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Constant Turnover of Arachidonic Acid and Inhibition of a Potassium Current in *Aplysia* Giant Neurons

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Summary. Steady-state currents at hyperpolarized membrane potentials were studied in the homologous giant neurons, LP1 and R2, of *Aplysia* using two-electrode voltage clamp. Nearly half of the steady-state current at voltages more hyperpolarized than −70 mV had characteristics similar to the inwardly rectifying potassium current (I_R) described previously in *Aplysia* neurons. The pharmacological agents 4-bromophenacylbromide, indomethacin, and the phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate were found to modulate I_R. I_R was stimulated with BPB and indomethacin and inhibited with TPA. These agents altered I_R by a mechanism independent of *c*AMP, which can also modulate I_R. The effects of these modulators are consistent with their actions on arachidonic acid (AA) metabolism in *Aplysia* nervous system, suggesting AA may constitutively inhibit I_R. When ganglia were perfused for 12 hr with medium containing BSA to absorb extracellular fatty acids, I_R was increased nearly twofold. This increase was partially inhibited by addition of AA to the perfusion medium, and completely inhibited by pretreatment of ganglia with BPB. Although no direct effect of short-term exposure to exogenous AA was observed, long term exposure to exogenous AA and several other unsaturated fatty acids was accompanied by a decrease in I_R.

Key Words arachidonic acid · eicosanoids · *Aplysia* · inward rectifier · phorbol ester · potassium current · protein kinase C

Introduction

Arachidonic acid and its metabolites, or eicosanoids, have been shown to be involved in regulation of secretory processes. The role of eicosanoids in stimulus-secretion coupling in the anterior pituitary is one of the best characterized systems. Lipoxygenase and epoxygenase products have been implicated in the mediation of hormone-induced release of the pituitary hormones adrenocorticotropin hormone [1, 40, 43], β-endorphin [3, 45], prolactin [8, 29, 43], luteinizing hormone [15, 16, 28, 43], and growth hormone [12, 13, 27, 43, 58]. The first direct demonstration that eicosanoids may regulate secretion through modulation of electrical activity came from work with identified neurons of the marine snail, *Aplysia*. At an identified synapse in *Aplysia*, eicosanoids have been implicated in the activation of a potassium current (S-current), which leads to inhibition of electrically-evoked transmitter release, or presynaptic inhibition [47, 48]. Modulation of potassium currents can alter cell excitability and thereby indirectly modulate neurotransmission or other secretory processes. Recently, several additional examples of AA- and eicosanoid-mediated modulation of potassium currents have been reported. Kim et al. [31] and Kurachi et al. [36] have implicated lipoxygenase metabolites in the regulation of muscarinic receptor-linked potassium channels. Also, AA[1] and other unsaturated fatty acids have been shown to directly activate potassium channels in ripped-off patches from heart [30] and smooth muscle cells [46].

In the accompanying paper [11], we demonstrated constant turnover of intracellular free AA in *Aplysia* ganglia. This turnover was accompanied by a constant and substantial release of free AA and eicosanoids to the extracellular medium. In this paper, we report our investigation of AA-mediated regulation of an inwardly rectifying potassium current in the giant neurons, LP1 and R2, of *Aplysia*. This current has previously been shown to be activated by serotonin [2, 6, 19] and *Aplysia* egg-laying hormone [39], through the *c*AMP cascade. We

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[1] *Abbreviations:* AA, arachidonic acid; AA-CoA, arachidonyl coenzyme A; TPA, 12-O-tetradecanoylphorbol 13-acetate; BPB, 4-bromophenacylbromide; PLA_2, phospholipase A_2; NDGA, nordihydroguaiaritic acid; 5HT, serotonin creatine sulfate; IBMX, isobutylmethylxanthine; DMSO, dimethylsulfoxide; I_R, inwardly rectifying potassium current.

show now, using the pharmacological agents described in the accompanying paper and techniques which alter the amount of AA in lipid storage, that AA can also modulate I_R, independent of *c*AMP.

Materials and Methods

Electrophysiology

Adult *Aplysia californica* (100 to 250 g) were supplied by Alacrity Marine Biological Services (Redondo Beach, CA). To anesthetize prior to dissection, *Aplysia* were injected with up to 100 ml of 0.4 M $MgCl_2$. Ganglia were removed and immediately placed in artificial seawater (ASW (control), in mM: 460 NaCl, 55 $MgCl_2$, 11 $CaCl_2$, 10 KCl, 10 NaHEPES, pH 7.3) with 1% (wt/vol) glucose.

Ganglia were incubated in 1% (wt/vol) dispase in ASW for 1 hr at room temperature to loosen the connective tissue sheath prior to desheathing. Dispase was washed away and the abdominal or left pleural and connected left pedal ganglion, or "pleuropedal" ganglion, was pinned into a 0.5-ml chamber of Sylgard in a plastic petri dish. The ganglion was desheathed and placed under perfusion with ASW at about 1 ml/min. Perfusion was used to supply all pharmacological agents to the ganglion. DMSO was used to solubilize all agents used (except 5HT) prior to addition to ASW. The final concentration of DMSO in ASW was normally 0.05% (vol/vol), but as much as 0.25% DMSO was used to solubilize the highest concentrations of indomethacin tested. No discernible electrophysiological effects were observed for control solutions containing up to 0.25% DMSO.

The giant neuron (R2 in the abdominal ganglion or LP1 in the pleural ganglion) was impaled with two glass microelectrodes (1–4 MΩ, filled with 0.5 M K_2SO_4) for intracellular recording and two-electrode voltage clamp. Two-electrode voltage clamp was performed with an Almost Perfect Electronics Voltage Clamp, a high-voltage Almost Perfect Electronics preamp (for current injection) and a W-P Instruments preamp (for monitoring voltage). Data were recorded on a Gould chart recorder and a four-channel Hewlett-Packard tape recorder for subsequent analysis. The giant neurons were clamped at −70 or −50 mV. Pulses from this holding potential to command potentials were used to generate current-voltage (*I-V*) curves. For command potentials more hyperpolarized than −30 mV, these pulses were normally 0.5 to 1 sec in duration. The current magnitude was measured at the end of the pulse and graphed as a function of command potential.

For experiments involving perfusion or dialysis and subsequent electrophysiological measurements over extended periods of time, aseptic cell culture techniques were employed. Ganglia were washed with several changes of sterile ASW, followed by incubation in a filter-sterilized solution of 1% (wt/vol) dispase in ASW for 2 hr. For extended perfusion, the sterile, dispase-treated abdominal and/or left pleuropedal ganglia, in groups of four to eight, were pinned to sterilized Sylgard-lined plastic 60-cm petri dishes, and desheathed using sterile instruments. The plate was then placed under perfusion with sterile, control ASW. The giant neurons were successively placed under voltage clamp, and the resting steady-state *I-V* curves were measured. The plate was then placed under perfusion (~1 ml/min) for 12 hr with perfusion medium: sterile, dilute L15 (a nutrient medium from Flow Laboratories (McLean, VA); present at 20% of normal concentration) in ASW in penicillin (10 IU/ml), streptomycin (10 μg/ml), fungizone (amphotericin B, 0.5 μg/ml) and 1% (wt/vol) glucose. (Antibiotics were from Flow Laboratories.) This perfusion was carried out in a laminar flow hood. Then the plate was again placed under perfusion with control ASW, and the giant neuron steady-state *I-V* curves were measured.

For dialysis, the dispase-treated abdominal and/or left pleuropedal ganglia (with sheaths intact), in groups of four to eight, were placed in sterile dialysis tubing (Spectro/Por 7, 25 K molecular weight cut-off) filled with sterile, control ASW. The ganglia were then dialyzed in 250–300 ml of medium identical to that used for extended perfusion. Dialysis was carried out in a stirred bath at 10°C, and the dialysis medium was changed every 24 hr. The ganglia were then removed from the dialysis tubing, and each group was pinned to a 60-cm, Sylgard-lined, plastic petri dish, and the ganglia were desheathed. The plate was then placed under perfusion with control ASW and the giant neuron steady-state *I-V* curves were measured.

All reagents were of highest grade available. 12-O-tetradecanoylphorbol-13-acetate, indomethacin, 4-bromo-phenacylbromide, nordihydroguaiaritic acid, acetylsalicylic acid, serotonin creatine sulfate and isobutylmethylxanthine were from Sigma. Forskolin was from Calbiochem. DMSO was from Aldrich. Dispase was from Boehringer-Mannheim. Inorganic salts were from Fisher. Fatty acids were from Cayman Chemicals (Ann Arbor, MI).

Biochemistry

Methods for analyzing AA metabolism are described in the accompanying paper [11].

Results

Giant Neurons Contain Inwardly Rectifying Potassium Current

Current-voltage curves in the giant neurons, R2 and LP1, of *Aplysia* were generated with 0.5-sec voltage steps from a holding potential of −70 to −50 mV, to command potentials from −110 to −40 mV. At rest, the *I-V* curves exhibited an inwardly rectifying current at potentials more hyperpolarized than −70 mV, which is the potassium equilibrium potential (E_K) in *Aplysia* neurons in ASW (*see I-V* curves in control ASW in Fig. 1). This inwardly rectifying current was abolished in the presence of 0.5 to 1 mM Ba^{2+} (Fig. 1). Also, lowering the potassium concentration from 10 mM to 2 mM removed the rectifying component of the current (Fig. 1). These results suggest this conductance is likely an inwardly rectifying potassium current (I_R) of the type described previously in *Aplysia* neurons [6, 22]. I_R is blocked selectively by a low concentration of Ba^{2+}, and is known to rectify inwardly at E_K [6, 23, 38].

We therefore defined I_R in giant neurons as the current present at potentials more hyperpolarized than −70 mV that was blocked with 0.5–1 mM Ba^{2+}.

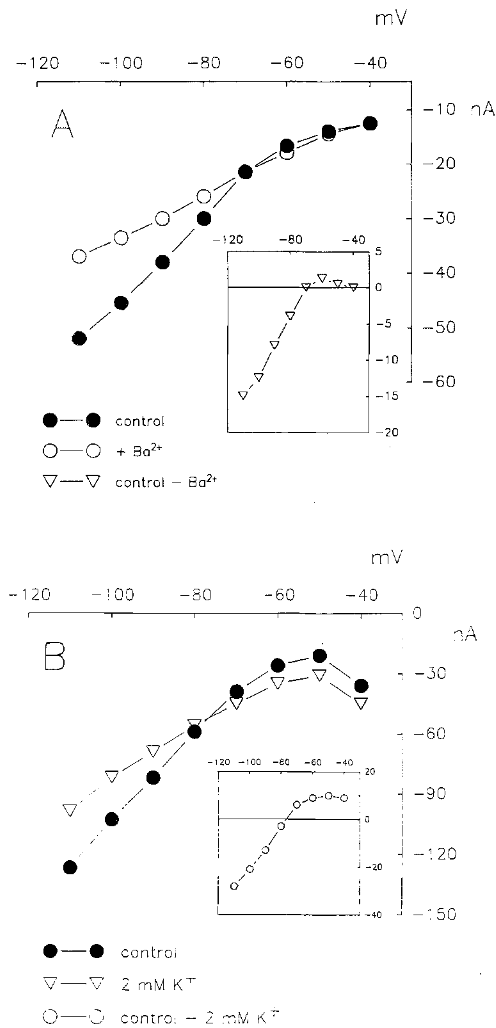

Fig. 1. Ba^{2+} or low extracellular K^+ reveal I_R in giant neurons. (*A*) Current-voltage curves were measured for the giant neuron, R2, under perfusion with control ASW (filled circles) or ASW + 0.5 mM Ba^{2+} (open circles). To determine the *I-V* curve, voltage was stepped from the holding potential of −50 mV to command potentials from −110 to −40 mV for 0.5 sec. The current magnitude was measured at the end of each pulse and graphed as function of the command potential. The current remaining in the presence of Ba^{2+} was designated "leak" current. In the inset graph, the *I-V* curve for this leak current was subtracted from the control ASW *I-V* curve to yield a leak-subtracted *I-V* curve for control ASW (open triangles). This leak-subtracted current was assumed to be due solely to I_R. In subsequent figures, the leak current in the presence of 0.5–1 mM Ba^{2+} was measured in this voltage range and subtracted from the total current to illustrate changes specific for I_R. (*B*) *I-V* curves for the giant neuron, LP1, were measured as described in *A* under perfusion of control ASW (10 mM K^+, filled circles), or ASW with 2 mM K^+ (open inverse triangles). The difference curve, or current change due to lowering $[K^+]$, is depicted in the inset graph (open circles)

The current not blocked by barium was designated undefined or "leak" current. Leak current measured in the presence of barium was subtracted from *I-V* curves to isolate I_R. This leak subtraction procedure (depicted in Fig. 1*A*) was used routinely to identify changes specific to I_R. Changes in I_R present at potentials more depolarized than −70 mV were not analyzed due to the presence of undefined contaminating currents. To determine the amount of current due to I_R in giant neurons at rest, the slope conductance of the *I-V* curve from −110 to −70 mV was compared before and after exposure to barium in control ASW (example in Fig. 1). At rest, I_R was found to account for 48 ± 2% (n = 32) of the total conductance at voltages more hyperpolarized than E_K. This proportion of I_R was found to be generally typical of other *Aplysia* neurons tested (e.g., medial cells of the pleural ganglia, B1 of the buccal ganglion, and R14 of the abdominal ganglion). A larger amount of resting I_R was found only in R15 [6, 33] and left upper quadrant neurons of the abdominal ganglion (this study).

Modulators of AA Metabolism Alter I_R

4-bromophenacylbromide

In the accompanying paper, BPB was shown to inhibit the liberation of arachidonic acid from phospholipid. Application of BPB by perfusion to the bath was found to stimulate I_R in giant neurons (n = 8, examples for 20 and 50 μM BPB are in Fig. 2). The current induced by BPB displayed the inward rectification characteristic of I_R (inset graph, Fig. 2*A*), and this induced current was inhibited with Ba^{2+} (Figs. 2*B* and 4). To determine the extent to which the BPB-induced conductance increase was due to a change in I_R, the slope conductance (from −110 to −70 mV) was measured for 0.5 mM Ba^{2+} alone and Ba^{2+} plus BPB. Over 96% of the slope conductance increase induced with 50 μM BPB was inhibited with Ba^{2+}. Also, the BPB-induced stimulation of I_R did not reverse with up to 2.5 hr of perfusion with control ASW (Fig. 2*B* shows the BPB-induced increase in I_R did not reverse with a 70 min wash). BPB is established as an irreversible inactivator of phospholipase A_2 [51, 61], which is consistent with this irreversible stimulation of I_R.

To test whether the effect of BPB was directly on the giant neuron, BPB was applied to the giant neuron cell body in a gentle stream flowing by gravity from the broken tip of a microelectrode pipette. The location of the stream was monitored by addition of 0.2% (wt/vol) fast green to the solution in the pipette. By altering the size of the pipette aperture, the stream width could be confined within the dimensions of the giant neuron cell body, and the rate of flow was adjusted to cause the stream to be diluted substantially (as determined by disappearance of the tracking die) within several hundred microm-

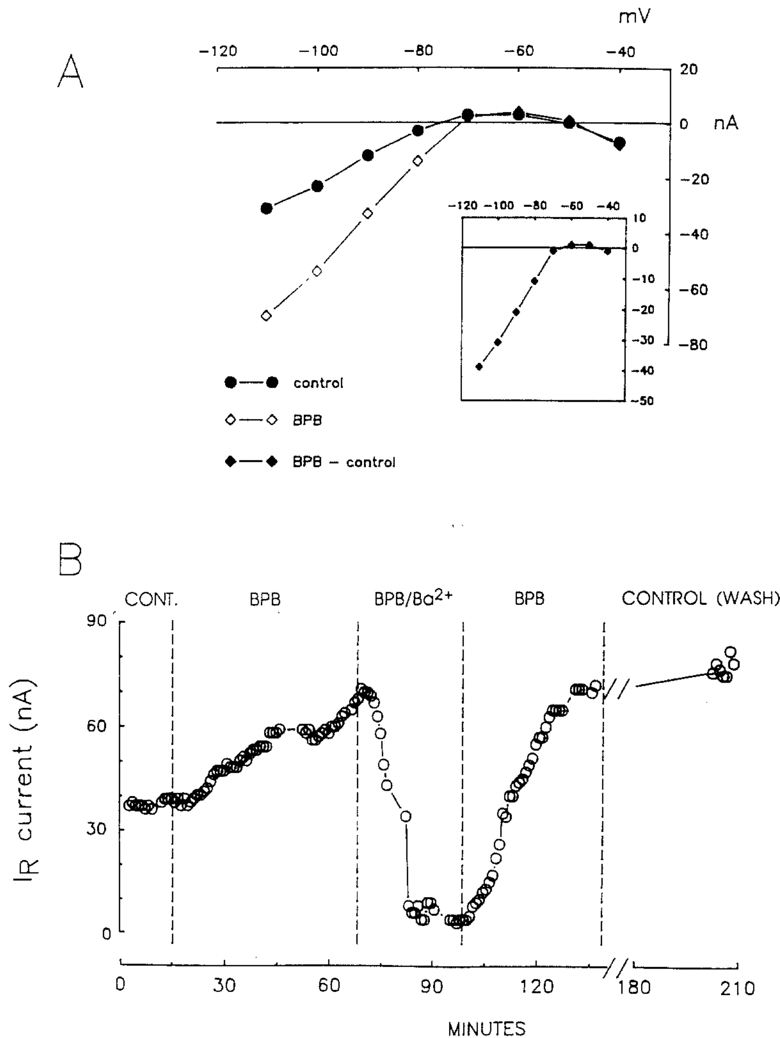

Fig. 2. Effect of BPB on I_R. (*A*) *I-V* curves for LP1 (held at −50 mV) under perfusion with control ASW (filled circles) or ASW + 20 μM BPB (open diamonds) were measured and leak-subtracted as described in Fig. 1. Therefore, the current in the *I-V* curves for this (and all subsequent figures) is due solely to I_R. The curve in the presence of BPB was measured 80 min after the start of perfusion with BPB. The difference curve, or BPB-induced current, is depicted in the inset graph (filled diamonds). (*B*) In a separate experiment, 0.5-sec pulses from −50 to −100 mV were used to follow the time course of the effect of BPB on I_R. The dotted lines indicate a change in experimental conditions. The labels above the graph depict the following sequence of conditions: control ASW; +50 μM BPB; +BPB + 0.5 mM Ba^{2+}; BPB alone; control ASW wash

eters away from the pipette tip. This created a small "plume" of perfusate that could be localized on the giant neuron cell body. Such a plume of BPB-containing perfusate (50–100 μM) was found to stimulate I_R in a manner identical to that described for bath-applied BPB (n = 14).

Indomethacin

Indomethacin was also found to increase I_R in giant neurons (n = 31, examples for 50–150 μM indomethacin in Figs. 3 and 4). The indomethacin-induced current was inwardly rectifying around E_K (inset graph, Fig. 3*A*), and was blocked by Ba^{2+} (Figs. 3*B* and 4), and by lowering potassium from 10 to 2 mM (*data not shown*). Determined as described for BPB, 93% of the indomethacin-induced slope conductance increase was due to an increase in I_R. Also, as described for BPB, indomethacin was equally effective when its application was confined to the giant neuron cell body (n = 5). However, unlike BPB, the effect of indomethacin was readily reversible when applied by bath (Figs. 3*B* and 4) or cell-body-localized perfusion. This reversibility allowed a test of the accuracy of cell body perfusion, which proved quite precise. Reversal of the indomethacin-induced stimulation of I_R began immediately upon removal of the perfusion plume, even if the plume was simply moved just out of range of the cell body.

The dose dependence for the effect of indomethacin on I_R is depicted in Fig. 5. Indomethacin was consistently stimulatory above 10 μM, and this effect saturated by 250 μM. At saturation, I_R was increased over fivefold. Also, a saturating dose of indomethacin occluded the effect of BPB, suggesting these agents are acting through the same pathway to modulate I_R.

Indomethacin is known to be an inhibitor of cyclooxygenase [20], which catalyzes the first step for conversion of AA to prostaglandins and thrombox-

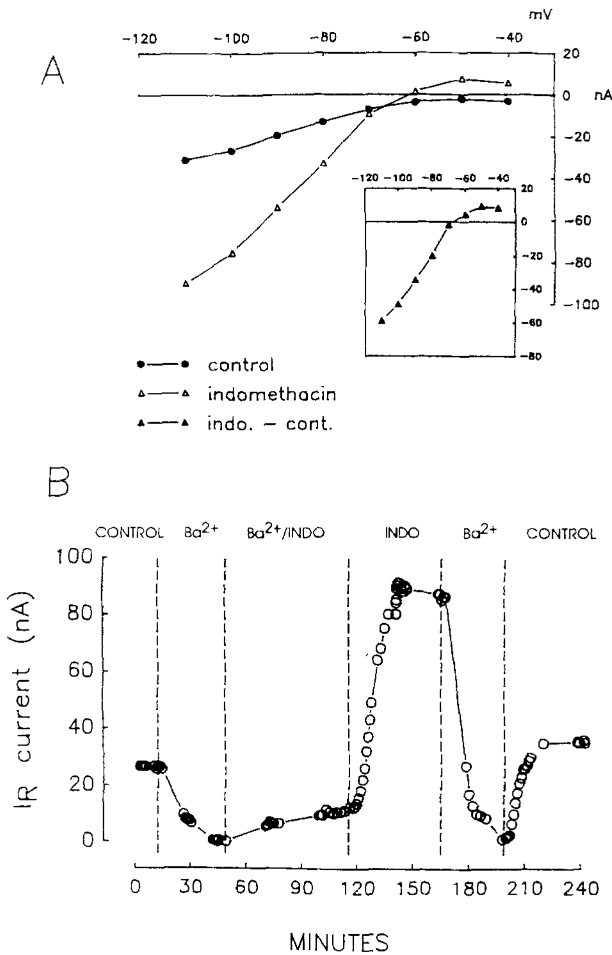

Fig. 3. Effect of indomethacin on I_R. (*A*) *I-V* curves for R2 (held at −50 mV) under perfusion with control ASW (filled circles), or ASW + 50 μM indomethacin (open triangles) were measured and leak subtracted as described in Fig. 1. The *I-V* curve for control ASW was measured just prior to the first dotted line in *B* (at ~10 min); the *I-V* curve in the presence of indomethacin was measured after ~2 hr of perfusion with indomethacin (in *B*, just prior to the fourth dashed line at ~170 min). The difference curve, or indomethacin-induced current, is depicted in the inset graph (filled triangles). (*B*) 0.5-sec pulses from −50 to −100 mV were used to follow the time course of the effect of indomethacin on I_R. The dotted lines indicate a change in conditions; the labels above the columns designated by the dotted line describe the following sequence of conditions: control ASW; +0.5 mM Ba^{2+}; Ba^{2+} + 50 μM indomethacin; 50 μM indomethacin alone; 0.5 mM Ba^{2+} alone; control ASW

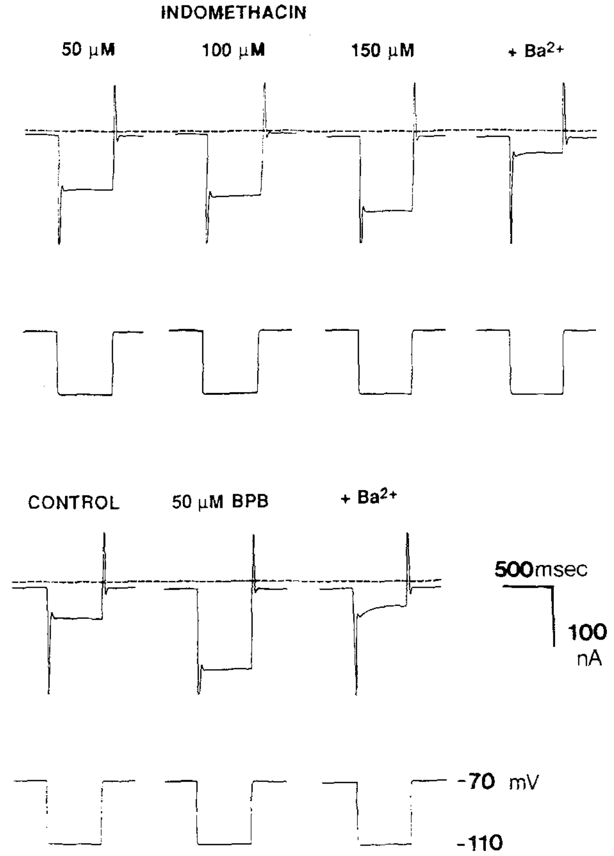

Fig. 4. Voltage-clamp records for effects of indomethacin and BPB. Voltage-clamp records for R2. Voltage steps for 500 msec from −70 to −110 mV were used to elicit inward current. The steady-state inward currents resulting from this voltage step are shown for each condition tested. Recording began under perfusion of 50 μM indomethacin (due to a previous experiment with indomethacin in an adjacent neuron in the same ganglion). The concentration of indomethacin for perfusion was increased to 100 μM and then to 150 μM. Approximately 30 min of perfusion was required to reach a new steady state in inward current for each change; much of this time was required for the bath to attain the new concentration. In the presence of 150 μM indomethacin, the addition of 0.5 mM $BaCl_2$ decreased the inward current by over 73%. Subtraction of the current magnitude at the end of the pulse, with and without Ba^{2+}, revealed that of the 134 nA of inward current elicited by hyperpolarization in the presence of 150 μM indomethacin, 98 nA were due to I_R. Subsequently, both indomethacin and Ba^{2+} were removed by perfusion with control ASW for about 1 hr. After this wash, the steady-state I_R was reduced to 21 nA out of a total of 57 nA of inward current. The giant neuron was then exposed to 50 μM BPB by perfusion for 1 hr, and the BPB was removed by perfusion with control AWS. Subsequent addition of 0.5 mM Ba^{2+} inhibited 72% of the inward current. Therefore, of the 147 nA of inward current induced by hyperpolarization after exposure to BPB (labeled 50 μM BPB), 106 nA due to I_R, which is a fivefold increase in I_R relative to control

anes. Acetylsalicylic acid is also known to inhibit cyclooxygenase [20]; however, we observed no effect of this inhibitor at up to 50 μM. We also tested the effect of nordihydroguaiaritic acid on I_R. NDGA is known to inhibit lipoxygenase catalyzed peroxylation and hydroxylation of AA [63] and has been reported to inhibit this activity in *Aplysia* [48]. We did not observe any effect of NDGA on I_R at up to 50 μM. In each case, after testing for the effects of these inhibitors, a robust effect of either indomethacin or BPB was observed.

Although a saturating dose of indomethacin occluded the effect of BPB on I_R, indomethacin did

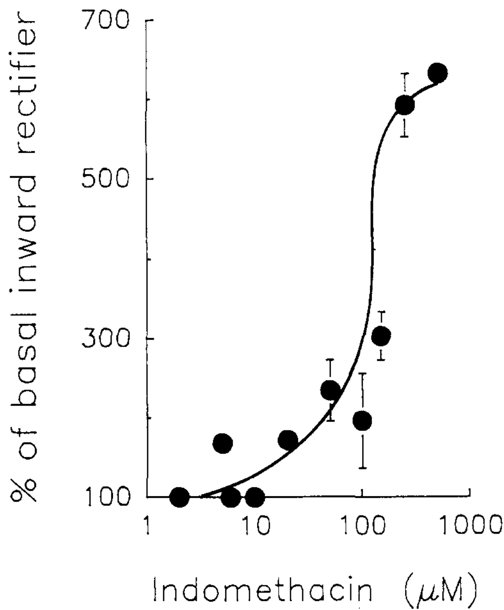

Fig. 5. Dose-response for stimulation of I_R by indomethacin. The effect of indomethacin on I_R was determined as function of concentration. *I-V* curves were measured and leak subtracted as described in Fig. 1. Slope conductances for leak-subtracted *I-V* curves from giant neurons were measured from −70 to −110 mV. The slope conductance is expressed as the percent of conductance due to I_R at rest. This normalization is termed *% of basal inward rectifier* on the ordinate axis. The curve was fitted by hand, and the depiction of saturation is supported by experiments in which an increase in indomethacin from 250 to 500 μM did not produce any increase in I_R (n = 2)

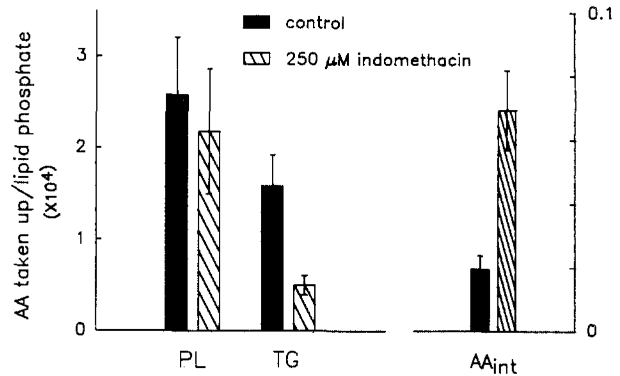

Fig. 6. Effect of indomethacin on uptake of AA. Pleuropedal ganglia were incubated in 0.5 μM AA (sp act 4.0 Ci/mmol) in ASW with 0.05% (wt/vol) BSA for 2 hr with 250 μM indomethacin (diagonal), or ASW with 0.1% (vol/vol) carrier DMSO (solid). AA uptake into total phospholipid, total neutral lipid, and internal free AA was normalized to ganglionic lipid phosphate. Bars and error bars are the mean and SD of measurements from four ganglia. For a description of the techniques used for lipid analysis, *see* Materials and Methods of the accompanying paper [11]

not have the same effect as BPB on AA metabolism in *Aplysia* ganglia. At concentrations up to 250 μM, indomethacin did not consistently alter the redistribution of radioactivity which occurred with 50 μM exogenous AA chase. However, indomethacin (250 μM) was found to inhibit uptake of AA into storage lipids. Figure 6 depicts the uptake of AA from medium containing 0.5 μM AA (sp act, 40 mCi/mmol), with and without indomethacin. Uptake into total phospholipid did not show a significant change, while uptake into neutral lipid was inhibited 68%, and the radioactivity in internal free AA was fourfold greater in the presence of indomethacin.

12-O-tetradecanoylphorbol-13-acetate

The phorbol ester, TPA, was found to inhibit I_R (500 nM, n = 8, example in Fig. 7*A*). 500 nM TPA has been shown to stimulate maximally the liberation of AA from phospholipid storage, which increases intracellular free AA and greatly increases the release of free AA and eicosanoids into the extracellular medium [11]. Over 85% of the conductance decrease with TPA was due to inhibition of I_R (determined with and without Ba^{2+}, as described for BPB). This inhibition was on average a 40 ± 25% reduction of I_R at rest and was completely reversible with wash.

To see if TPA could inhibit the effect of indomethacin, a ganglion was first exposed to perfusion with TPA, then TPA and indomethacin, and finally indomethacin alone. Steady state *I-V* curves for each of those conditions are depicted for a representative experiment in Fig. 7*B*. The stimulatory effect of indomethacin was decreased 64 ± 35% in the presence of TPA (n = 4 for 50–60 μM indomethacin and 500 nM TPA), suggesting indomethacin (and BPB, by inference) and TPA are acting through a common pathway to affect I_R.

Interaction Between Inhibitors of AA Metabolism and cAMP

In *Aplysia* neuron R15, serotonin (5HT) has been shown previously to modulate I_R through cAMP [2, 6, 19]. 5HT binds cell surface receptors to activate adenylate cyclase and thereby increase intracellular cAMP, which initiates a cascade of steps leading to an increase in I_R. We also found I_R in the giant neurons was increased with 5HT, as Gunning [22] reported previously. This stimulatory effect of 5HT was found to saturate at 50 μM.

We were interested in the possibility that the pharmacological agents used in this study were act-

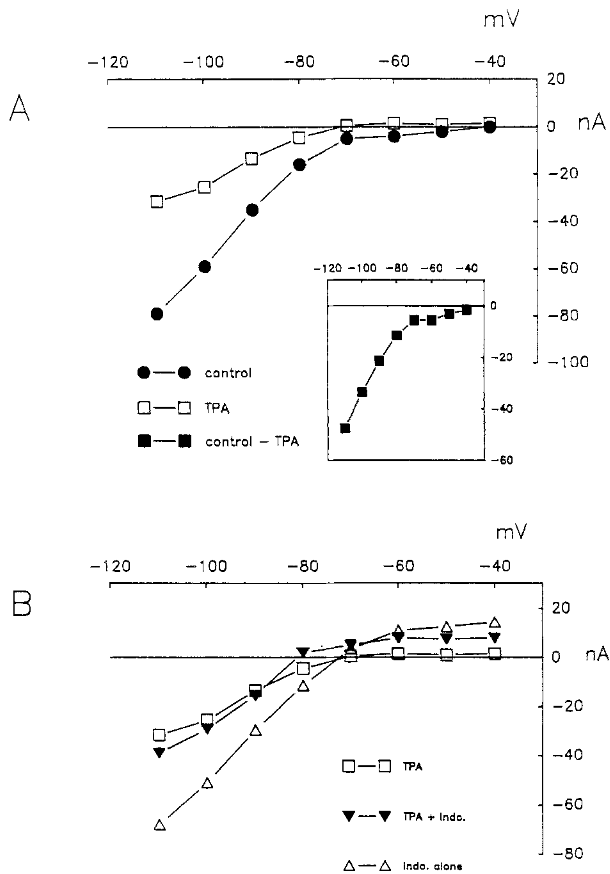

Fig. 7. Effect of TPA on I_R. (*A*) *I-V* curves for LP1 (held at −50 mV) under perfusion with control ASW (filled circles) or 500 nM TPA (open squares) were measured and leak subtracted as described in Fig. 1. *I-V* curve in the presence of TPA was measured after 45 min of perfusion with TPA. The difference curve, or TPA-inhibited current, is depicted in the inset graph (filled squares). (*B*) The effect of indomethacin in the presence of 500 nM TPA was determined. *I-V* curves were measured for LP1 in the presence of the following conditions (in sequence): 500 nM TPA (open squares, same as in *A*); 500 nM TPA + 60 μM indomethacin (filled inverse triangles); 60 μM indomethacin alone (open triangles)

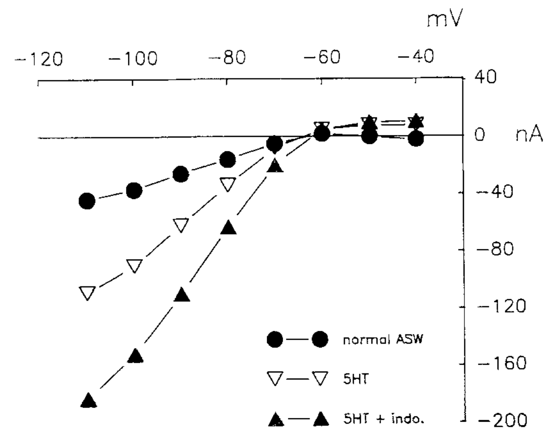

Fig. 8. Effect of indomethacin with saturating 5HT. *I-V* curves for R2 under perfusion with control ASW (filled circles), ASW + 50 μM 5HT (open inverse triangles), or 50 μM 5HT + 50 μM indomethacin (filled triangles) were measured and leak subtracted as described in Fig. 1. 50 μM 5HT was determined to be a saturating dose for stimulation of I_R (*data not shown*)

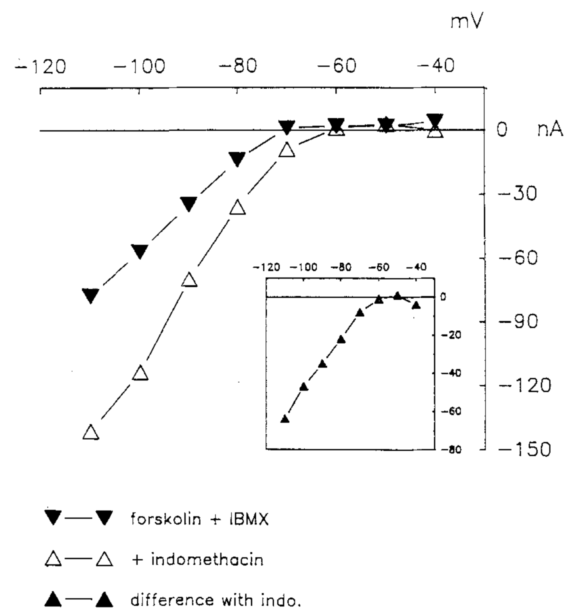

Fig. 9. Effect of indomethacin with maximally stimulated adenylate cyclase. *I-V* curves for R2 in the presence of forskolin (50 μM) and IBMX (200 μM) in concentrations sufficient to occlude the stimulatory effect of 50 μM 5HT (*data not shown*). *I-V* curves (measured and leak-subtracted as described in Fig. 1) in the presence of forskolin and IBMX (filled inverse triangles), or forskolin and IBMX + 150 μM indomethacin (open triangles) are shown. Indomethacin-induced current is depicted in the inset graph (filled triangles)

ing through the *c*AMP cascade to modulate I_R. Interaction at the level of the 5HT receptor was addressed first. Saturating 5HT did not block the indomethacin- or BPB-induced increase in I_R. In fact, the stimulatory effect of indomethacin was apparently unattenuated in the presence of saturating 5HT (Fig. 8). These results preclude the 5HT receptor as the site of action of indomethacin, or, by inference, of the other modulators of AA metabolism. However, these agents could be acting at a point downstream from 5HT receptor-linked adenylate cyclase activation. A combination of the adenylate cyclase activator forskolin and the phosphodiesterase inhibitor isobutylmethylxanthine was used to bypass cell surface receptor activation of adenylate cyclase. Doses of these drugs, sufficient to occlude the effect of a saturing dose of 5HT, did not block the response of I_R to indomethacin (50–150 μM, Fig. 9). Therefore, under conditions of po-

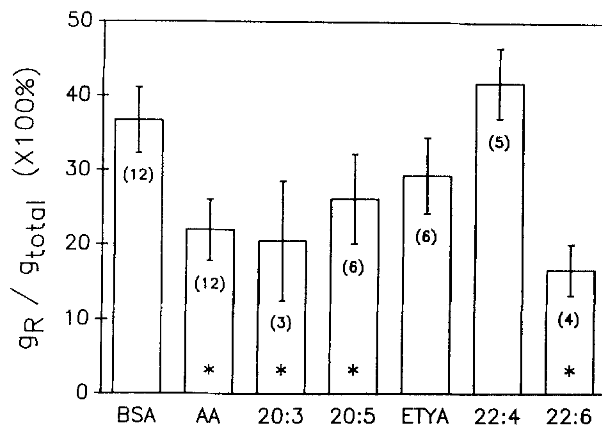

Fig. 10. The effect of dialysis with AA and other unsaturated fatty acids on I_R. Groups of four to eight abdominal and/or left pleuropedal ganglia were placed in dialysis tubing and dialyzed in perfusion medium for 40–46 hr as described in Materials and Methods. The mean inward rectifier conductance (expressed as percent of the total conductance to normalize for cell size differences) was determined for dialysis in perfusion medium alone (g_R/g_{total} = 31.1 + 2.1 SE %, n = 6, not graphed), or with 0.05% (wt/vol) BSA, or BSA with 20 μM of fatty acid. The abbreviated names of the fatty acids tested are listed under the graph: *BSA* = BSA alone; *20:3* = eicosatrienoic acid (ω9); *20:5* = eicosapentaenoic acid (ω3); *ETYA* = eicosatetraynoic acid; *22:4* = docosatetrenoic acid (ω3); *22:6* = docosahexaenoic acid (ω3). The number of measurements averaged are listed in parentheses. An asterisk (*) indicates the mean g_R was significantly different from the control at the 99% confidence level (BSA only) as determined by student's t test

tent adenylate cyclase activation and phosphodiesterase inhibition, I_R was still stimulated with indomethacin, suggesting a mechanism of activation of I_R independent of cAMP metabolism.

Dialysis with AA and Other Fatty Acids

Direct application of AA (up to 50 μM) did not consistently produce changes in I_R. However, long-term incubation in AA was found to inhibit I_R. Ganglia were placed in dialysis tubing for dialysis in ASW supplemented with a diluted nutrient medium (termed perfusion medium; described in Materials and Methods), either alone, with BSA, or with BSA and 20 μM AA or other unsaturated fatty acids (at 10°C for 40–46 hr). Following dialysis, the ganglia were desheathed and the inward rectifier conductance of the giant neurons was measured. The mean inward rectifier conductances (expressed as percent of total conductance to normalize for differences in cell sizes) for each condition are compared in Fig. 10. In the presence of 20 μM AA, I_R was inhibited ~40% relative to dialysis with BSA alone. However, several other unsaturated fatty acids were equally as effective for inhibition of I_R, including eicosatrienoic (20:3ω9), eicosapentaenoic (20:5ω3), and docosahexaenoic (22:6ω3) acid. Eicosatetraynoic acid (ETYA) and docosatetraenoic acid (22:4ω6) were the only fatty acids tested which did not produce a significant change in I_R.

Effects of Prolonged Perfusion on I_R

TPA and BPB altered I_R in ways consistent with the activity of these substances on AA metabolism [11]. The fact that BPB had a potent effect on I_R in the resting state suggested that the constant AA turnover (described in the companion paper [11]) may be providing free AA to mediate constitutive inhibition of I_R. To test this idea, we attempted to deplete the giant neuron of stored AA, reasoning that if AA was involved in inhibition of I_R in the resting state, then loss of stored AA might reveal some of this constitutively inhibited I_R. With the knowledge that AA is continuously released from storage in ganglia, we used extended perfusion with BSA to strip the giant neurons of AA, and tested the effect of this perfusion on the magnitude of I_R.

Steady-state I_R was measured in giant neurons in freshly dissected and desheathed ganglia, and then the ganglia were placed under perfusion with perfusion medium for ~12 hr. Finally, the ganglia were placed under perfusion with control ASW, and the steady-state I_R of the giant neurons was measured again. As shown in Fig. 11, after this protocol with perfusion medium alone, the mean I_R was decreased. However, if 0.5 mg/ml BSA was added to the perfusion medium, I_R was significantly larger after 12 hr of perfusion (Fig. 11).

To test whether this effect of perfusion with BSA could be due to loss of AA, the protocol was repeated with perfusion medium containing BSA and 5–10 μM AA. As depicted in Fig. 11, the addition of AA substantially decreased the stimulatory effect of perfusion with BSA alone. Conversely, we hypothesized that a decrease in phospholipase activity might limit the amount of AA lost and thereby decrease the stimulatory effect of perfusion with BSA. In fact, pretreatment of giant neurons with BPB completely abolished the effect of perfusion with BSA (Fig. 11).

Discussion

Pharmacological Agents Alter AA Metabolism and I_R

In the preceding paper [11], constant turnover of internal free AA was demonstrated in *Aplysia* gan-

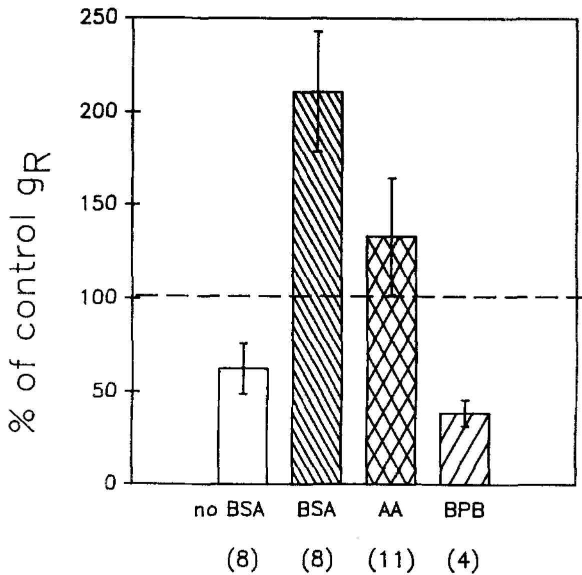

Fig. 11. The effect of extended perfusion on I_R. Ganglia were prepared for extended perfusion as described in Materials and Methods. Groups of four to eight abdominal and/or left pleuropedal ganglia were pinned out and desheathed on a single plate. The plate was placed under perfusion with sterile, control ASW. The giant neurons were successively placed under voltage clamp (held at −50 mV), the time for the start of voltage clamp for each was noted, and the resting steady-state inward rectifier conductance was measured. This initial conductance was compared to the inward rectifier conductance measured again in the same cell after perfusion for 12 hr with sterile perfusion medium (described in Materials and Methods) alone (blank), or containing 0.05% (wt/vol) BSA (left diagonal), or BSA and 5–10 μM AA (cross-hatch), or with BSA after pretreatment with 100 μM BPB for 30 min (right diagonal). Inward rectifier conductance for giant neurons treated with BPB was measured initially *before* exposure to BPB

glia. We concluded the constant release of free AA and eicosanoids that accompanied this turnover was too large to be solely the result of futile cycling. Therefore, we hypothesized that the free AA or eicosanoids provided by this constant turnover must have a physiological role. In this paper, we have identified constitutive inhibition of I_R as a potential role. This conclusion is based in part upon a correlation between the effects of several pharmacological agents on I_R on the one hand, and on AA metabolism on the other. BPB, an inhibitor of AA turnover, activated I_R, and TPA, a stimulator of AA turnover, inhibited I_R.

BPB has been shown to inhibit liberation of AA from phospholipid and decrease internal free AA in *Aplysia* [11]. At rest, I_R was stimulated with BPB, suggesting that inhibition of phospholipase activity and a concomitant decrease in availability of free AA resulted in stimulation of I_R. Therefore, resting phospholipase activity must be high enough to provide sufficient free AA to maintain constitutive inhibition of I_R, which is revealed in the presence of BPB. Although BPB is a fairly nonspecific alkylating agent, the widespread evidence that BPB is an effective inhibitor of PLA_2, both in vivo and in vitro (*see* accompanying paper [11] for references), supports the likelihood that BPB is acting through this pathway in the giant neurons.

The results with TPA support this model. TPA has been shown to stimulate liberation of AA from phospholipid in *Aplysia* nervous system and TPA has been shown to inhibit I_R. If TPA acts by stimulating phospholipase activity, this means that the resting activity, although high, is not maximal. That is, the resting phospholipase activity is high enough to shut down most, but not all, of the potentially available I_R. If all of the constitutively inhibited I_R is revealed with a saturating dose of indomethacin, then over 80% of the potentially available I_R is inhibited at rest.

Indomethacin is often used to selectively inhibit prostaglandin synthesis. Piomelli et al. [48] reported that prostaglandin E_2 production in *Aplysia* nervous system was inhibited by indomethacin with an IC_{50} value of 0.5 μM. We found the half maximal concentration for indomethacin-induced stimulation of I_R was about 100 μM, which is not consistent with indomethacin acting as selective inhibitor of prostaglandin production as the mechanism for stimulation of I_R. High concentrations (100–500 μM) of indomethacin have been shown to inhibit phospholipase activity [21, 25, 34, 50], diacylglycerol lipase activity [49] and AA uptake [35]. With 250 μM indomethacin (saturating with respect to I_R stimulation) we were only able to detect an effect on AA uptake. After two hours incubation in 0.5 μM AA in the presence of indomethacin, radioactivity in the internal free AA pool was fourfold greater, and uptake into neutral lipid was decreased 68%, while uptake into phospholipid was unchanged. If indomethacin specifically inhibited the fatty-acyl transferase which catalyzes esterification of AA-CoA into neutral lipid (*see* accompanying paper [11], Fig. 1, pathway *III*), we would expect to see an *increase* in uptake into phospholipid, and a smaller increase in internal free AA than observed. Therefore, specific inhibition of the fatty acyl-CoA synthetase (*see* accompanying paper [11], Fig. 1, pathway *II*) accounts best for the effect of indomethacin. We would expect to see decreased AA-CoA in ganglia commensurate with the magnitude of inhibition of uptake into neutral lipid, a hypothesis soon to be tested. Neufield et al. [44] demonstrated that a mutant cell line lacking synthetase activity specific for AA was also deficient in prostaglandin production

and bradykinin-stimulated liberation of AA from phospholipid. Therefore, acyl-CoA synthetase activity can be very important for regulation of free AA and the production of eicosanoids.

The conclusion that indomethacin is not likely acting as a specific cyclooxygenase inhibitor to stimulate I_R is supported by the lack of effect of acetylsalicylic acid, another inhibitor of cyclooxygenase. Furthermore, this means prostaglandins are not likely responsible for inhibition of I_R. At concentrations from 25–100 μM, indomethacin has been shown to inhibit both cyclooxygenase and lipoxygenase activity [7, 10, 53, 55, 57]. However, if indomethacin is acting through inhibition of lipoxygenases, the lipoxygenase inhibitor NDGA also should have stimulated I_R, but it was found to be ineffective. The available evidence is most consistent with the conclusion that free AA is involved in constitutive inhibition of I_R, and that the eicosanoids produced likely have another physiological role.

Altering AA in Storage Affects I_R

AA has been shown to have direct effects on cellular physiology, particularly with regard to AA-induced mobilization of intracellular calcium [4, 14, 17, 32, 42, 52, 62, 64]. In permeabilized pancreatic islet cells, intracellular calcium was mobilized with AA in a manner similar to that produced by IP_3 [60, 62]. Direct application of AA has been shown to activate potassium currents in several systems. In patch-clamp studies of *Aplysia* sensory neurons, AA increased the open probability of individual S-channels [48]. Most recently, outwardly rectifying potassium currents of heart and smooth muscle cells under patch clamp were observed to be activated directly by AA and other unsaturated fatty acids [30, 46].

In addition to the pharmacological evidence, a direct indication of AA involvement in inhibition of I_R came from the experiments using extensive perfusion. The observed constitutive release of AA [11] suggested extensive perfusion of ganglia with BSA should remove stored AA, and disinhibit I_R. Such perfusion did in fact increase I_R, and this stimulation was decreased by including AA in the perfusion medium, or by pretreatment with BPB. Paradoxically, extensive perfusion *without* BSA led to a *decrease* in I_R. We do not know the cause of this; however, we plan to investigate the possibility that extensive perfusion may lead to "calcium-loading" due to decreased membrane resistance. Extensive perfusion was often observed to increase the leak current in the giant neuron (*data not shown*). The increase in intracellular calcium which likely accompanies the increased leak current could activate phospholipase, leading to an increase in internal free AA and inhibition of I_R.

The effect of BPB on the perfusion-induced activation of I_R was determined by comparing inward rectifier conductance *before* exposure to BPB to the conductance after BPB pretreatment and perfusion with BSA for ~12 hr. Like perfusion with no BSA, the inward rectifier conductance in giant neurons treated with BPB was actually smaller after overnight perfusion with BSA. Again, we cannot explain this phenomenon, but BPB clearly prevents any increase in conductance, consistent with inhibition of phospholipase.

Exogenous AA did not affect I_R when applied acutely during electrophysiological recording, consistent with the finding that exogenous AA did not increase the apparent size of the internal free AA pool [11]. Kim and Clapham also found that AA was ineffective for activation of an atrial potassium channel when added extracellularly or added to the pipette in cell-attached patches; this potassium channel was activated only with application of AA to inside-out patches [30]. However, we found that I_R was smaller after extensive dialysis in AA-containing medium. With AA, inhibition of I_R was observed after 24 hr of dialysis (*data not shown*), although the inhibition was more significant after ~40 hr. This time dependence may be explained by a gradual increase in the size of the AA storage pools with dialysis, which may lead to elevated internal free AA and inhibition of I_R. Cells in culture have been shown to slowly increase the AA content of lipids when excess AA is available in the medium [59]. McGee et al. [41] recently reported that enrichment of phospholipids with AA altered the gating kinetics of a delayed rectifier potassium channel in a neuroblastoma *x* glioma cell line.

I_R was also found to be decreased after dialysis with other polyunsaturated fatty acids. These fatty acids were chosen for testing because of their similarity to AA, based upon their ability to undergo catalysis or compete with AA for catalysis by eicosanoid-producing enzymes [9, 18, 37], and because of their reported presence in *Aplysia* nervous system [48]. Therefore, although AA may not exclusively mediate inhibition of I_R, the predominance of AA in phospholipids in *Aplysia* relative to the other fatty acids tested [48] suggests AA likely has the most significant physiological role.

AA- and *c*AMP-Mediated Modulation of I_R

The *c*AMP-mediated activation and AA-mediated inhibition of I_R that we report is reminiscent of findings for the serotonin-sensitive potassium current

(I_S) in *Aplysia* sensory neurons. In these cells, 5HT acts through *c*AMP to inhibit I_S [54, 56], and the tetrapeptide FMFRamide acts through AA to activate the same current [48, 47]. AA and *c*AMP can interact at the level of the channel to modulate the same population of I_S channels in sensory neurons, as demonstrated in cell-attached patch recordings in which FMFRamide could open S-channels closed by 5HT-induced elevation of *c*AMP [5]. In the present paper, modulators of AA metabolism were found to alter I_R without acting through *c*AMP metabolism. One explanation for the additivity of the effects of *c*AMP and the modulators of AA metabolism is that AA may mediate inhibition of a population of I_R channels distinct from the population regulated by *c*AMP. However, the molecular mechanism of AA-mediated inhibition of I_R remains to be determined.

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