CMLS, Cell. mol. life sci. 53 (1997) 61–68
1420-682X/97/010061-08 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Arachidonic acid as an endogenous signal for the glutathione-induced feeding response in *Hydra*

P. Pierobon[a,*], L. De Petrocellis[a], R. Minei[a] and V. Di Marzo[b]

[a]*Istituto di Cibernetica, C.N.R., via Toiano 6, I-80072 Arco Felice, Naples (Italy), Fax +39 81 526 7654, e-mail: pierobon@cell.na.cnr.it*
[b]*Istituto per la Chimica di Molecole di Interesse Biologico, C.N.R., I-80072 Arco Felice, Naples (Italy)*
Received 30 July 1996; received after revision 27 September 1996; accepted 18 October 1996

Abstract. Phospholipase A_2-derived arachidonic acid (AA) and related metabolic products represent an important pathway involved in the regulation of growth and morphogenesis as well as in oxidative processes in cnidarian tissues. Here we present data on the participation of AA in the glutathione (GSH)-induced feeding response in *Hydra vulgaris*. Under conditions in which it produces the feeding response (which consists mainly of mouth opening followed by mouth closure), GSH dose-dependently induced the release of free arachidonic acid from live polyps. Phospholipase A_2 inhibitors blocked this release and enhanced the duration of GSH-induced *Hydra* mouth opening. Accordingly, AA and, to a smaller extent, α-linolenic acid, were found to reduce the duration of the feeding response in a 10–100 μM concentration range, and this effect resulted mainly from earlier times of mouth closure. 11-(*R*)-hydroxy-eicosa-5*Z*,8*Z*,12*E*,14*Z*-tetraenoic acid, a lipoxygenase metabolite of AA in *H. vulgaris*, reproduced the effects of its precursor fatty acid at lower concentrations. The kinetics of GSH-induced arachidonate release and GSH-induced feeding response were correlated, suggesting that AA might modulate the mechanisms controlling mouth closure. Finally, the role of AA in the facilitation of the mouth-closing phase of the feeding response was further supported by the finding that: (1) exogenous AA reversed the effect on the feeding response of γ-aminobutyric acid, which normally delays the times of mouth closure, and (2) endogenous AA was also released in the absence of GSH from live polyps stimulated with antho-RFamide, a neuropeptide abundant in hydrozoans. We suggest that in *Hydra* AA may act as a second messenger following chemical stimulation.
Key words. Arachidonic acid; glutathione; γ-aminobutyric acid; signal transduction; *Hydra*.

Abbreviations. AA = arachidonic acid; BPB = 4-bromophenacyl bromide; cGMP = guanosine 3′:5′-cyclic monophosphate; CNS = central nervous system; DMSO = dimethyl sulfoxide; GABA = γ-aminobutyric acid; GTP = guanosine 5′-triphosphate; (11-*R*)-HETE = 11-(*R*)-hydroxy-eicosa-5*Z*,8*Z*,12*E*,14*Z*-tetraenoic acid; (9-*R*)-HOTrE = 9-(*R*)-hydroxy-octadeca-10*E*,12*Z*,15*Z*-trienoic acid; (11-*R*)-HPETE = 11-(*R*)-hydroperoxy-eicosa-5*Z*,8*Z*,12*E*,14*Z*-tetraenoic acid; HPLC = high-pressure liquid chromatography; (9-*R*)-HPOTrE = 9-(*R*)-hydroperoxy-octadeca-10*E*,12*Z*,15*Z*-trienoic acid; α-LA = α-linolenic acid; OOPC = oleyloxyethylphosphorylcholine; PLA_2 = phospholipase A_2; PUFAs = polyunsaturated fatty acids; TBPS = *t*-butylbicyclophosphorothionate.

The nerve net of the freshwater polyp *Hydra* (Cnidaria, Hydrozoa) is currently regarded as one of the most primitive nervous systems to have evolved [1]. Neurons are dispersed in the epithelia and are interconnected by neurites into a functional network, or to non-neuronal effectors by uni- and bidirectional synapses (see ref. 2 for a concise review). The nervous net spreads homogeneously over the entire body except in the head region, where the fibres are condensed to form a circular ring, possibly representing a rudimentary cephalization of the system [3]. This seemingly simple, two-dimensional nervous system shows neurophysiological and cellular specializations as sophisticated as those of many higher invertebrates in terms of electrogenesis, coordination of sensory and motor functions, plasticity, development and regulation of growth [4].

Neurotransmission in *Hydra* has been considered to be essentially peptidergic, owing to the presence of several neuropeptides [5, 6]. Progress in identifying nonpeptidergic neurotransmitters in cnidarians has been slow, and the results confusing or even contradictory [7–9]. On the other hand, recent evidence has been obtained of the occurrence of dopamine in *Hydra* tissues [10]. Dopaminergic agonists and antagonists have been shown [11] to modify a typical behaviour of *Hydra*, the feeding response, where polyps stimulated by glutathione (GSH) respond with tentacle writhing and mouth opening followed eventually by mouth closure. Moreover, we have recently shown that γ-aminobutyric acid (GABA) and its positive allosteric modulators prolong the duration of the feeding response in *H. vulgaris* through a receptor-mediated action [12]. The enhancement of the response induced by GABA is potentiated by benzodiazepines and suppressed by the specific $GABA_A$ antagonist bicuculline. Neuroactive steroids, picrotoxin and the specific Cl^- channel ligand *t*-butylbicyclophosphorothionate (TBPS) respectively prolong or decrease response duration, suggesting that a population of $GABA_A$-like recep-

* Corresponding author.

tors and/or GABA-gated Cl^- channels are involved in the regulation of the feeding response (A. Concas, P. Pierobon, M. C. Mostallino, G. Marino, R. Minei and G. Biggio, unpublished results).

Arachidonic acid (AA) and its metabolites as well as other polyunsaturated fatty acids (PUFAs) are known to affect ion channels, protein kinases and other key regulatory enzymes, thus suggesting a role in neurotransmitter signal transduction in both mammalian and invertebrate nervous systems (for recent reviews see [13, 14]). AA also affects motility and contraction in *H. oligactis*, indicating a possible short-term action exerted by these compounds on excitable cnidarian tissues [15]. AA, α-linolenic acid (α-LA) and their lipoxygenase metabolites have been found in different *Hydra* species [16] where they appear to be involved in the control of metamorphosis, tentacle regeneration and budding. In particular, an ω10-lipoxygenase responsible for the enantioselective peroxidation of AA, α-LA and other C_{18} and C_{20} PUFAs has been characterized in *H. vulgaris* [17–19]. In this species high levels of the enzyme mostly responsible for PUFA release from membrane phospholipids, i.e. phospholipase A_2 (PLA_2), have also been found [20]. Recently, much attention has focussed on the ability of PUFAs directly or indirectly to regulate the activity of $GABA_A$ receptor-coupled Cl^- channels in mammalian central nervous system (CNS) [21, 22] and to produce long-term structural and functional changes in invertebrate neurons [23]. Based on this background, and bearing in mind the recently described effect of GABA on the feeding behaviour induced by GSH in *Hydra* [12], we have undertaken an in vivo study on the participation of AA in this chemoreceptor-mediated response. We present data on the role of AA as a possible modulator of the final phase of the GSH-induced feeding response in *H. vulgaris*.

Materials and methods

Materials. *H. vulgaris* (attenuata) were originally obtained from Prof. P. Tardent, Zurich, and cultured asexually in our laboratory by a modification of the method of Loomis and Lenhoff [24]. All the reagents were purchased from Sigma except oleyloxyethylphosphorylcholine (OOPC), which was purchased from Biomol, and 9-(*R*)-hydroxy-octadeca-10*E*,12*Z*,15*Z*-trienoic acid [(9-*R*)-HOTrE], which was purified from *Hydra* homogenates as previously described [18].

Study of the effect of various substances on *Hydra* feeding response. The feeding reaction was studied by the procedure described by Lenhoff [24], modified as follows: polyps from 'homogeneous' populations, about 3 weeks old, carrying one or two buds, and starved for at least 3 days before the trial, were equilibrated to room temperature in 3.5-cm-diameter Falcon dishes containing 1 ml of physiological solution (1 mM $CaCl_2$, 0.1 mM $NaHCO_3$, buffered with 1 mM Tris-HCl, pH 7.35) (controls); the dishes were divided into four chambers by glass partitions in order to allow simultaneous testing and recording of individual mouth opening (T_i) and closing (T_f) times for each animal. In other series of such dishes AA, α-LA, 11-(*R*)-hydroxy-eicosa-5*Z*,8*Z*,12*E*,14*Z*-tetraenoic acid [(11-*R*)-HETE], (9-*R*)-HOTrE, 4-bromophenacyl bromide (BPB), OOPC, GABA or TBPS were added immediately before the test. Lipophilic molecules were dissolved in small amounts of dimethyl sulfoxide (DMSO) and diluted to working concentrations in physiological solution, so that final DMSO content in the test solution did not exceed 1 µl/ml. In these experiments equal amounts of DMSO were added to control solutions. All PUFAs were freshly prepared and added to test solutions immediately before use in order to avoid degradation of the molecules. The test was started by adding reduced GSH at different concentrations (1–10 µM); six to eight animals were tested per group and per GSH concentration. Scoring of mouth opening and closing was performed using a cold light stereo microscope (Wild) and times were recorded by independent observers. After the first mouth closure healthy animals could reopen and close their mouths repeatedly, so T_f was always measured at the first mouth closing. In every experiment a control series was included; for each substance tested the experiments were repeated several times. All the experiments were carried out in an air-conditioned environment at 22 °C.

Behavioural data were analysed as follows: in each experiment the duration of the response to different GSH doses in the absence or presence of different drugs was measured and the kinetics of response analysed by linear regression (fig. 1). When different regression curves were obtained, percentages of increase (or, as negative values, of decrease) versus respective control samples were calculated for a given GSH concentration. Statistical significance of differences was evaluated by ANOVA followed by Scheffè's test. A P value of <0.05 was considered statistically significant.

Study of the effect of GSH on arachidonate release from *Hydra* polyps. Release of [^{3}H]AA in vivo was monitored by the following procedure: 'standard' polyps, starved for 4 days before the test, were incubated for 8 h with 3.7 MBq/l [^{3}H]AA (NEN, 8510 TBq/mol), washed three to five times in physiological solution and then placed in groups of 50 in Falcon dishes containing 2 ml of physiological solution. Incubation of live polyps with [^{3}H]AA for 8 h resulted in the incorporation of 23.2% of the fatty acid in the *sn*-2 position of phosphoglycerides [20]. Incubations with live polyps for less than 30 min (as in the feeding response assay conditions) did not lead to significant incorporation of AA into membrane phosphoglycerides. The test was started by adding GSH at the desired concentration; 500-µl

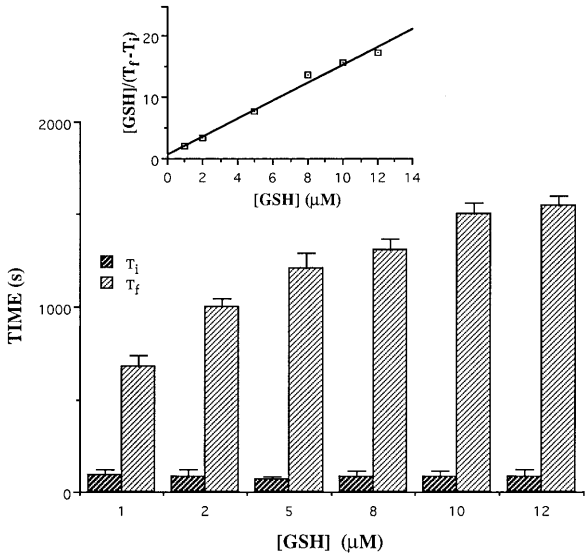

Figure 1. Response to GSH. Columns represent times of mouth opening (T_i) and mouth closure (T_f) at different GSH concentrations, expressed in seconds. Duration of the response (i.e. the time interval $T_f - T_i$) increases linearly with increasing GSH concentrations, and the increase is due to prolonged T_f times, while T_i values do not vary significantly. Data are shown from a typical experiment; vertical bars represent individual variability within sample groups. Kinetics of response are calculated by a modified Lineweaver-Burk plot [24]; the curve in the inset is obtained by linear regression analysis of $[GSH]/T_f - T_i$ ratios from several experiments.

aliquots of the test solutions were collected at different times (0, 5 and 15 to 20 min) after GSH administration. The radioactivity released was measured by a liquid scintillation counter (Packard, USA). Samples collected at corresponding times from animals not treated with GSH were used as controls. Aliquots were analysed by high-pressure liquid chromatography (HPLC) as described previously [20] in order to determine the contribution of AA and its possible metabolites to total radioactivity released from polyps. As determined in a previous study [20], AA release from live polyps is directly proportional to AA released inside *H. vulgaris* cells and can be used as a measure for PLA_2-mediated release of AA from membrane phospholipids.

Results

Participation of PLA_2 and of endogenous AA in GSH-induced feeding response. In normal, otherwise untreated polyps, the duration of the response to GSH, i.e. the time interval between mouth opening (T_i) and mouth closure (T_f), varies from about 10 min at 1 μM GSH to 20–25 min at 10 μM GSH (fig. 1), and is linearly correlated to GSH concentration in the 1–10 μM range of concentrations.

In order to assess whether endogenous AA was involved in the control of GSH-induced feeding response, BPB and OOPC, two different inhibitors of PLA_2, the enzyme catalysing the release of free arachidonate from membrane phospholipids, were tested. Both BPB and OOPC induced an increase in duration of the response at 1 μM concentrations (fig. 2). This effect was exerted by prolonging times of mouth closure up to 30 min, and was potentiated (in the case of OOPC, significantly) by co-incubation with 100 μM GABA, which alone is also capable of increasing the response duration (fig. 2).

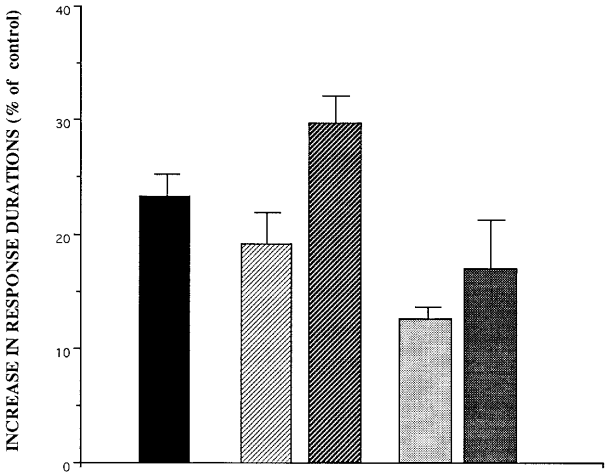

Figure 2. Effects of GABA 100 μM (■), OOPC 1 μM (▨), BPB 1 μM (▨), OOPC plus GABA (▨) and BPB plus GABA (■) on the duration of the feeding response induced by 10 μM GSH. The effects are expressed as the percent increase of the response induced by 10 μM GSH (100%, see fig. 1), and correspond to response durations statistically different from those to GSH only ($P < 0.001$). Both GABA and OOPC versus OOPC plus GABA are also significantly different ($P < 0.05$), while differences between GABA or BPB versus BPB plus GABA are not significant.

Next, we wanted to determine whether GSH treatment would induce AA release from *Hydra* membrane phospholipids. Exposure to GSH of polyps prelabelled overnight with [^{3}H]AA produced, under the same incubation conditions used to monitor the feeding response, a significant release of AA-bound radioactivity (figs 3 and 4). HPLC analysis of samples showed that free [^{3}H]AA was the component mostly responsible for the radioactivity (data not shown). The kinetics of release varied according to GSH doses (fig. 3): in samples treated with 10 μM GSH, where mouth closure is normally observed after 20–25 min, the increase in radioactivity levels was detectable upon mouth opening and progressed with time. At 1 μM GSH, where mouth closure is normally observed after 10 min, an initial peak of release was observed after 5 min. In the presence of OOPC, radioactivity levels remained unchanged with respect to control throughout the time of the experiment (fig. 4). Finally, a significant, albeit smaller, release of free [^{3}H]AA (fig. 4) was also observed, in the absence of GSH, in animals treated with 1 μM pGlu-Gly-Arg-Phe-NH_2 (anthoRFamide).

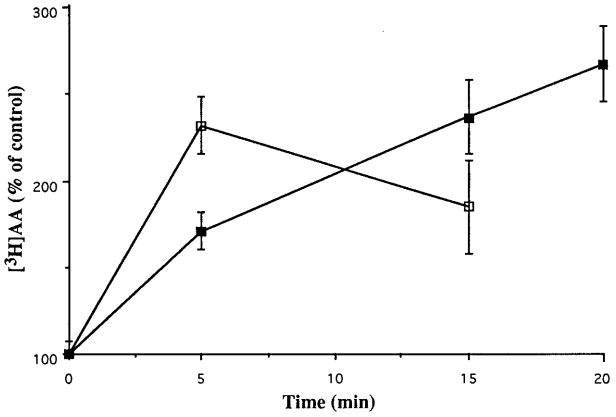

Figure 3. Release of [^{3}H]AA from intact polyps, prelabelled with [^{3}H]AA, 0, 5, 15 and 20 min after treatment with 1 μM (□) and 10 μM GSH (■). The release is measured as percent of [^{3}H]AA released from control (unstimulated) polyps. For basal [^{3}H]AA release see figure 4. Data of [^{3}H]AA release at 1 μM GSH after 15 min and at 10 μM GSH after 20 min are biased by the onset of a new cycle of response. Data are means ± SD of three separate experiments.

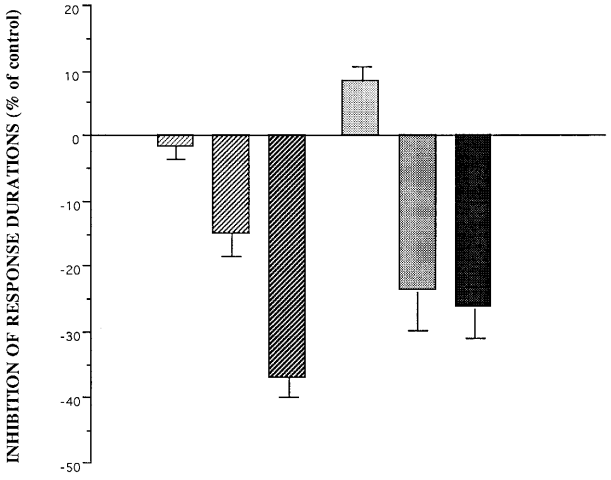

Figure 5. Effect of 1 μM (▨), 10 μM (▨) and 100 μM (▨) AA; 1 μM (▩), 10 μM (▩) and 100 μM (■) α-LA on the feeding reaction induced by 10 μM GSH. The effects are expressed as the percent inhibition of the response induced by 10 μM GSH (100%, see fig. 1) and correspond to response durations statistically different ($P < 0.001$) from those to GSH only, except with 1 μM AA and α-LA.

Effect of AA and other PUFAs and of their derivatives on GSH-induced feeding response. Exposure to AA dose-dependently reduced duration of the feeding response (fig. 5); the degree of inhibition also depended on GSH concentrations (not shown), with a maximum of about −38% at 10 μM GSH. 1 μM AA, which did not modify response duration when administered simultaneously with GSH, was able to shorten times of the response when polyps were pretreated with the fatty acid for 30 min before the test (fig. 6). The degree of inhibition (about −20%) by 1 μM AA following pretreatment was comparable to that obtained when stimulating the polyps with GSH in the presence of 1 μM (11-*R*)-HETE (fig. 6), the most abundant derivative of AA in *H. vulgaris*. The effect of (11-*R*)-HETE was enantioselective since its enantiomer (11-*S*)-HETE was not active (fig. 6). In all cases, shortening of the response resulted from earlier times of mouth closing with respect to control; delayed mouth opening times were observed only occasionally (data not shown).

Administration of α-LA also produced a dose-dependent decrease in duration of the GSH-induced feeding

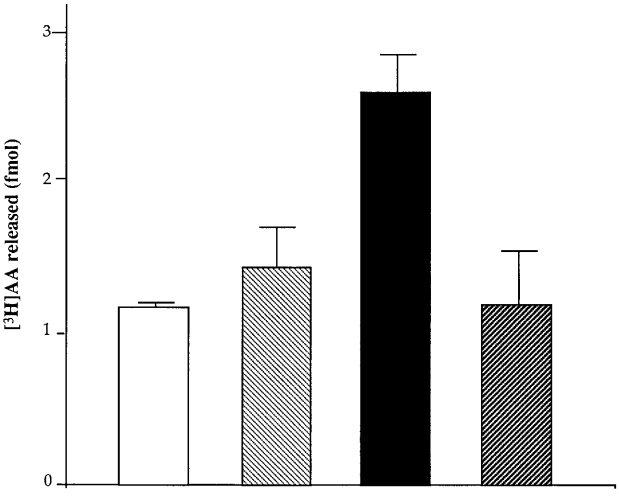

Figure 4. Release of [^{3}H]AA (fmol) from intact polyps after 15-min treatment with 10 μM GSH (■), 10 μM GSH + 1 μM OOPC (▨), and 1 μM antho-RFamide (▧). White column represents basal [^{3}H]AA release from control animals. The effects of antho-RFamide and GSH are statistically different from controls ($P < 0.05$ and 0.001, respectively). Data are means ± SD of three separate experiments.

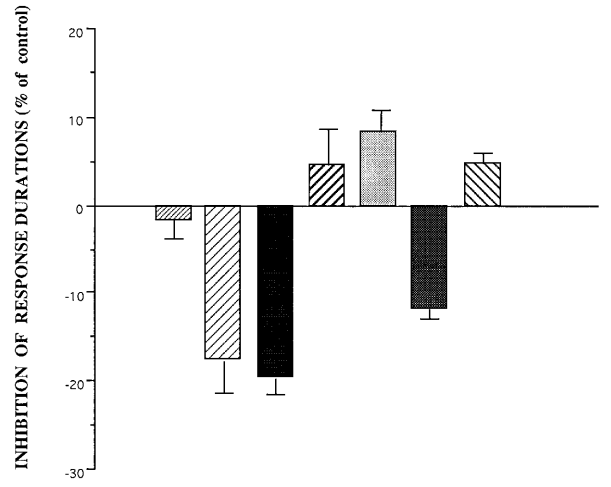

Figure 6. Effects of 1 μM AA, without (▨) or with (▨) 30-min pretreatment of polyps, and of 1 μM (11-*R*)-HETE (■), 1 μM (11-*S*)-HETE (▨), 1 μM α-LA (▩), 1 μM (9-*R*)-HOTrE (■) and 1μM 9−keto-HOTrE (▧) on the feeding response to 10 μM GSH. The effects are expressed as the percent inhibition of the response induced by 10 μM GSH (100%, see fig. 1) and correspond to response durations statistically different ($P < 0.001$) from those to GSH only, except for 1 μM AA, 9-keto-HOTrE and 1 μM (11-*S*)-HETE.

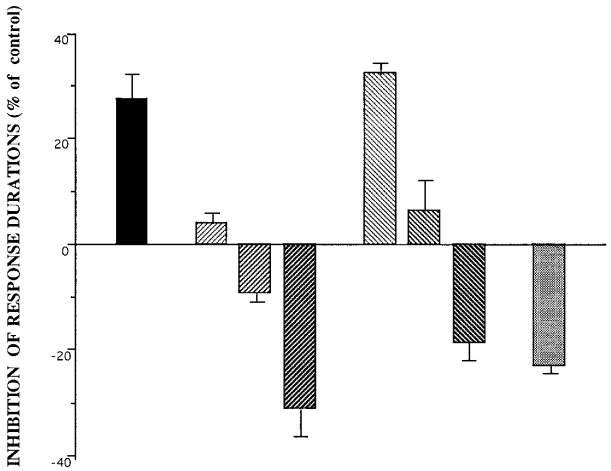

Figure 7. Effects of 1 μM (▨), 10 μM (▨) and 100 μM (▨) AA; 1 μM (▧), 10 μM (▧) and 100 μM (▧) α-LA; and of 1 μM TBPS (▩) in the presence of 100 μM GABA on the response induced by 10 μM GSH. The effect of 100 μM GABA is also shown (■). The effects are expressed as the percent inhibition of the response at 10 μM GSH (100%, see fig. 1); differences in response durations are statistically significant ($P < 0.001$) versus GSH + GABA, except for 1 μM α-LA.

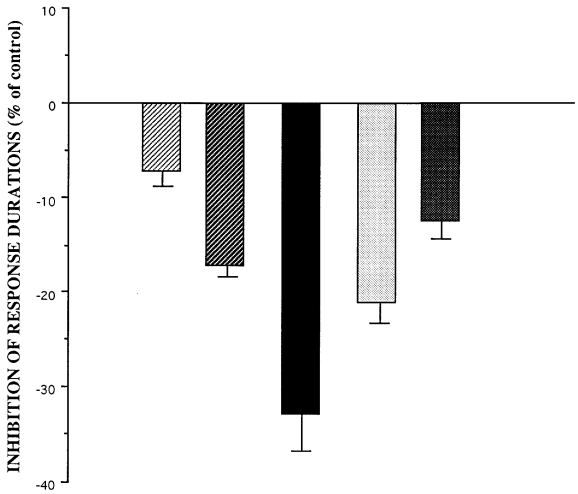

Figure 8. Effects of 10 μM (▨) and 100 μM (▨) AA plus 1 μM TBPS. The effects are expressed as the percent inhibition of the response induced by 10 μM GSH (100%, see fig. 1). 100 μM AA and TBPS association produce a significant difference ($P < 0.001$) versus 1 μM TBPS alone (■), but not versus 100 μM AA. The effects of 10 μM AA, though not significant, are clearly not additive to TBPS. 10 μM (□) and 100 μM (■) AA are also shown in the figure. 1 μM TBPS significantly inhibits the response ($P < 0.001$).

response, albeit to a smaller extent (fig. 5). In this case the amount of reduction also depended on GSH concentrations (not shown), with a maximum of about −26% at 10 μM GSH. A reduction (−12%), smaller than that obtained with 1 μM (11-*R*)-HETE (−20%), was also obtained with the endogenous α-LA metabolite (9-*R*)-HOTrE, but not with its keto derivative, at 1 μM concentrations (fig. 6).

AA administration suppressed the increase in times of response produced by 100 μM GABA. The effect was already pronounced at 1 μM AA, was dose-dependent at a 1–100 μM concentration range, and was similar to that exerted by 1 μM TBPS (fig. 7). In the presence of 100 μM GABA, treatment with 10 and 100 μM α-LA also suppressed the GABA-induced enhancement of the response; maximal effect was observed with 100 μM α-LA, whereas 1 μM α-LA was not effective (fig. 7). Similar results were obtained with the endogenous AA and α-LA lipoxygenase metabolites (11-*R*)-HETE and (9-*R*)-HOTrE at 1 μM concentrations (−12% and −6% reduction respectively, data not shown).

Finally, the effect of 100 μM AA was comparable to that obtained in the presence of the convulsant drug TBPS (1 μM). Simultaneous TBPS and AA administration never resulted in an additive response (fig. 8) but in fact reduced the inhibitory action of TBPS. This effect was dose-dependent over a 1–100 μM AA concentration range with a statistically significant effect at 100 μM AA. The inhibition of the feeding response observed with 100 μM AA plus 1 μM TBPS was not significantly different from that observed with 100 μM AA alone.

Discussion

The feeding response has received much attention since its first description by Loomis [25], since it represents a simple and reliable model for the study of chemoreception. It consists of a sequence of events following prey capture, i.e. tentacle writhing, mouth opening, ingestion of prey and closing of the mouth. It is initiated by the association of reduced GSH with an external chemoreceptor [26]. Intensity and duration of the GSH-induced response are dose-dependent, saturable and partially antagonized by L-glutamic acid [27]. The feeding behaviour is activated by GSH or by a limited number of amino acids (proline, valine) in different cnidarian species; in *Hydra*, GSH is the specific activator of the feeding response [28]. The metabolism of GSH is not believed to play a role in eliciting the feeding behaviour, since treatment with azaserine, an inhibitor of γ-glutamyl transpeptidase, does not affect the GSH-induced response [29]. Moreover, the glutamyl residue and the tripeptide backbone have been reported to be essential for activation [30].

The anatomical pathways leading from chemoreceptors to the effector cells involved in the feeding response have been only partially identified. GSH receptors have been tentatively localized on nematocytes of tentacles [31, 32]. Characterization of the receptor protein(s) is in progress [33], but the molecular mechanisms underlying receptor activation are unknown. Formation and opening of the mouth are ultimately achieved by contraction of the longitudinal myofibrils embedded in the epitheliomuscular cells of the head, while mouth closure prob-

ably results from parallel relaxation of ectodermal longitudinal myofibrils and contraction of the circular myofibrils of the endoderm [34, 35], a process requiring nervous system coordination. Again, the molecular events by which termination of the response is achieved are still poorly understood, though recent findings that inhibitors of the nitric oxide/Guanosine 3′:5′-cyclic monophosphate (cGMP) pathway prolong duration of the response [36] suggest that its modulation may depend on complex inhibitory mechanisms rather than simple dissociation of GSH from its receptors.

Starting from the knowledge that (1) AA and its lipoxygenase metabolites are abundant in *Hydra* species [16], (2) GABA, possibly via $GABA_A$ receptors, delays the termination of *H. vulgaris* feeding response [12] and (3) AA and other PUFAs are known to modulate $GABA_A$ receptor-coupled Cl^- channels in mammals [21, 22], we have examined the possibility that PUFAs are involved as endogenous modulators of the GSH-induced feeding response in *H. vulgaris*. At least three prerequisites must be satisfied for a substance in order to suggest its role as endogenous mediator for a given response in a certain organism: (1) the molecular mechanisms for its biosynthesis and metabolism must be available to the organism; (2) inhibitors of its biosynthesis must counteract the response under study; (3) when administered exogenously, the substance must be able to reproduce this response. In addition to AA-metabolizing enzymes, *H. vulgaris* also contains a membrane-bound PLA_2 [20], as well as high levels of its preferential substrates, i.e. 1-acyl-2-*sn*-arachidonoyl-phosphoglycerides [37]. This enzyme was suggested to be activated upon the onset of the cellular processes occurring during *H. vulgaris* tentacle regeneration, and to be regulated by protein kinase C and pertussis-toxin sensitive Guanosine 5′-triphosphate (GTP)-binding proteins [20, 37]. We decided to assess the possible participation of PLA_2-catalysed endogenous AA release in the mechanisms controlling GSH-induced mouth opening and closure by studying the effect on this response of two PLA_2 inhibitors, OOPC and BPB. OOPC is a site-specific inhibitor of the low molecular weight PLA_2 which selectively hydrolyses AA-containing phospholipids [38]; BPB is an inhibitor of both PLA_2 and phospholipase C, which in some cells is involved in AA release in combination with diacylglycerol-lipase [38]. These two substances have been previously shown to inhibit *H. vulgaris* PLA_2 both in vivo and in vitro [20, 37]. Both inhibitors prolonged the feeding response by delaying the times of GSH-induced mouth closure, thus leading to the hypothesis that termination of the response may be attained at least in part through stimulation of PLA_2 and subsequent liberation of AA, following the binding of GSH to its receptor. It is noteworthy that the effect of OOPC was further potentiated by GABA, suggesting that the PLA_2 inhibitor and the neurotransmitter independently contribute to prolonging response duration.

In order to verify the hypotheses described above, we examined first the effect of GSH on the release of radiolabelled AA from live polyps previously labelled with [^{3}H]AA, under conditions where the tripeptide produces the feeding response. We found that GSH stimulation always resulted in [^{3}H]AA release in an OOPC-inhibitable fashion, and that the kinetics of this release were dependent on GSH doses and, therefore, upon the time of mouth closure, with higher AA levels always being found before mouth closure.

We then studied the effects of AA and α-LA, the two most abundant PUFAs esterified to membrane phosphoglycerides in *H. vulgaris* [19, 20], on the GSH-induced feeding response. We found that, over a 10–100 μM range of concentration, AA and, to a smaller extent, α-LA (1) shorten GSH-induced feeding response by causing an earlier closure of the mouth, and (2) counteract the prolongation of GSH-induced feeding response induced by GABA, which acts by delaying GSH-induced mouth closure. Both these effects were exhibited, at tenfold lower concentrations; also by (11-*R*)-HETE and, to a much smaller extent, by (9-*R*)-HOTrE which are the most abundant metabolites of AA and α-LA respectively in *H. vulgaris*. This finding might explain why AA, at a 1 μM concentration, is capable of inhibiting GSH-induced feeding response only after a 30-min pretreatment of live polyps with the PUFA. In fact, it can be envisaged that AA may rapidly diffuse through *Hydra* ectodermal cell membranes and be enzymatically oxidized to (11-*R*)-HETE during the incubation. Accordingly, 30-min incubations of AA with *H. vulgaris* homogenates have been previously shown to be sufficient to observe the formation of (11-*R*)-HETE [17].

Finally, the effect of AA on GSH response was comparable to that exerted by TBPS, which inhibits Cl^- ion fluxes by means of selective binding to Cl^- channels coupled to the $GABA_A$ receptor [39]. Moreover, treatment with maximal doses (100 μM) of AA reduced the inhibitory effect of a maximal dose (1 μM) of TBPS to a degree similar to that observed with AA alone, thus suggesting that the fatty acid may displace TBPS from its site of action. This would lead to the hypothesis that AA [and possibly (11-*R*)-HETE] interferes with the putative mouth closure-inducing endogenous mechanisms at the level of $GABA_A$-coupled Cl^- channels [12]. Indeed, inhibition of these channels by AA has been repeatedly reported in the mammalian CNS [21, 22]. However, the data presented here do not allow any definitive conclusion to be drawn as to whether this effect also occurs in *Hydra*.

On the basis of the data described here it is possible to suggest that endogenous AA, produced following GSH-

induced stimulation of PLA_2, might intervene in the final phase of the GSH-induced feeding response, i.e. mouth closure. The time interval of mouth opening may thus be determined at the molecular level by a metabolic interplay between GABA and AA. The latter may be a necessary component of the cellular feedback mechanisms started by GSH stimulation in order to achieve termination of the feeding response. This hypothesis is supported by the finding, reported here, that antho-RFamide, which is thought to modulate neuromuscular transmission in *Hydra* [40], and at 1–10 μM concentrations shortens the GSH-induced feeding response [41], also stimulates AA release from *H. vulgaris*. Other components of the RFamide family of neuropeptides have been previously shown to act through AA and its lipoxygenase metabolites on stimulation of PLA_2 [42]. Unfortunately, in our case it was not possible to provide evidence for the possible contribution to the control of the feeding response by endogenous (11-*R*)-HETE, whose levels may be raised in *Hydra* also by the direct stimulatory action of GSH on GSH-peroxidase, the enzyme which catalyses (11-*R*)-HETE formation from (11-*R*)-HPETE. In fact, inhibitors of mammalian lipoxygenases do not appear to influence the activity of the lipoxygenase responsible for (11-*R*)-HETE formation in *Hydra* [17, 43], and could not be used to assess (11-*R*)-HETE participation in AA-induced shortening of the feeding response. Moreover, only very small amounts of AA oxidation products were found in the radioactive material (which consisted mostly of [^{3}H]AA) released by live polyps of *H. vulgaris* upon stimulation with either GSH or antho-RFamide.

In conclusion, we have presented evidence, obtained in vivo under physiological conditions, suggesting that AA represents one of the endogenous chemical signals intervening in the chemoreceptor-induced feeding behaviour of *Hydra*. Results obtained with the second most abundant PUFA in *Hydra* phosphoglycerides, i.e. α-LA, suggest that this function, although not restricted to AA, can be effected by other PUFAs only to a limited extent and at higher concentrations. To the best of our knowledge, this is the first report that AA, released via PLA_2 activation, may act as a second messenger of the natural tripeptide GSH, either directly or through the production of specific lipoxygenase metabolites. Further studies are now needed in order to clarify the molecular mechanism by which AA causes the shortening of the GSH-induced *Hydra* feeding response.

Acknowledgments. We wish to thank G. Marino, S. Piscitelli and F. Castelluccio for skillful technical assistance. This work was partly supported by the CNR strategic project 'Tecnologie Chimiche Innovative' (to VDM).

1 Parker G. H. (1919) The Elementary Nervous System. Lippincott, Philadelphia
2 Westfall J. A. (1987) Ultrastructure of invertebrate synapses. In: Nervous Systems in Invertebrates, pp. 3–28, Ali M. A. (ed.), Plenum Press, New York
3 Koizumi O., Itazawa M., Mizumoto H., Minobe S., Javois L. C., Grimmelikhuijzen C. J. P. et al. (1992) Nerve ring of the hypostome in Hydra. I Its structure, development and maintenance. J. Comp. Neurol. **326:** 7–21
4 Mackie G. O. (1990) The elementary nervous system revisited. Am. Zool. **30:** 907–920
5 Grimmelikhuijzen C. J. P., Graff D., Koizumi O., Westfall J. A. and Mcfarlane I. D. (1989) Neurons and their peptide transmitters in Coelenterates. In: Evolution of the First Nervous Systems, pp. 95–109, Anderson P. A V. (ed.), Plenum Press, New York
6 Grimmelikhuijzen C. J. P., Carstensen K., Darmer D., Moosler A., Nothacker H. P., Reinscheid R. K., Schmutzler C. et al. (1992) Coelenterate neuropeptides: structure, action and biosynthesis. Am. Zool. **32:** 1–12
7 Kass-Simon G. and Passano L. M. (1978) A neuropharmacological analysis of the pacemakers and conducting tissues of *Hydra attenuata*. J. Comp. Physiol. A **128:** 71–79
8 Martin S. M. and Spencer A. N. (1983) Neurotransmitters in Coelenterates. Comp. Biochem. Physiol. **74C:** 1–14
9 Carlberg M. and Anctil M. (1993) Biogenic amines in Coelenterates. Comp. Biochem. Physiol. **106C:** 1–9
10 Carlberg M. (1992) Localisation of dopamine in the fresh water hydrozoan *Hydra attenuata*. Cell Tissue Res. **270:** 601–607
11 Venturini G. and Carolei A. (1992) Dopaminergic receptors in *Hydra*: pharmacological and biochemical observations. Comp. Biochem. Physiol. **1O2C:** 39–43.
12 Pierobon P., Concas A., Santoro G., Marino G., Minei R., Pannaccione A. et al. (1995) Biochemical and functional identification of GABA receptors in *Hydra vulgaris*. Life Sci. **56:** 1485–1497
13 Piomelli D. (1993) Arachidonic acid in cell signalling. Curr. Opin. Cell Biol. **5:** 274–280
14 Di Marzo V. (1995) Arachidonic acid and eicosanoids as targets and effectors in second messenger interactions. Prostag. Leukotr. Ess. Fatty Acids **53:** 239–254.
15 Flanagan S., Muller W. and Kass-Simon G. (1995) Effects of arachidonic acid, diacylglycerol and 5-*S*-HPETE on Hydra behavior. Proc. 6th Int. Workshop on Hydroid Development, Tutzing, p. 78
16 De Petrocellis L. and Di Marzo V. (1994) Aquatic invertebrates open up new perspectives in eicosanoid research: biosynthesis and bioactivity. Prostag. Leukotr. Ess. Fatty Acids **51:** 215–229.
17 Di Marzo V., De Petrocellis L., Gianfrani C. and Cimino G. (1994) Biosynthesis, structure and biological activity of hydroxyeicosatetraenoic acids in *Hydra vulgaris*. Biochem. J. **295:** 23–29
18 Di Marzo V., Gianfrani C., De Petrocellis L., Milone A. and Cimino G. (1994) Polyunsaturated-fatty-acid oxidation in *Hydra*: regioselectivity, substrate-dependent enantioselectivity and possible biological role. Biochem. J. **300:** 501–507
19 Gianfrani C., Di Marzo V., De Petrocellis L. and Cimino G. (1995) *Hydra vulgaris* ω10-lipoxygenase is used in vivo to synthesize new α-linolenic acid metabolites. Experientia **51:** 48–56
20 Borrelli L., Carginale V., Capasso A., Schneider T., Leitz T., De Petrocellis L. et al. (1995) Phospholipase A_2 and protein kinase C enzymatic activities and their interactions in *Hydra vulgaris*. Comp. Biochem. Physiol. **111B:** 211–219
21 Schwartz R. D. and Yu X. (1992) Inhibition of GABA-gated chloride channel function by arachidonic acid. Brain Res. **585:** 405–410
22 Samochocki M. and Strosznajder J. (1994) Age-related changes of GABA-activated chloride channel properties in brain cortex synaptic plasma membrane: evidence for phospholipase involvement. J. Neurochem. **63:** 1522–1528
23 Schacher S., Kandel E. R. and Montarolo P. G. (1993) cAMP and arachidonic acid simulate long-term structural and functional changes produced by neurotransmitters in Aplysia sensory neurons. Neuron **10:** 1079–1088

24 Lenhoff H. M. (1983) Water, culture solutions, and buffers. In: *Hydra* Research Methods, pp. 29–34, Lenhoff H. M. (ed.), Plenum Press, New York
25 Loomis W. F. (1955) Glutathione control of the specific feeding reaction of Hydra. Ann. N.Y. Acad. Sci. **62:** 209–228
26 Bellis S. L., Kass-Simon G. and Rhoads D. E (1992) Partial characterization and detergent solubilization of the putative glutathione chemoreceptor from Hydra. Biochemistry **31:** 9838–9843
27 Grosvenor W., Bellis S. L., Kass-Simon G. and Rhoads D. E. (1992) Chemoreception in Hydra: specific binding of glutathione to a membrane fraction. Biochim. Biophys. Acta **1117:** 120–125
28 Lenhoff H. M. (1974) On the mechanism of action and evolution of receptors associated with feeding and digestion. In: Coelenterate Biology, pp. 211–243, Muscatine M. and Lenhoff H. M. (eds), Academic Press, New York
29 Heagy W., Danner J., Lenhoff H., Cobb M. H. and Marshall G. (1982) Azaserine affinity labelling of γ-glutamyl transferase of *Hydra attenuata* without inactivation of the glutathione receptor. J. Exp. Biol. **101:** 287–293
30 Lenhoff H. M. and Bovaird J. (1961) Action of glutamic acid and glutathione analogues on the hydra glutathione receptor. Nature **169:** 486–487
31 Sakaguchi M., Hanai K., Ohta K., Kitajima M., Matsuhashi S., Hori K. (1991) Monoclonal antibody that depresses a specific subset of multiple components of the glutathione-induced response of Hydra. J. Comp. Physiol. A **168:** 409–416
32 Golz R. and Thurm U. (1992) Monoclonal antibodies against surface components of the mechano- and chemosensitive cnidocil complex of *Hydra vulgaris*. Cell Tissue Res. **268:** 327–333
33 Bellis S. L., Laux D. C., Rhoads D. E. (1994) Affinity purification of *Hydra* glutathione binding proteins. FEBS Lett. **354:** 320–324
34 Campbell R. D. (1987) Structure of the mouth of *Hydra* spp. A breach in the epithelium that disappears when it closes. Cell Tissue Res. **249:** 189–197
35 Technau U. and Holstein T. W. (1995) Boundary cells of endodermal origin define the mouth of *Hydra vulgaris* (Cnidaria). Cell Tissue Res. **280:** 235–242
36 Colasanti M., Lauro G. M. and Venturini G. (1995) NO in hydra feeding response. Nature **374:** 505
37 De Petrocellis L., Di Marzo V. and Cimino G. (1993) The possible involvement of protein kinase C and phospholipase A_2 in *Hydra* tentacle regeneration. Experientia 49: 57–64
38 Van den Bosch H. (1980) Intercellular phospholipases A. Biochim. Biophys. Acta **604:** 191–246
39 Squires R. F., Casida J. E., Richardson M. and Saederup E. (1983) [^{35}S]*t*-butylbicyclophosphorothionate binds with high affinity to brain-specific sites coupled to γ-aminobutyric acid-A and ion recognition sites. Mol. Pharmacol. **23:** 326–336
40 Koizumi O., Wilson J. D., Grimmelikhuijzen C. J. P. and Westfall J. A. (1989) Ultrastructural localization of RFamide-like peptides in neuronal dense-cored vesicles in the peduncle of Hydra. J. Exp. Zool. **249:** 17–22
41 Pierobon P., De Petrocellis L. and Minei R. (1996) Effects of antho-RFamide on the action of GABA in *Hydra vulgaris* Pallas 1766. In: Proc. 6th ICCB 1995, J. C. den Hartog et al. (eds), National Natuurhistorisch Museum, Leiden (in press)
42 Piomelli D., Volterra A., Dale N., Siegelbaum S. A., Kandel E. R., Schwartz J. H. et al. (1987) Lipoxygenase metabolites of arachidonic acid as second messengers for presynaptic inhibition of *Aplysia* sensory cells. Nature **328:** 38–43
43 Leitz T., Muller W., De Petrocellis L. and Di Marzo V. (1994) Enantiospecific synthesis of bioactive hydroxy-eicosatetraenoic acids (HETEs) in *Hydra magnipapillata*. Biochim. Biophys. Acta **1213:** 215–223

.