Regional modulation of the response to glutathione in *Hydra vulgaris* (Pallas, 1766).

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ABSTRACT
In the presence of prey, or upon exposure to reduced glutathione (GSH), Hydra polyps open a mouth to ingest the captured prey and close it after feeding; at rest the mouth is not evident. In previous papers we have shown that GABA, glycine and NMDA modulate the mechanisms of mouth closure through Ligand-Gated-Ion-Channel receptors that compare to their mammalian analogues in terms of biochemical and pharmacological properties. In order to study the regional distribution of these receptors, we have applied the GSH assay to polyps amputated at different levels of the body column. The response to 1-10 μM GSH of polyps lacking either peduncle and foot or the entire body columns (heads) was not different from control, whole animals. In the presence of GABA or muscimol duration of the response was significantly decreased in heads; the decrease was suppressed by the GABA antagonists gabazine and bicuculline. By contrast, in animals lacking peduncle and foot, duration of the response did not vary upon GABA administration. Conversely, in the presence of glycine, duration of the response in heads preparations was similar to control, while in footless polyps it was significantly reduced. The decrease was mimicked by the glycine agonists taurine and β-alanine, and counteracted by strychnine. These results suggest a regional distribution of receptors to GABA and glycine in the neuromuscular circuitry modulating the feeding behaviour.
INTRODUCTION

In the fresh-water polyp *Hydra vulgaris* (Cnidaria, Hydrozoa) neurons are connected to one another to form a net spreading homogeneously throughout the body, except at the head and foot regions, where the fibres are condensed into a circular nerve ring (Koizumi et al., 1992). Different types of synapses, with their complement of clear and dense cored vesicles, have been described in Hydra and in all cnidarian classes (Westfall, 1996). The apparent lack of centralized ganglia and the occurrence of diffuse epithelial conduction via gap junctions have long favoured the view that in Hydra the electrical signal may pass from contractile myoepithelium to unpolarized nerve net to non contracting epithelium (Anderson, 1980). Current knowledge indicates that in Hydra species the nerve net, one of the most primitive nervous systems to have evolved, shows a greater structural and functional complexity than previously acknowledged, modulating different behavioural responses through a variety of cellular effectors (Mackie, 1990; Koizumi, 2007; Koizumi et al., 2004, 2015; reviewed in Kass-Simon and Pierobon, 2007). Neuronal signalling relies largely on neuropeptides; recently a peptide-gated ion channel has been cloned and functionally characterized (Assmann et al., 2014; Durrnagel et al., 2010; Golubovic et al., 2007; reviewed in Grunder and Assmann, 2015).

The freshwater polyp *Hydra vulgaris* (Cnidaria, Hydrozoa) feeds on live prey. Hydra tentacles sense vibrations of nearby swimming prey through mechanoreceptors. This leads to activation of the stinging cells, or nematocytes, which discharge the nematocyst tubule into the prey, capturing and paralyzing it onto the tentacles. A sequence of events follows prey capture, namely tentacle writhing, opening of a mouth (Campbell, 1987; Technau and Holstein, 1995), ingestion of prey, closing of the mouth. The feeding response is initiated by the association of reduced glutathione (GSH), outflowing from the wounded prey, with an external chemoreceptor (Grosvenor et al., 1992; Venturini, 1984).

Part of the response, tentacle writhing and mouth opening, can be produced in vitro by polyps’ exposure to GSH, which is the specific stimulant of the feeding behaviour in several cnidarian species (Loomis, 1955, Lenhoff, 1961). GSH is the specific activator of the feeding response in Hydra; intensity and duration of the GSH-induced response are dose-dependent, saturable, and antagonized by L-glutamic acid (Lenhoff 1974, 1977). Measurement of response duration, i.e. the time interval between mouth opening and mouth closure, in basal conditions or in the presence of additional drugs, provides a quantitative assay for determining the activity of different substances on the feeding response.

By using the GSH assay, we have shown that amino acid neurotransmitters, GABA, glycine, NMDA, and related ligands, acting through their ionotropic receptors, are able to modulate the duration of the response to GSH by delaying or anticipating respectively the time of mouth closure.
(Concas et al., 1998; Pierobon et al., 1995, 2001, 2004a). Differently from glutamate (Bellis et al., 1991), these ligands do not modify times of mouth opening, suggesting that their effect is exerted on the neuromuscular circuitry underlying the feeding behaviour, rather than on the GSH receptor itself (reviewed in Pierobon, 2012). Therefore, neurotransmitter modulation of the feeding behaviour seems to be attained by multiple complex chemical and/or cellular pathways.

In order to obtain functional evidence on the regional distribution of receptors to GABA and glycine in hydra tissues, we studied the effects of these ligands in amputated polyps exposed to GSH. Two types of preparations were used: isolated hypostomes with their tentacles (heads), or polyps amputated of peduncle and foot (footless). In a preliminary series of experiments we examined the response to GSH in heads and footless polyps at different times after cutting. In this paper we present the results obtained upon exposure to GSH in the absence or in the presence of the different drugs.

RESULTS

The GSH response

In our experimental conditions the duration of mouth opening in response to GSH varied from about 10 minutes at 1 μM GSH to about 20 minutes at 10 μM GSH in whole polyps. Ablated heads obtained identical results upon GSH administration, provided the test was effected within 3-5 minutes after cutting: both times of mouth opening and response duration were comparable to control, i.e. whole animals (Table 1; Fig. 1A). However, duration of the response of heads treated at different times after cutting decreased significantly, starting at 15 minutes and for the following two hours, returning to control values three hours after the cut (Fig. 1C). The decrease depended on a shortened duration of the response, while times of mouth opening were not different from control. Finally, duration of mouth opening in heads undergoing the GSH test 20 hours after cutting was equal to control (Table 1; Fig. 1A). On the basis of these results, in the following experiments we only used heads either immediately after cutting (Hds 0) or 20 hours after cutting (Hds 20).

Similarly, duration of the response to GSH in animals lacking peduncle and foot (footless) was equal to control, whole polyps (Table 1; Fig 1B). In this case, however, duration of the response to GSH remained equal to control when the assay was performed at different times after cutting (Fig. 1C). In order to maintain comparable parameters, in the following experiments we used footless polyps either immediately (Ftl 0), or 20 hours after cutting (Ftl 20).

GABA, agonists and antagonists.

Administration of 100 μM GABA to isolated heads significantly reduced duration of the response to all GSH concentrations, by anticipating times of mouth closure (~ -35% of control, with a maximum of -38.8% at 10 μM GSH; Fig. A1, appendix). The effects of GABA were dose-
dependent: 10 μM, 50 μM and 100 μM GABA reduced response duration, while 1 μM GABA was not effective (Fig. 2A). Conversely, in footless polyps, GABA did not modify duration of the response in a 1-100 μM concentration range (Fig. 2A). In whole animals, 50 μM and 100 μM GABA produced a significant increase in response duration (+21.5% and +31.5% respectively at 10 μM GSH), as expected. 1 μM and 10 μM GABA did not significantly modify response duration.

The GABA<sub>A</sub> antagonist gabazine, *per se* reducing response duration at 10 μM and 5-10 μM doses in heads as well as in whole animals, respectively, suppressed the decrease in response duration produced by 100 μM GABA in heads preparations (Fig. A2, appendix). Co-administration of 10 μM bicuculline methiodide, another GABA<sub>B</sub>R antagonist, completely counteracted the decrease of response duration produced by 100 μM GABA (Fig. 3A). The Cl<sup>-</sup> channel blocker picrotoxin at 1 μM concentration, *per se* reducing response duration, partially antagonized the GABA-induced decrease of response (Fig. 3A).

The action of GABA was mimicked by the GABA<sub>A</sub> agonist muscimol that also decreased duration of the response in isolated heads at 10 μM and 100 μM doses. The effects of muscimol were counteracted by 5-10 μM gabazine (Fig. 3B).

Finally, the specific GABA<sub>B</sub>R agonist baclofen was able to reduce response duration at 10 μM and 100 μM doses. The decrease was antagonized by the GABA<sub>B</sub>R antagonist phaclofen, *per se* ineffective at 10 μM or 100 μM concentrations (Fig. 3C). In the presence of 100 μM GABA, 10 μM phaclofen caused a significant reduction of the decrease in response duration; conversely, it did not modify the decrease produced by 100 μM muscimol (Fig. 3D). In whole animals, neither baclofen in a 0.05 – 100 μM concentration range, nor 1-100 μM phaclofen significantly modified duration of the response to GSH (data not shown; see Pierobon et al., 1995).

**Glycine, agonists and antagonists**

Administration of 10 μM or 100 μM glycine to amputated heads did not obtain significant differences in duration of the response to all GSH doses (99.5 ± 8.9 % and 105.6 ± 5.3% of control, respectively, at 10 μM GSH). In these experiments, glycine administration to whole animals resulted in a dose-dependent increase of response duration, as expected (Fig. 2B).

Conversely, in the presence of 10 μM or 100 μM glycine, response duration of footless polyps was significantly reduced in a dose-dependent manner, with a maximum of ~ 43% at 10 μM GSH for 100 μM glycine (Fig. 2B). Co-administration of the GlyR antagonist strychnine, *per se* inactive at 1 μM concentration, and 1-100 μM glycine, suppressed the agonist-induced decrease, duration of the response returning to control values (Fig. 4A). The GlyR agonists taurine and β-alanine at 10 μM doses mimicked the effects of glycine (~48% and -26% respectively); the decrease
was antagonized by 1 μM strychnine (Fig. 4B). Table 2 summarizes the results of GABA and glycine administration in intact, footless polyps and isolated heads.

DISCUSSION

Our results show that amputated Hydra polyps react to GSH stimulation similarly to intact animals. Both polyps lacking peduncle and foot (footless) and heads lacking the entire body column open the mouth in response to GSH: times of mouth opening and response duration do not differ from control. These findings suggest that a) the cellular GSH transduction pathway is localised in the hypostome and tentacles; b) the neuronal and neuromuscular circuitry involved in mouth opening and closing also resides in the head; c) the body column does not apparently contribute to the GSH response.

In fact, early electrophysiological experiments provided evidence that, upon GSH stimulation, both body column and tentacle contractions are inhibited, as well as the corresponding electrical coordinates, namely tentacle pulses and contraction burst pulses (Rushforth and Hofman, 1972); at the same time, monophasic potentials associated to asymmetric, GSH-induced movements start in the tentacles (Rushforth and Burke, 1971). These data suggest that the sequence of cellular events prompted by feeding is regionally restricted to the head. Further experiments directed to studying the roles of neuromuscular and epithelial conduction in Hydra electrical activities, again indicate a regional distribution of pacemakers and their conducting systems (Kass-Simon, 1973, 1976). The mechanisms underlying the feeding behaviour in Hydra could be explained as “linked sequences of local responses, each component being initiated by the results of the preceding one” (Josephson, 1965).

The decrease of response duration observed in heads, but not in footless polyps, during the two hours after the cut could depend on amputation. In fact, the wound may cause loss of signal molecules, nutrients, (ions, cAMP, etc.). As a consequence, the electrical coordinates of the neuromuscular circuitry involved in mouth opening and closure would change. This hypothesis could tentatively explain the shortening of response duration in heads, but not in footless polyps, in the first 2 hours after cutting: in footless, in fact, the wound distance from the hypostomal region may be sufficient to prevent perturbation of the response. It is interesting to note that wounding triggers stretching of endodermal and ectodermal epithelial layers to close the wound within two hours; the wound healing process requires the contractile activity of myoepithelial cells (Wenger et al., 2014), thus contributing to further alteration of the conducting systems involved in modulation of mouth closure. In addition, regeneration and reorganization of the neuromuscular circuitry following amputation per se may affect the extant excitable structures on which the GSH response relies. Further studies are needed in order to clarify this issue.
In Hydra the classical amino acid neurotransmitters GABA and glutamate exert an inhibitory and excitatory action respectively on the pacemaker systems (Kass-Simon et al., 2003). In previous papers we have shown that the response to GSH is finely tuned by inhibitory and excitatory amino acid neurotransmitters, indicating that the cellular components leading to mouth closure are modulated by the nerve net. However, GABA and glycine prolong response duration, while NMDA reduces it; this finding was tentatively explained with the hypothesis of potentiation or inhibition, respectively, of a chain of multiple sequential inhibitory loci, which modulate contraction/relaxation of ectodermal and endodermal myofibrils (reviewed in Pierobon, 2012).

Here we show that administration of GABA to isolated heads obtains an opposite effect to whole animals, in that it significantly reduces duration of the response to GSH, with a dose-dependent effect in a 10-100 μM GABA concentration range (Fig. 3). Conversely, GABA administration does not modify times of the GSH response in footless polyps. A working hypothesis to understand these results could be that part of the GABAergic inhibitory circuit localises into the gastric region, peduncle and/or foot; the interruption of neural circuits obtained by ablation of the body column and/or peduncle may result in removing one or more inhibitory loci, thus reversing or suppressing the local action of GABA.

In heads the pharmacology of GABA was consistent with previous findings (Concas et al., 1998; Pierobon et al., 1995). Muscimol, the specific GABA\textsubscript{A}R agonist, mimicked the effects of GABA in the same concentration range. The GABA\textsubscript{A}R antagonist gabazine suppressed the GABA-induced or the muscimol-induced decrease of the response, the latter in a dose-dependent manner. Bicuculline completely antagonized the action of GABA, while picrotoxin was the least effective antagonist. These findings provide further evidence that the action of GABA depends on activation of the specific ionotropic receptors, blocked by the corresponding receptor antagonists; they also indicate that different types of GABARs by their subunit structure may be involved in modulation of the response to GSH.

The finding that baclofen, the specific GABA\textsubscript{B}R agonist, and its antagonist phaclofen are able to modulate the feeding response of amputated heads, though surprising, is not entirely unexpected. In previous works we failed to find an action of baclofen either on GABA binding or on the feeding behaviour (Pierobon et al., 1995, 2004b). However, more recent studies have now provided evidence that putative GABA\textsubscript{B} receptors are present in *Hydra vulgaris*, where they modulate nematocyst discharge (Scappaticci et al., 2008) and in tentacles nematocytes and ganglion cells of another cnidarian, the sea fan *Eunicella cavolini* (Girosi et al., 2007). It is tempting to speculate that the relative abundance of receptors to GABA of the LGIC superfamily in Hydra tissues (4.75 pmol/mg of protein) may contribute to masking the activity of GABA\textsubscript{B} receptor
ligands in whole animals, that only becomes evident upon surgical removal of major body portions. Studies directed at investigating the issue are currently in progress.

The results of glycine administration to heads and footless polyps, though quite preliminary, still point to a diversified regional distribution of receptors to glycine in Hydra tissues. In footless, 10 μM and 100 μM glycine significantly reduces duration of the response to GSH; 1 μM strychnine, the specific GlyR antagonist, reverses the decrease. The GlyR agonists taurine and β-alanine also decrease response duration, with taurine being more potent than glycine and β-alanine. Again, the pharmacological findings are in keeping with previous results (Pierobon et al., 2001), but the effects of glycine administration are reversed in footless with respect to whole animals. In heads, glycine administration does not significantly modify response duration. The lack of an effect could depend on the removal of glycinergic loci, or on the presence of an insufficient receptor density in the hypostome and tentacles. In fact the estimated $B_{\text{max}}$ of the Hydra strychnine-sensitive GlyR population is quite low (79 fmol/mg of protein) compared to that of GABA$_{\text{A}}$Rs. The data suggest localization of GlyRs in the gastric region and/or in the peduncle or foot. In this case also interrupting the circuitry would result in an opposite effect with respect to whole animals.

In conclusion, the amputation of different body regions of Hydra polyps shows that the complex behavioural response to GSH is positioned in the head. The effects of GABA or glycine administration are reversed in heads and in footless animals respectively, as compared to control, whole polyps. These findings hint at a possible modulation of the response by the gastric and foot neural circuitry that actively participates in the feeding behaviour through different LGIC receptor populations. Studies directed at investigating the contribution of different types of LGICs to the electrical activity of Hydra conducting systems in intact and regenerating animals could help to a better understanding of this controversial subject.

MATERIALS AND METHODS

Animals

*Hydra vulgaris* (Pallas, 1766) were originally obtained from Prof. P. Tardent (University of Zurich, Switzerland), and cultured asexually in our laboratories by the method of Loomis and Lenhoff (1956), with minor modifications. GSH assays were carried out on animals that were kept at 18 ± 1°C under artificial 12-h-light, 12-h-dark cycle in physiological solution [1 mM CaCl$_2$, 0.1 mM NaHCO$_3$ (pH 7.3 to 7.4)], and fed three times a week with freshly hatched nauplii of the brine shrimp *Artemia salina*; culture solution was changed 1 hour after feeding. Homogeneous sample populations were obtained from freshly detached buds collected on the same day and cultured in separate dishes until use.

The GSH assay
The feeding reaction was studied by the procedure described by Lenhoff et al., (1983), with minor modifications. Polyps from homogeneous populations, ~3 weeks old and carrying one or two buds, were starved for at least 3 days before the trial. On the day of the experiment polyps were transferred in physiological solution buffered with 1 mM Tris-HCl (pH 7.4) and equilibrated at room temperature for 1 hour. 4 or 3 + 3 animals at a time were placed in 3.5-cm-diameter Falcon dishes divided into four chambers by glass partitions and allowed to relax under the stereo microscope (2 to 3 minutes). The test was initiated by removing the physiological solution and gently pipetting 1 ml of buffered physiological solution containing GSH (1 to 10 μM) or GSH plus ligands at different concentrations. Animals were then monitored for mouth opening and mouth closure times. As usual in behavioural experiments, independent observers monitored mouth opening and closing, recorded the corresponding times, and dispensed the different solutions. This procedure permits simultaneous testing and recording of individual mouth opening (T_i) and closing (T_f) times for each animal. Scoring of mouth opening and closing was performed with a cold-light Wild stereo microscope. All experiments were performed in an air-conditioned environment at 22°C.

In order to obtain heads, namely hypostomes and tentacles, groups of 5-6 polyps were allowed to relax briefly under the microscope light before head excision; the cut was effected by transverse section immediately below tentacle insertion. Heads were then collected, rinsed in buffered physiological solution and either used immediately (within 3-5 minutes after cutting) or stored at 18 ± 1°C and used 20 hours later. In some experiments heads were tested at different times after cutting, i.e. 15 min, 30 min, 1 hr, 2 hr, 3 hr; in the interval the physiological solution was changed repeatedly. The same procedure was used for preparing footless samples: in this case the cut was effected immediately below the gastric region, i.e. along the distinct border between dense (budding region) and clear (peduncle) tissues.

All the drugs were dissolved in distilled water at a 100x concentration and used immediately. Lipophilic molecules (phaclofen) were initially dissolved in dimethylsulfoxide (DMSO) so that its final concentration did not exceed 1 μl/ml; in these experiments, the equivalent amount of DMSO was added to control. Six to eight animals were tested per group and per GSH concentration. Mouths opened within 1 to 2 min of GSH administration, and healthy animals were able to respond repeatedly. T_i and T_f were always measured for the first response. A control series of 4–5 groups treated with GSH only was performed in all experiments, which were repeated three to several times for each substance tested. GSH, GABA, muscimol, gabazine, bicuculline methiodide, picrotoxin, baclofen, phaclofen, glycine, taurine, β-alanine and strychnine were obtained from Sigma (Milan, Italy) or from Tocris Cookson, Inc. (Ballwin, MO, USA).
Data analysis.

Behavioural data were analysed as follows: in each experiment the duration of the response to different GSH doses in the absence or in the presence of the various drugs was measured. The kinetics of the response was determined by linear regression analysis of all the data obtained in different experiments, using a modified Lineweaver-Burk equation.

Since only a limited number of animals could be tested in a single experiment (100 – 120 polyps), in the assays where several groups were required (direct comparison of two or more drugs, drug association, etc.), both the number of polyps and of GSH doses had to be reduced, thus preventing linear regression analysis. In order to compare data from these experiments, percentages of decrease or increase versus the maximal control value were calculated for each treatment at all GSH doses. Differences were then analyzed by ANOVA followed by Scheffé’s test. Data are presented as means ± s.e.m. from experiments repeated at least three times. Since the number of post-hoc comparisons did not exceed the degrees of freedom, no correction in the alpha level was made, and a $P$ value of <0.05 was considered statistically significant. The software used was StatView 4.5, Abacus.

LIST OF SYMBOLS AND ABBREVIATIONS

DMSO: dimethylsulfoxide  
Ftl: footless  
GABA: $\gamma$-amino butyric acid  
GABAR: GABA receptor  
GlyR: glycine receptor  
GSH: $\gamma$-glutamyl-cysteinyl-glycine (reduced glutathione)  
Hds: heads  
LGIC: Ligand-Gated Ion channel  
NMDA: N-methyl-D-aspartic acid

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COMPETING INTERESTS

The authors declare no competing financial interests.

FUNDING

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REFERENCES


Figure 1A

Figure 1B

Figure 1A, 1B. Response to 1-10 μM GSH.

Linear regression analysis of the response to different GSH concentrations in heads (A) and footless polyps (B). The kinetics of the response are calculated by a modified Lineweaver-Burk equation of [GSH]/(T_f-T_i) ratios, where [GSH] represents the stimulus concentration and (T_f - T_i) is...
the time measured at the corresponding GSH dose. **A.** Control (square), isolated heads 3-5 minutes after cutting (circle), or 20 hours after cutting (triangle). Curves are obtained from 10 experiments. **B.** Control (square), footless polyps 3-5 minutes after cutting (circle), or 20 hours after cutting (triangle). Curves are obtained from 8 experiments.

**Figure 1C. Time course of response to 10 μM GSH.**

Duration of response in isolated heads (circle) and footless polyps (triangle) at 15’, 30’, 60’, 120’ and 180’ after cutting. Data are expressed as the percentage variation in response duration relative to respective whole control value (10 μM GSH) and are the means ± s.e.m. of 4 separate experiments for each time setting (heads), and 3 separate experiments for each time setting (footless). ANOVA followed by Scheffe’s test; *P<0.05
Figure 2A, 2B. Effects of different GABA or glycine doses on response duration in whole polyps, footless and heads.

A. 50 μM and 100 μM GABA significantly increased response duration in whole animals; 1 μM and 10 μM GABA were not effective. Conversely, 10 μM, 50 μM and 100 μM GABA
significantly reduced response duration in isolated heads (Hds 0); the decrease produced by 50 μM and 100 μM GABA was also significantly greater than that obtained by 1 μM and 10 μM GABA. GABA did not significantly modify response duration in footless polyps at all concentrations. Here and in the following figures data are expressed as the percentage variation in response duration relative to respective whole control value (10 μM GSH) and are the means ± s.e.m. of 9-10 separate experiments. ANOVA followed by Scheffe’s test; *P<0.05

B. 10 μM and 100 μM glycine significantly increased response duration in whole animals, while 1 μM glycine was not effective. Conversely, 10 μM and 100 μM glycine significantly reduced response duration in footless polyps (Ftl 0). Glycine did not significantly modify response duration in isolated heads at all concentrations. Data are the means ± s.e.m. of 9-10 separate experiments. ANOVA followed by Scheffe’s test; *P<0.05
Figure 3A, 3B, 3C, 3D. Effects of GABA agonists and antagonists on response duration.

A. The GABA\textsubscript{A}R antagonists gabazine (10 \textmu M), bicuculline (10 \textmu M) or picrotoxin (1 \textmu M) suppressed the decrease in response duration produced by 100 \textmu M GABA in isolated heads (Hds 0). Data are the means ± s.e.m. of 3 separate experiments for each drug. ANOVA followed by Scheffe’s test. *P<0.05 vs control; ^ = P<0.05 vs 100 \textmu M GABA-treated heads.

B. The GABA\textsubscript{A} agonist muscimol at 10 \textmu M and 100 \textmu M doses mimicked the effects of GABA on heads (Hds 0). The muscimol-induced decrease was suppressed by 5-10 \textmu M gabazine. Data are the means ± s.e.m. of 4-6 separate experiments. ANOVA followed by Scheffe’s test. *P<0.05 vs control; ^ = P<0.05 vs 100 \textmu M muscimol-treated heads.

C. In the presence of 10 – 100 \textmu M baclofen, a specific GABA\textsubscript{B}R agonist, duration of the GSH response significantly decreased in heads preparations (Hds 20). The decrease was completely abolished by concomitant administration of 10 \textmu M phaclofen, per se not effective. Data are the means ± s.e.m. of 4 separate experiments. ANOVA followed by Scheffe’s test. *P<0.05 vs control.

D. The specific GABA\textsubscript{B}R antagonist phaclofen at 10 \textmu M concentration significantly reduced the GABA-induced decrease of response duration in isolated heads (Hds 20) but did not counteract the muscimol –induced decrease. Data are the means ± s.e.m. of 6 separate experiments. ANOVA followed by Scheffe’s test. *P<0.05 vs control; ^ = P<0.05 vs 100 \textmu M GABA-treated heads.
Figure 4A, 4B. Effects of glycine agonists and antagonists on response duration.

A. The GlyR specific antagonist strychnine, *per se* inactive at 1 μM concentration, suppressed the decrease in response duration produced by 10-100 μM glycine in footless polyps (Ft
0). Data are the means ± s.e.m. of 4 separate experiments. ANOVA followed by Scheffe’s test; *P<0.05 vs control; ^ = P<0.05 vs respective glycine-treated footless value.

B. The GlyR agonists taurine and β-alanine significantly reduced response duration in footless polyps (Ftl 0) at 10 μM concentration (white bars). The relative potency of ligands was taurine > glycine > β-alanine, similarly to results obtained in whole animals. 1 μM strychnine completely suppressed the decrease produced by 10 μM glycine, taurine or β-alanine. Data are the means ± s.e.m. of 4-6 separate experiments. ANOVA followed by Scheffe’s test; *P<0.05 vs control.
Table 1. **Times of mouth opening and closing after 10μM GSH administration.** Data are expressed in minutes and seconds, and are the means ± S.D. from a typical experiment for each time setting. $T_i$ = time of mouth opening; $T_f$ = time of mouth closure. Duration of the response, i.e. the time interval ($T_f - T_i$), was calculated for each polyp in all sample groups. Average values were used for linear regression analysis or as a percentage of maximal control value (ANOVA).

<table>
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<tr>
<th>Drug</th>
<th>$T_i$</th>
<th>$T_f$</th>
<th>$T_f - T_i$</th>
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<tr>
<td>Whole (control)</td>
<td>46” ± 11”</td>
<td>22’05” ± 1’13”</td>
<td>21’04” ± 1’04”</td>
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<tr>
<td>Heads: 5’ after cut</td>
<td>40” ± 14”</td>
<td>22’19” ± 1’31”</td>
<td>21’39” ± 1’27”</td>
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<td>Heads: 20 hr after cut</td>
<td>47” ± 13”</td>
<td>22’00” ± 1’14”</td>
<td>21’13” ± 1’16”</td>
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<tr>
<td>Footless: 5’ after cut</td>
<td>22” ± 9”</td>
<td>20’53” ± 28”</td>
<td>20’31” ± 36”</td>
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<tr>
<td>Footless: 20 hr after cut</td>
<td>34” ± 14”</td>
<td>21’49” ± 38”</td>
<td>21’15” ± 37”</td>
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Table 2. **Summary of different effects of GABA and glycine on the response to GSH in intact polyps, heads, footless.** Data are expressed as percentage variations of the response to 10 μM GSH (control). Figures in bold represent significant differences (ANOVA followed by Scheffe’s test; *P<0.05 vs control)

<table>
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<th>drug</th>
<th>whole</th>
<th>heads</th>
<th>footless</th>
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<tr>
<td></td>
<td>solvent</td>
<td>+10 μM gabazine</td>
<td>solvent</td>
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<td>100 μM GABA</td>
<td>+32.2 ± 3.0</td>
<td>-28.5 ± 0.3</td>
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<td>100 μM muscimol</td>
<td>+22.2 ± 2.9</td>
<td>-23.9 ± 7.4</td>
<td>-48.0 ± 8.2</td>
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<tr>
<td></td>
<td>solvent</td>
<td>+1 μM strychnine</td>
<td>solvent</td>
</tr>
<tr>
<td>10 μM glycine</td>
<td>+18.4 ± 1.5</td>
<td>0.1 ± 1.9</td>
<td>+7.5 ± 5.3</td>
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<tr>
<td>10 μM taurine</td>
<td>+18.9 ± 3.6</td>
<td>3.3 ± 5.7</td>
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</tr>
<tr>
<td>10 μM β-alanine</td>
<td>+17.7 ± 4.9</td>
<td>14.5 ± 3.3</td>
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