible because, once initiated, the I_M effect of SS or AA did not show desensitization over the complete testing period. In some cells, the antagonist was superfused alone to test possible effects on baseline properties. In a few other cells, the inhibitor was applied first (for 6 – 7 min), followed by addition of the agonist.

Somatostatin was obtained from Drs. J. Rivier and W. Vale at the Salk Institute and M. Buchmeier at The Scripps Research Institute. Arachidonic acid (AA; Sigma), hydroperoxy acids (Cayman Chemical), leukotrienes (Sigma), 5-LOX inhibitors (Biomol), and prostaglandins (Sigma) were stored at -80°C and used immediately after suspension; the lipids were dried under nitrogen and dissolved in the ACSF by sonication. Preparations of lipids showing precipitate or droplets after sonication (indicating lack of solution in the ACSF) were discarded. We dissolved nordihydroguaiaretic acid (NDGA; Sigma), 4-bromophenacyl bromide (4BPB; Sigma), baicalein (Biomol), and leukotrienes in 0.1% dimethyl sulfoxide (DMSO), and AA in 0.25% DMSO. DMSO alone in the superfusion medium at the concentration used during our experiments had no significant effect on seven cells. However, we observed a small decrease in I_M and a small inward current slowly developing when testing 0.5% DMSO. Quinacrine (mepacrine, Sigma) was dissolved directly in the ACSF. Indomethacin (Sigma) was dissolved in ACSF containing 16 mM ethanol, which alone did not affect I_M (see Moore et al., 1990). 8-Bromo-cAMP (8-Br-cAMP), dibutyryl-cAMP, and carbachol (CCh) were obtained from Sigma; QX-314 was a gift of Astra Pharmaceuticals.

Results

We studied a total of 145 CA1 pyramidal neurons. These neurons had an average resting membrane potential (RMP) of -66.7 ± 0.5 mV (mean $\pm$ SEM) and action potential amplitude of 102 ± 0.6 mV ($n = 87$); mean resistance was 56 M Ω ($n = 72$) at RMP. Stable recordings could be maintained for up to 4 hr, suggesting a relative lack of injury by the electrode penetration. We tested I_M using voltage clamp at holding potentials of -38 to -50 mV (mean, -42.7 ± 0.4 mV). The mean (outward) holding current reached at these depolarized potentials was 635 ± 31 pA. The I_M relaxation was voltage dependent with a maximum amplitude of 70 – 150 pA (103 ± 4 pA) in control condition, using commands of -5 to -20 mV. Several lines of evidence suggest, as reported previously (Halliwell and Adams, 1982; Moore et al., 1988), that the relaxations represent I_M : (1) the relaxations are suppressed (leaving only a "rectangular" ohmic current response) by the muscarinic agonists CCh or muscarine (10 – 40 μ M) or by 1 mM Ba^{2+} , (2) the current–voltage curves for the relaxations are shifted in a positive direction by an increase of K^+ in the medium, (3) the magnitude, kinetics, and voltage dependence of the relaxations appear equivalent to those of the pyramidal cell M-current reported previously.

SS effects on I_M and I_Q

In accord with our previous reports (Moore et al., 1988; Schweitzer et al., 1990), superfusion of SS (1 μ M) elicited in CA1 hippocampal pyramidal neurons an outward current with a concomitant increase in I_M amplitude and input conductance (Fig. 3). The current recording (Fig. 3A) exhibits a slow inward deflection (relaxation) at the onset of each hyperpolarizing step. These relaxations are due to the inactivation of the I_M for the steps positive to -70 mV, and activation of I_Q for the hyperpolarizing steps more negative than -70 mV. Superfusion of SS elicits an outward current in the depolarized range with no effect at more hyperpolarized potentials. A plot of the steady-state current versus voltage (Fig. 3B) reveals the outward rectifying nature of I_M in the -40 to -70 mV range and the inward rectifying behavior of I_Q in the -70 to -95 mV range. Measurements of the relaxation amplitudes show that SS increases

Figure 2. I_M protocol and relaxation fitting. **A**, Current recording of a CA1 pyramidal neuron clamped at -43 mV and submitted to a 5 mV hyperpolarizing step. The slow inward (downward) relaxation reflects the reduction of the noninactivating time- and voltage-dependent M-current (I_M), carried by the outward flow of K^+ . I_M relaxation amplitude is estimated from the peak of the relaxation (I_{inst} , instantaneous current) to the end of the voltage step where a steady baseline current is reached (I_{stst} , steady-state current). We estimated the I_M relaxation amplitude by two methods (see Materials and Methods): **a** shows fitting to peak (with magnification of the current trace shown in the broken circle in **A**); **b**, fitting with extrapolation back to command onset. **B**, Graph of I_M amplitude (average of 39 cells) plotted against hyperpolarizing step command (5 , 10 , 15 , and 20 mV; holding potential, -43 mV). **a**, M-current fitting to the peak of the relaxation; **b**, I_M fitting with extrapolation to command onset. Observe the similarity of shape of the peak and extrapolated graphs and the I_M increase by SS, except that I_M amplitudes are 17% larger when extrapolating the fit to command onset (note the different y-axis scales in **a** and **b**).

I_M without affecting I_Q (Fig. 3C), as seen in most neurons (although three cells showed an increase in the I_Q relaxation).

Of 53 cells tested with SS, 39 responded with an increased I_M amplitude of 55 – 60% across all voltage commands (Fig. 4A). This was associated with an outward (steady-state) current shift relative to control (Fig. 4B) at both resting (61 ± 8 pA) and depolarized potentials (221 ± 20 pA at -43 mV). These effects usually occurred within 1 – 3 min after SS reached the inner slice chamber, with peak responses occurring at 4 – 6 min. Recovery to control measures usually required about 15 min of SS washout with ACSF alone. Among the 14 neurons that did not show an I_M augmentation with SS, 8 revealed a small outward current in the presence of SS (40 – 80 pA from -70 to -40 mV holding potentials).

SS effects on depolarization-evoked inward currents

We examined the possibility that large inward conductances such as Na^+ or Ca^{2+} might be involved in the SS effects, by treating the CA1 neurons with agents that block these currents. Injection or leakage of QX-314, the local anesthetic Na^+ channel blocker (Connors and Prince, 1982), into the cells from pipettes containing high concentrations (50 – 100 mM) of the drug often appeared to lead to smaller M-current relaxations, suggesting nonspecific actions on K^+ channels. Use of pipettes containing a 25 mM concentration of the agent had no obvious effect on I_M or other K^+ conductances such as those underlying the post-burst afterhyperpolarizations (AHPs), but Na^+ -dependent spikes recorded in current-clamp mode were blocked within 10 – 15 min

Figure 3. Effect of SS on current-voltage relationships of a CA1 pyramidal neuron. **A**, Superimposed current recording of a neuron held at -40 mV and hyperpolarized, by -5 mV incrementally increasing steps, to -95 mV. RMP was -66 mV for this cell. The current relaxation seen from -45 to -70 mV is due to I_M ; note the outward steady-state current in the depolarized range (broken line, control holding current) and the I_M increase observed in the presence of SS (6 min after onset). The relaxation appearing between -75 and -95 mV is due to the anomalous (inward) rectifier or Q-current. **B**, Plots of instantaneous current (I_{inst} ; see Fig. 2A) and steady-state current (I_{stst}) before (control) and during 1μ M SS superfusion, taken from data in **A**. Note convergence of the I_{inst} and I_{stst} curves between -70 to -80 mV, indicating lack of SS effect in the hyperpolarized range. **C**, Curve of voltage dependence of relaxation amplitude (I_{inst} minus I_{stst}) measured as in Figure 2A shows I_M and I_Q amplitudes, with overlap of the curves from about -75 mV to -95 mV showing that I_Q is not affected by SS. By contrast, SS elicits separation of the curves from -70 mV into the depolarized (I_M) range.

of cell penetration. At this point, increasing the strength of depolarizing current steps evoked broad, slowly developing spikes typical of Ca^{2+} -dependent action potentials (Fig. 5A). As these spikes were always completely blocked by subsequent superfusion of 200μ M Cd^{2+} , they were likely produced primarily by Ca^{2+} currents. Subsequent superfusion of TTX (0.5 – 1μ M) had little or not effect on the shape of these spikes.

SS (1μ M) superfusion, whether with or without TTX, had little or no effect on the shape or size of the Ca^{2+} -dependent spikes (Fig. 5A) in seven of eight neurons (five recorded with pipettes containing 25 mM QX-314, two with 50 mM, one with 80 mM). Only one neuron showed a small reversible decrease in the Ca^{2+} -dependent spike. In patch-clamp studies, neurons recorded in the whole-cell configuration using pipettes containing Cs^+ and TEA to block K^+ channels, and with TEA in the superfusate, showed pronounced inward currents on depolarization from holding potentials of -60 mV with command steps from -44 to $+12$ mV. These currents are likely to be carried

by Ca^{2+} , because they are activated at around -35 mV, show current-voltage characteristics typical of the high-threshold Ca^{2+} current (see Fig. 5B), and are blocked by Cd^{2+} at 50 – 200μ M. Superfusion of SS at 1μ M had little consistent effect on the amplitude of the inward currents in four of six cells (Fig. 5B). In the two other cells an apparent small reduction of the currents by SS could not be reversed by washout with ACSF alone; rather, the inward currents continued to get smaller, suggesting a “run-down” effect probably due to the whole-cell recording configuration.

To examine further the possibility that large remote Na^+ and/or Ca^{2+} conductances contribute to the SS effects, in eight cells QX-314 (25 – 100 mM in the pipette) was injected intracellularly and TTX and Cd^{2+} (200μ M) applied extracellularly during recording of I_M relaxations. Under these pharmacological conditions, the I_M relaxations were generally smaller than in TTX alone (especially with higher QX-314 concentrations) and often slowly decreased over time (about 1 hr), probably as a result of

Figure 4. SS effects averaged across 39 CA1 pyramidal neurons. *A*, SS increases the mean ($\pm$ SEM) I_M relaxation amplitudes averaged from 39 cells, by 55–60% (from 104 to 163 pA for the maximum amplitude reached). Mean holding potential was -43 mV. *B*, Average current–voltage relationship of 35 CA1 pyramidal neurons. SS elicits a mean outward steady-state current that increases on depolarization, with recovery on washout. Error bars in this and all subsequent figures represent $\pm$ SEM values.

Figure 5. SS has little effect on calcium-dependent action potentials or high-threshold Ca^{2+} currents. *A*, Superimposed voltage traces of CA1 neuronal responses to depolarizing current injection in current-clamp mode, recorded with a sharp pipette containing QX-314 (25 mM) and with TTX $1 \mu M$ in the bathing medium. Superfusion of $1 \mu M$ SS resulted in a very slight decrease in the amplitude of the Ca^{2+} -dependent action potential that never recovered with washout. RMP was -68 mV. *B*, Different CA1 pyramidal neuron recorded in the whole-cell, voltage-clamp configuration. The patch pipette contained 130 mM Cs-gluconate and 2 mM TEA to block K^+ channels; the superfusion medium also contained TEA at 20 mM, and $1 \mu M$ TTX to block Na^+ channels. Selected inward currents resulting from two (to -12 mV and $+6$ mV; voltage protocol on lower left) of several (not shown) depolarizing steps from a -60 mV holding potential, subtracted for leak currents. SS superfusion ($1 \mu M$) had no significant effect on the inward currents. RMP = -47 mV (depolarized due to intracellular Cs^+).

nonspecific actions of QX-314 or Cd^{2+} . Nonetheless, in seven cells with a measurable I_M relaxation (25–50 mM QX-314 in the pipette), SS ($1 \mu M$) superfusion increased the I_M relaxations in four cells by 25–250% (mean: 105%). The other three cells either showed no change of I_M with SS superfusion or exhibited a slow I_M “rundown” over time.

cAMP

To examine a possible mediator role of cAMP in the SS- I_M effect, we tested 8-Br-cAMP (0.5–2.5 mM) on six cells. On average, 8-Br-cAMP evoked an early (1–2 min), small (25 pA) outward current, which reversed to a small inward current (-35 pA) after 3–4 min of superfusion. These effects were associated with decreased conductance (8–10%) and a slight (10%) reduction in I_M . One cell showed a small I_M increase (20%) associated with little outward steady-state current. Dibutyryl-cAMP (1 mM) was tested in two cells. One responded with an I_M increase (20%) and an outward steady-state current (70 pA) at depolarized potentials, with recovery on washout. The other cell did not show a measurable response.

Involvement of PLA_2 in SS effects

To test the involvement of the PLA_2 system (Fig. 1) in the SS effects, we superfused two substances that can inhibit PLA_2 (see Fig. 1 caption for drug references), 4BPB (10 μM ; seven cells) and quinacrine (8 μM ; eight cells). Both abolished all SS effects: the augmentation of I_M (Fig. 6*A*) and the outward current shift (Fig. 6*B*) returned to near control levels. To identify the current effects of these compounds, we subtracted the current–voltage relationship obtained during drug application from that obtained in the control condition. Thus, Figure 6*C* shows the net outward current elicited by SS, which is fully abolished in the presence of 4BPB. The difference between these two currents is the 4BPB-sensitive component. 4BPB, like quinacrine (data not shown), completely blocked SS-induced effects. Neither 4BPB (four cells) nor quinacrine (four cells) alone in the superfusion medium had a measurable effect on control I_M or steady-state current (data not shown).

We also tested mellitin (1–5 $\mu g/ml$), an activator of PLA_2

Figure 6. SS effects are blocked by the PLA₂ inhibitor 4BPB. *A*, Mean I_M amplitude is increased by SS, and this effect is completely abolished by application of 4BPB together with SS. *B*, Current-voltage curve showing that the SS-induced outward steady-state current is also completely reversed by concomitant superfusion of 4BPB. *C*, Graph of net SS induced current before and during 4BPB; data were obtained from *B* by subtracting the control from the SS and SS+4BPB currents. Unlike NDGA (see Fig. 11*A*), 4BPB reverses all SS effects, as indicated by the 4BPB-sensitive curve (4BPB sens., broken curve).

(Shier, 1979), on four cells. In one cell melittin increased I_M by 23% with a 10% increase in input conductance; in the three other cells, only a slight outward current could be detected (about 30 pA). In all cases, these effects were short-lived (a few minutes) and followed by an inward current without recovery that was associated with either a conductance increase (two cells), decrease (one cell) or no change (one cell).

AA mimics SS

Inhibition of SS effects by phospholipase inhibitors led us to examine the effects of AA, the initial product of the PLA₂ pathway (see Fig. 1). Figure 7*A* shows the current records of a CA1 neuron superfused with AA. In this experiment we superfused ACSF containing 2 mM CsCl to block I_Q . Like SS, AA elicited I_M augmentation and an outward steady-state current in three of three neurons bathed with 2 mM Cs⁺. With I_Q blocked, we were able to determine the I_M reversal potential (Fig. 7*B*), and estimate the M-conductance variation (Fig. 7*C*, caption). The I_M reversal potential was -80 mV for this cell, and the M-conductance increased from 3.7 nS in the control condition to 7 nS in the presence of AA.

In 33 of 45 cells, AA elicited a 55–75% increase in I_M amplitude (Fig. 8*A*), and caused a net outward current at both resting potential (66 ± 10 pA) and at depolarized potentials (233 ± 16 pA at -43 mV) (Fig. 8*B*). The time course for these AA effects (1–3 min for response onset; peak effect, 5–6 min; 12–15 min for recovery on washout) was equivalent to that for SS. To verify that the AA effect indeed involved I_M , we added CCh, a muscarinic agonist, in the presence of AA. As shown in Figure 8*C*, CCh (25 μ M) blocked I_M and the concomitant outward current produced by AA, supporting the notion that M-channels are the source of the relaxation increase, as previously demonstrated for SS (Moore et al., 1988). Of the 12 neurons in which AA did not modify I_M , six exhibited an AA-induced outward current (40–90 pA from -70 to -40 mV

holding potentials). This current amplitude was equivalent to that induced by SS in neurons in which SS did not increase I_M .

SS and AA increase M-current time constant

The time constant (τ) of the I_M relaxation increased with superfusion of either SS or AA. This augmentation reached significance ($P < 0.001$ for SS, $P < 0.002$ for AA; Student's *t* test) when averaged across 32 SS- and 28 AA-responding cells (Fig. 9). Average control τ values at -53 mV (during a 10 mV hyperpolarizing step from -43 mV holding potential) were 173 ± 12 msec (mean $\pm$ SEM) for the group subsequently tested with SS and 177 ± 13 msec for the AA group, diminishing with further hyperpolarization as previously described (Adams and Brown, 1982; Halliwell and Adams, 1982). Mean τ (at the same average membrane potential, -53 mV) increased to 219 ± 17 msec with superfusion of SS (a 20–27% increase across all four voltage steps; mean, 24%) and 218 ± 18 msec with AA (23–31% increase across the four voltage steps; mean, 27%). The average τ returned to near control levels on washout. Interestingly, all drugs blocking I_M augmentation (PLA₂ and LOX inhibitors: see sections on LOXs below) also abolished the τ increase.

COX pathway

A major route for AA metabolism is the COX pathway, leading to formation of prostaglandins and thromboxanes (Fig. 1). However, superfusion (5–8 min) of indomethacin (8–10 μ M), a COX inhibitor, did not significantly antagonize SS-induced (10 cells) or AA-induced (5 cells) I_M augmentation or outward steady-state current (data not shown). In fact, in some cells the SS (five cells) and AA effects (two cells) were clearly increased by indomethacin, including a more pronounced outward steady-state current. However, prolonged indomethacin superfusion (15 min) reduced SS effects in three cells but never reduced AA effects. Superfusion of indomethacin onto cells that did not present SS-

Figure 7. AA increases I_M in a CA1 pyramidal neuron. **A**, Current recording of a neuron held at -40 mV and hyperpolarized to -95 mV by -5 mV incrementally increasing steps. The superfusion medium contained 2 mM cesium to block I_Q (note the absence of relaxation at hyperpolarized potentials compared to Fig. 3A). Superfusion of AA ($130 \mu\text{M}$) for 7 min, like SS (see Fig. 3) elicits an outward steady-state current at the depolarized potentials, increases I_M , and augments input conductance (larger currents are evoked by equivalent voltage steps). RMP was -68 mV for this cell. **B**, Same cell: plot of I_M amplitude versus voltage obtained from records in **A**. Note the large increase of I_M evoked by AA, with no change in the reversal potential (-80 mV). **C**, Data obtained from the same cell showing the changes in the M-conductance underlying the current relaxation (closing of M-channels), calculated by dividing the amplitude of relaxation (inverted across the range of voltage steps) by its driving force (holding potential minus reversal potential). This M-conductance is roughly doubled by AA.

($n = 4$) or AA- ($n = 4$) induced I_M augmentation did not cause any measurable effect (e.g., I_M potentiation). Indomethacin alone had no effect on I_M relaxations or steady-state current (three cells).

We further investigated a possible involvement of the COX pathway by testing the prostanoids, prostaglandin E_2 (PGE_2), $\text{PGF}_{2\alpha}$, or PGI_2 . PGE_2 (20 – $60 \mu\text{M}$; three cells) had no effect on two cells and provoked a small inward current (30 pA) in one cell with an input conductance decrease of 20% but no effect on I_M . $\text{PGF}_{2\alpha}$ (2 – $3 \mu\text{M}$; two cells) superfusion also caused a small depolarization in one cell with a 15% decrease in input conductance without affecting I_M . There was no $\text{PGF}_{2\alpha}$ effect on the other cell. Both cells tested with PGI_2 responded with some inward current at depolarized potentials; one exhibited a 35% decrease in I_M . Thus, COX products did not consistently alter

the CA1 pyramidal neuron current–voltage relationships; rather, the effects observed involved a small inward current with no change in I_M .

LOX pathways

LOXs provide other routes for AA metabolism. Superfusion of NDGA ($10 \mu\text{M}$), a general inhibitor of LOXs, had no significant effect when applied alone ($n = 4$). However, it abolished both the SS-induced (7 of 8 cells) and the AA-induced (11 of 11 cells) I_M augmentation (Fig. 10A), bringing I_M amplitudes back to control level. Nonetheless, NDGA superfusion did not completely reverse the overall SS- or AA-induced net outward current (Fig. 10B): a residual outward current remained with both SS and AA at the potentials tested. To separate these different components, we subtracted the current–voltage curve during

Figure 8. AA effects averaged across 33 CA1 pyramidal neurons. *A*, AA increases mean I_M by 55–75% (from 91 to 152 pA for the maximum amplitude reached). Mean holding potential was -43 mV. *B*, AA elicits an outward steady-state current that increases with depolarization and is completely reversed after washout of the lipid. *C*, I_M relaxations, their increase by AA, and the AA induced outward steady-state current (broken lines) are all completely blocked by CCh, a muscarinic agonist. CCh also causes an additional steady-state inward current. These effects of CCh began within 1 min after the perfusate reached the slice chamber (peak effect within 2 min); recovery to control levels required 15–20 min of washout with ACSF alone (see Pontzer et al., 1992). RMP was -70 mV.

NDGA application from the control curve, as described for 4BPB. Figure 11*A* shows the net outward currents elicited by SS (left panel) or AA (right panel) alone and in the presence of NDGA (contrast to Fig. 6*C*). The difference between these two currents reveals the component blocked by NDGA, that is, the NDGA-sensitive current (broken lines in Fig. 11*A*). Figure 11*B* shows the conductance calculations relative to this NDGA-sensitive current: it activates at about -65 mV, shows voltage dependency, and exhibits a net conductance augmentation of about 3.5–4 nS around -40 mV (Fig. 11*B*); the extrapolated reversal potential is estimated to be -82 mV with SS and -83 mV with AA. Thus, addition of NDGA inhibits SS- or AA-induced I_M augmentation and much of the induced outward current (15–200 pA over the voltages tested), and reveals a residual SS- or AA-induced outward current of about 30–50 pA remaining over the same range of potentials.

We also tested NDGA on those few neurons in which superfusion of SS or AA did not increase I_M but still induced a small outward current. In these cells, NDGA did not antagonize the SS ($n = 3$) or AA ($n = 2$) effects.

12-LOX

Because 12-LOX-like immunoreactivity recently has been shown to exist in canine hippocampus (Nishiyama et al., 1992), we tested the effect of the specific 12-LOX inhibitor baicalein (4 μ M) on SS responses. Baicalein affected neither the SS-induced outward steady-state current nor the I_M increase in the five pyramidal neurons tested (Fig. 12). We also tested the primary metabolite of the 12-LOX pathway, 12-hydroperoxyeicosatetraenoic acid (12-HPETE). In five cells examined, two exhibited an outward current but without change in I_M amplitude. The three remaining cells did not exhibit any measurable effect.

Involvement of 5-LOX and leukotrienes in I_M increase

To investigate further the LOX pathways, we superfused two specific 5-LOX inhibitors, 5,6-methanoleukotriene A₄ methyl ester (MLTA; 10 μ M) and 5,6-dehydroarachidonic acid (DHAA; 10 μ M). Both substances completely blocked the I_M -augmenting effects of SS (five cells; Fig. 13*A,B*) and AA (four cells; Fig. 13*B*); however, like NDGA, they did not fully reverse the overall SS-

Figure 9. Averaged effects of SS ($n = 33$) or AA ($n = 28$) on I_M kinetics. Time constants measured from I_M relaxations induced by hyperpolarizing steps from a mean holding potential of -43 mV. I_M augmentation elicited by SS or AA is associated with a 23–30% increase in the I_M inactivation time constant. This effect was reversed on washout (not shown).

Figure 10. Action of NDGA, an LOX inhibitor, on SS and AA effects. *A*, NDGA completely reverses SS- (*left*) or AA- (*right*) induced I_M increase, implying that some further metabolite(s) of AA mediates the I_M increase. *B*, Although NDGA reduces the outward steady-state current induced by SS (*left*) or AA (*right*), it does not fully bring the current back to control level. Some residual outward current remains within the range of potentials tested (see also Fig. 12).

Figure 11. NDGA reveals more than one component in SS and AA effects. *A*, Graph of the net outward current induced by SS (left) and AA (right) before and during NDGA (data obtained from Fig. 11*B* by subtracting steady-state currents). Application of SS or AA together with NDGA leaves a residual current. The NDGA-sensitive current (NDGA sens., broken line) represents the amount of SS or AA induced outward current blocked by NDGA, and is obtained by subtracting the residual current (SS+NDGA or AA+NDGA) from the total outward current evoked by either SS or AA. *B*, Conductance calculated from the NDGA-sensitive component of SS and AA effects (NDGA sens. from *A*), with an extrapolated reversal potential of -82 and -83 mV for SS and AA, respectively. This component of SS and AA effects exhibits a conductance increase of up to 3.5 nS, is voltage dependent, and activates around -65 mV, implying equivalence to I_M .

and AA-induced outward current (SS: four of five cells, Fig. 13*C*; AA: four of four cells, data not shown), leaving a residual current of 25 – 45 pA (compare Figs. 11*A*, 13*C*).

The involvement of the 5-LOX pathway in I_M augmentation led us to test the leukotrienes. The initial product, leukotriene A_4 (LTA $_4$), can be transformed to LTB $_4$ or LTC $_4$. Superfusion of LTC $_4$ (5 – 8 μ M), even in the presence of NDGA (copperfused to prevent endogenous generation of AA metabolites), mimicked the action of SS and AA in augmenting both I_M and input conductance, and in eliciting an outward steady-state current at depolarized potentials (four of five cells; Fig. 14). LTB $_4$ (15 – 30 μ M) had no significant effect on these measures in three of four cells (data not shown), although it clearly increased I_M in one cell.

Discussion

SS hyperpolarizes central neurons in several preparations by activating a K $^+$ conductance. This K $^+$ current is outwardly rectifying in solitary tract complex (STC) (Jacquin et al., 1988), hippocampus (Schweitzer et al., 1990), and neocortex (Wang et al., 1989) and involves I_M in the STC and hippocampus but not in the neocortex. SS acts on an inward-rectifying conductance in locus coeruleus (Inoue et al., 1988) and possibly in dorso-lateral septal nucleus (Twery and Gallagher, 1989). In the present study, we have analyzed the overall effects of SS in hippocampal pyramidal neurons and have (1) quantified the SS augmentation of I_M and the outward rectification, and detected an increase in its kinetics by SS and AA; (2) performed studies that largely exclude SS actions on some other conductances; (3) uncovered another outward-current effect of SS in hippocampus that may be activated by AA itself; and (4) provided further evidence for mediation of the I_M effect by an AA metabolite, probably a leukotriene.

Selectivity of SS effects on the I_M -current

Three major points demonstrate that the I_M effect of SS does not arise from actions on I_Q : (1) in contrast to I_Q , the SS-enhanced inward I_M relaxations are larger at more depolarized potentials and show a greater ohmic step at hyperpolarizing command onset than at command offset; (2) Cs $^+$ blocks I_Q but not I_M nor the augmenting effect of SS on I_M ; (3) CCh (and Ba $^{2+}$; Moore et al., 1988) block the I_M relaxation and its augmentation by SS, but not the I_Q relaxation. Another potential site of action of SS is on an anomalous or inwardly rectifying K $^+$ current that could be distinct from I_Q . Such inward rectifying currents are increased in some central neurons by SS, but these currents are sensitive to Cs $^+$ (Inoue et al., 1988). Indeed, we now have evidence that both SS and AA can elicit an outward current with properties distinct from I_M . However, this current is rather small (30 – 50 pA), and current–voltage relationships before and during SS superfusion do not show much difference in the hyperpolarized voltage range. This contrasts with the effects of the GABA $_B$ receptor agonist baclofen, which augments inward rectification and elicits a pronounced divergence of the current–voltage curves in the hyperpolarized range (S. Madamba and G. R. Siggins, unpublished observations; see Andrade et al., 1986). SS has also been shown to increase an outward K $^+$ current in neocortical neurons in culture (Wang et al., 1989). However, this effect is insensitive to CCh, whereas the SS effect in hippocampus is blocked by this muscarinic agonist.

Other possible sites of SS action are large inward currents carried by, for example, Ca $^{2+}$ or Na $^+$, also possibly unclamped at remote (dendritic) sites. SS has been reported to diminish Ca $^{2+}$ currents in cell lines (Ikeda and Schofield, 1989) and, more recently, in neocortical neurons in culture (Wang et al., 1990). AA has also been reported to inhibit Ca $^{2+}$ currents in CA1

Figure 12. Effect of baicalein (*Bai*), a 12-LOX inhibitor, on SS effects. *A*, Current records: baicalein 4 μ M, added for 7 min to the perfusate containing SS, does not alter the SS-induced I_M or conductance increase, or the outward steady-state current. Subsequent washout of both SS and baicalein with ACSF alone for 16 min reverses the SS effects. *B*, Plots of average I_M relaxations obtained with several different voltage commands. Baicalein did not reduce the SS-induced I_M increase in any of these five cells. In four of these cells, baicalein was applied after SS as in *A*. In one of these cells, baicalein was applied for 8 min before the coadministration of SS; this cell also showed no alteration of the SS induced I_M increase. Washout of all drugs returned the mean I_M to control levels.

neurons in culture, possibly via protein kinase C activation (Keyser and Alger, 1990). However, in our studies, CA1 pyramidal neurons still showed SS augmentation of I_M after blockade of Na^+ - and Ca^{2+} -dependent spikes with TTX, QX-314, and Cd^{2+} . Furthermore, our observations that SS did not consistently alter Ca^{2+} -dependent spikes in current clamp, or the inward currents (thought to be I_L) evoked by depolarizing commands in the whole-cell configuration, also argue against a SS effect on Ca^{2+} conductances.

Another possible site of SS action is on conductances under-

lying the posttrain AHP, such as I_{AHP} or I_C . However, in previous studies we did not see a consistent effect of SS on the AHPs themselves (Moore et al., 1988). Furthermore, SS effects and I_M itself, but not I_C (Madison et al., 1987), are blocked by CCh. In the present study, superfusion of Cd^{2+} , known to block several types of AHP currents (as well as other Ca^{2+} -dependent processes), did not alter SS-induced I_M increases. Nonetheless, a thorough voltage-clamp ("hybrid-clamp") study of the underlying currents has not been carried out, partly because of the likelihood that there will also be a contribution of I_M to such conductances (Storm, 1989). Thus, we cannot completely eliminate the possibility that an AHP current is involved.

Second messenger mediation of SS effects

SS has been shown to inhibit adenylate cyclase in many preparations (see, e.g., Chneiweiss et al., 1985; Wang et al., 1989). Thus, cAMP would seem an obvious candidate for mediation of SS effects. However, analogs of cAMP did not markedly alter resting properties or the I_M of CA1 cells in our studies. They induced a small inward current similar to that previously described for hippocampal neurons (Madison and Nicoll, 1986). Alternatively, the early, small steady-state outward current elicited by 8-Br-cAMP could be due to a brief effect on extracellular adenosine receptors. It should be noted that the electrophysiological effects of SS in neocortical neurons described by Wang et al. (1989) also were independent of cAMP.

Thus, cAMP does not seem to be involved in the mediation of SS-effects in hippocampus. Other investigators have examined the role of additional second messenger systems in I_M regulation. Diacylglycerol and protein kinase C do not affect I_M (Malenka et al., 1986), but inositol triphosphate, a product of PLC, has been shown to decrease I_M and is thought to be the mediator for the muscarinic inactivation of I_M (Dutar and Nicoll, 1988). A role for intracellular Ca^{2+} in I_M regulation is also now becoming apparent (see Further aspects of second messenger mediation, below).

AA and its metabolites mediate SS effects. Our combined data (see also Schweitzer et al., 1990) implicates AA and its metabolites as strong candidates for mediation of SS effects. The parallel mimicry of all SS effects by exogenously applied AA is consistent with a role for AA as an intracellular mediator. Thus, SS and AA superfusion augments I_M amplitude (55–60% and 55–75%, respectively) and kinetics (20–27% and 23–31%, respectively) to the same extent. SS and AA elicit a similar outward steady-state current increasing with depolarization (221 pA and 233 pA, respectively, at -43 mV holding potential). Although it could be argued that the concentration of AA we used is high, it should be noted that because of the adsorption of fatty acids, the actual concentrations of AA surrounding the cell in our experiments are certainly lower than the calculated concentrations in the chambers. It is likely that the amount of AA gaining access to the neuronal membrane is <5% of that in the buffer (Freeman et al., 1990). Thus, presumably less than 6 μ M AA reached the neuronal membrane.

Our other pharmacological findings further support the involvement of AA in mediating SS effects. Thus, both the PLA_2 inhibitors 4BPB and quinacrine completely blocked all the SS effects. The antagonism appeared identical, in spite of differences in the site of action of each of these inhibitors: 4BPB inhibits PLA_2 (but not PLC in hippocampus; see Okada et al., 1989) by binding to the enzyme, whereas quinacrine interacts with the phospholipid substrate (see Blackwell and Flower, 1983).

Figure 13. 5-LOX metabolites are implicated in SS-induced I_M augmentation. *A*, Current recording of a CA1 neuron held at -43 mV. I_M amplitude is increased by SS superfusion (6 min), together with a pronounced outward steady-state current (broken line is control holding current). Subsequent superfusion of MLTA, a specific 5-LOX inhibitor (together with SS), abolishes I_M augmentation but does not completely bring the steady-state current back to control level. RMP was -67 mV for this cell. Voltage protocol is shown at lower left. *B*, I_M augmentation by either SS (left; five cells) or AA (right; four cells) is completely abolished by either of the specific 5-LOX inhibitors (5block; MLTA or DHAA). *C*, As with NDGA (see Fig. 12*A*), the net SS-induced outward current is partially reversed by the 5-LOX inhibitors. Current subtraction reveals a voltage-dependent component sensitive to 5-LOX inhibitors that activates around -65 mV (i.e., I_M).

Figure 14. LTC₄ mimics AA and SS effects on I_M . *A*, Digitized current records from a CA1 pyramidal neuron. Superfusion of LTC₄ (5μ M) in the presence of NDGA (10μ M) augments I_M and causes an outward steady-state current (broken line is control holding current) associated with an increase in input conductance. Washout brings all current measures back to near normal levels. Voltage protocol for all current records shown at lower left. *B*, Average of I_M amplitudes for five cells tested with LTC₄, using holding potentials of about -45 mV, and the same drug protocol as in *A*. Average I_M is significantly increased by LTC₄ ($P < 0.05$, two-factor ANOVA for repeated measures, Newman-Keuls post hoc test).

However, the PLA₂ activator mellitin did not elicit a consistent effect on I_M , but evoked a delayed dramatic inward current without recovery, possibly due to a lysis of the membrane lipids by mellitin (see Katsu et al., 1989). Further support of the role for AA in SS effects is a recent report showing that SS activates PLA₂ and AA release in astrocytes (Marin et al., 1991).

LOX metabolites mediate I_M augmentation. Our studies of the AA pathways suggest a role for LOX metabolites of AA in the SS- I_M effect. The LOX inhibitor NDGA completely antagonizes SS- and AA-induced I_M augmentation. Concomitantly, NDGA greatly reduces the SS- and AA-induced outward steady-state current. Thus, it seems that the NDGA-sensitive outward steady-state current is entirely due to I_M , since the threshold of activation, voltage dependency, and NDGA blockade of this outward current correlate with these properties of the I_M . Furthermore, our conductance calculations reveal an increase of M-conductance of 3–4 nS with AA (Fig. 7C) or SS (Schweitzer et al., 1990), values very similar to those found for the SS- or AA-induced outward current sensitive to NDGA (Fig. 11B). The different inhibitors of the AA cascade we used only blocked the SS-induced outward current and I_M increase, without affecting the basal currents. Thus, AA and its metabolites may be involved only in the SS effects and do not seem to control resting properties.

There are several LOXs; the two major ones are 5-LOX and 12-LOX. Our findings that specific 5-LOX but not 12-LOX inhibitors antagonize both SS- and AA-induced I_M effects are consistent with mediation of SS- I_M effects by 5-LOX metabolites. As with NDGA, a residual outward current remains when 5-LOX blockers are added together with SS or AA, suggesting again some effect of AA itself. The role of 5-LOX and leukotrienes in the SS- I_M effect is supported further by our finding that LTC₄ application leads to I_M effects similar to those obtained with AA and SS. Leukotrienes are synthesized in several brain regions, including hippocampus (Dembinska-Kiec et al., 1984; Lindgren et al., 1984). LTC₄ can be metabolized further to LTD₄ and LTE₄ (reviewed by Samuelsson et al., 1987), which could also be involved in the SS- I_M effect. However, binding sites for LTB₄ and LTE₄ are weak and those for LTD₄ undetectable, suggesting that LTC₄ may represent the most active metabolite in brain. Interestingly, specific LTC₄ binding sites are found in several brain areas and are among the most numerous in brainstem and hippocampus (Schalling et al., 1986), the two areas where SS has been shown to increase I_M (Jacquin et al., 1988; Moore et al., 1988).

The 12-LOX metabolites have been shown to be involved in FMRamide and histamine effects in *Aplysia* (Piomelli et al., 1987a,b). Here, the FMRamide effect could be mediated directly by 12-HPETE (Buttner et al., 1989) or a heme-catalyzed product (Belardetti et al., 1989). However, in our experiments, the 12-LOX inhibitor baicalein had no effect on SS responses. In accord with this finding, superfusion of the 12-LOX product 12-HPETE gave inconsistent results, and in no case did it alter I_M . Still, given the transient chemical nature of this short-lived intermediate metabolite (Piomelli and Greengard, 1990) compared to AA and LTC₄ (Samuelsson et al., 1987), conclusions regarding these negative findings should be tentative when using a brain slice preparation, where drugs need 1–2 min to reach the recorded cell. Other studies have shown that AA causes hyperpolarization and increased posttrain AHP size in CA1 hippocampal neurons, but without change in input resistance (Carlen et al., 1989). These effects were reproduced by the pep-

tidyl form of 8-HEpETE (8-hydroxy-11,12-epoxy-5,9,14-icosatrienoic acid or hepoxillin A₃), a 12-LOX metabolite, although without recovery on washout. Interestingly, the formation of this compound is analogous to that of LTC₄, in that both products are produced by a glutathione-conjugating system (Pace-Asciak et al., 1990).

A second SS effect. Effects of the LOX inhibitor NDGA, as well as MLTA and DHAA, also reveal that SS acts on another conductance. The persistence of a small current in the presence of LOX inhibitors and the lack of effect of the COX inhibitor indomethacin suggest that this effect is not mediated by an AA metabolite, but most likely by the fatty acid itself. AA and certain fatty acids have been shown to alter electrophysiological cell properties in peripheral tissues by acting on ionic currents (reviewed by Ordway et al., 1991). Therefore, the NDGA-resistant hyperpolarizing effect of AA could be due to a direct interaction with a channel or alteration of the membrane lipid bilayer. This current is rather small but appears to play a major role in the hyperpolarizing effect seen at rest. Interestingly, the hyperpolarizing effect (40–90 pA outward current) of SS and AA on a few neurons not responding with I_M augmentation resembles the NDGA-resistant current, and indeed also was resistant to LOX and COX inhibition. The properties of the NDGA-resistant current are still unclear: it could be an inwardly rectifying current, or perhaps a leak current such as that reduced by muscarinic agonists in hippocampal neurons (Madison et al., 1987). The unusual nonlinearity of this current could also be related to some uncharacterized dendritic conductance (see Benson et al., 1988). In fact, it is possible that if the underlying conductance is generated far away on the dendritic tree, it may not be properly clamped, especially in the depolarized potentials.

We also examined a possible role of the COX pathway. The lack of effect of the prostaglandins as well as indomethacin alone on I_M suggests that COX metabolites are probably not linked to the I_M augmentations elicited by SS or AA. Interestingly, indomethacin has been shown to increase LTC₄ levels in rat brain (Dembinska-Kiec et al., 1984). Thus, the potentiations we saw with indomethacin applied together with SS or AA could arise from COX inhibition, leading to shunting of increased AA toward the LOX pathway (see Higgs and Vane, 1983). However, we cannot disregard a possible effect on I_M of some other COX metabolites not yet tested, or possible inhibitory effects of prostanooids on the LOX pathway. A dual role of COX and LOX metabolites has been described for the modulation of an inwardly rectifying K⁺ current in atrial cells (Scherer and Brei-twieser, 1990). The partial later inhibition of SS effects by indomethacin we saw in some cells may reflect nonspecific inhibition of PLA₂ (Irvine, 1982), since long indomethacin superfusions never reduced AA-induced I_M increase.

The data reported here and elsewhere (Schweitzer et al., 1990) provide new electrophysiological evidence for eicosanoid mediation of a transmitter effect in a vertebrate central neuron. Piomelli et al. (1987a,b) previously showed that 12-LOX metabolites may mediate FMRamide effects on a K⁺ current, and hyperpolarizations elicited by histamine, in *Aplysia* neurons. Prostaglandins have been implicated in mediation of vasoactive intestinal peptide (VIP) biochemical effects in brain slices (Schaad et al., 1987), whereas LOX products may regulate VIP release (Martin and Magistretti, 1989). Similarly, a product of the ep-oxygenase pathway for AA, 5,6-epoxyeicosatrienoic acid, induces SS release in hypothalamus (Capdevila et al., 1983). Thus,

as with VIP, two different AA pathways appear to be involved in the release of SS on the one hand and its electrophysiological effects on the other.

M-current kinetics. Considering that our studies were done at a higher temperature, the I_M time constant (τ) values in the present study are consistent with those of Halliwell and Adams (1982). These authors reported values of 267–303 msec at -50 mV and 118–176 msec at -60 mV (temperature of 23°C) in guinea pig hippocampus, with a Q_{10} of about 5 around 25°C . We found that both SS and AA significantly increased τ for the I_M relaxations in CA1 pyramidal neurons. The increase with SS or AA compared to control conditions did not reach significance in our previous report (Schweitzer et al., 1990), because of the variability in τ -values and the smaller number of cells in our earlier sample. Interestingly, the I_M increase evoked by isoproterenol in smooth muscle is also associated with a τ increase (Sims et al., 1988), as is the I_M augmentation (termed over-recovery) observed on washout of muscarine in sympathetic neurons (Marrion et al., 1991). The τ increase for I_M we observe in pyramidal neurons with both SS and AA might represent a change of M-channel properties due to a variation in intracellular Ca^{2+} concentrations (see Marrion et al., 1991). The isoproterenol effect on τ in smooth muscle cells appears to be mediated by cAMP, and is postulated to reflect a decrease in the closing rate constant for M-channels (Sims et al., 1988).

Further aspects of second messenger mediation

Using fura-2 measurements, Marrion et al. (1991) observed that I_M augmentation (over-recovery) in sympathetic ganglia after muscarine washout is associated with a moderate increase of intracellular Ca^{2+} concentration (higher internal Ca^{2+} elevation blocks I_M). It is possible that in a process of muscarinic activation of both PLC and PLA_2 (Kanterman et al., 1990), where internal Ca^{2+} would rise and then fall, PLC may inactivate first because of a lower sensitivity to intracellular Ca^{2+} than PLA_2 . This would reveal a transient, exclusive PLA_2 effect (the “over-recovery”), before inactivation of this enzyme as the internal Ca^{2+} concentration returns to basal levels. More recent data also have shown augmentation of I_M by intracellular Ca^{2+} injection; this effect is blocked by NDGA and mimicked by AA (Yu et al., 1991). Thus, internal Ca^{2+} seems to play a key role in I_M regulation, especially when considering possible differential sensitivities to Ca^{2+} of the enzymes that may be involved (PLA_2 , PLC, protein kinase C), acting in opposite directions through the metabolites they generate (in hippocampal CA1 neurons, PLC and inositol triphosphate vs PLA_2 and AA metabolites).

It is also possible that a GTP-binding protein may be involved in the SS activation of PLA_2 (North, 1989; Axelrod, 1990). Biochemical studies have shown that SS receptors are linked to G-proteins in brain (see Law et al. 1991). Further studies will be required to determine if the SS effects described here involve one or more G-proteins. Furthermore, the exact metabolite or metabolites involved in hippocampal SS effects on I_M are not known, although our data with LTC_4 suggest that a leukotriene is the best candidate. Additional support of this idea must await localization (e.g., by immunocytochemistry or *in situ* hybridization) of the relevant enzymes or their products in hippocampal neurons. Given their great membrane permeability, eicosanoids, unlike most other messengers, can leave the cell in which they are generated and act as first messengers on neighboring cells (Piomelli et al., 1987a; Piomelli and Greengard, 1990; Shimizu and Wolfe, 1990). Thus, they could act on the M-chan-

nels and other conductances by either an intracellular or an extracellular route. Therefore, presynaptic or feedback effects cannot be excluded. Studies using intracellular drug application are planned to evaluate these alternatives.

In this regard, there is now considerable evidence that AA and/or PLA_2 (and possibly some LOX metabolites) play some role in hippocampal CA1 long-term potentiation (LTP), the electrophysiological model of learning and memory. Thus, (1) AA produces a long-lasting enhancement of synaptic transmission (Williams et al., 1989; Drapeau et al., 1990) and increases NMDA currents (Miller et al., 1992); (2) inhibitors of PLA_2 can prevent expression of LTP (Okada et al., 1989; Williams and Bliss, 1989; Drapeau et al., 1990); and (3) both NMDA (thought to be the trigger for LTP) and LTP itself increase the release and postsynaptic membrane levels of AA in hippocampal tissue (Sanfeliu et al., 1990; Clements et al., 1991). The view of some researchers (see, e.g., Clements et al., 1991) is that AA or a metabolite is generated postsynaptically but diffuses to presynaptic glutaminergic terminals to enhance transmitter release (but see O'Dell et al., 1991, for another mechanism involving nitric oxide). Although our findings of inhibitory (hyperpolarizing) effects of AA and LTC_4 are not entirely consistent with enhancement of synaptic transmission, the AA metabolite(s) may have a different effect on presynaptic terminals or elsewhere than at postsynaptic CA1 pyramidal neurons. The recent finding that AA (and also SS via PLA_2 ; Marin et al., 1991) causes a prolonged inhibition of glutamate uptake into glia (Barbour et al., 1989) is consistent with this interpretation.

The exact physiological role and sites of action of the relevant lipid(s) in hippocampus cannot be stated at this time. However, our findings do suggest that, as in some invertebrate neurons (Piomelli et al., 1987a; Belardetti and Siegelbaum, 1988), a single ion channel type (the M-channel) in mammalian central neurons can be regulated in opposing or reciprocal directions by two different ligands—SS and ACh—acting through different second messenger systems. These bidirectional regulatory mechanisms should provide sensitive biasing of ion channel activity, and thus of neuronal excitability. Possible cross-talk between the intracellular mediators adds further complexity. In addition, the present studies now suggest a second SS-sensitive conductance (NDGA resistant) in most of the same cells exhibiting I_M augmentation. Hence, in these cells SS receptors may not only converge with another receptor class (muscarinic) onto a single channel type; SS may also diverge to act on another SS-receptor-ion channel system with different, but still hyperpolarizing, properties. This latter SS substrate appears to require at least one less enzyme for its function than the M-channel effect, and therefore may be subject to quite different regulatory factors.

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