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Different roles of GABA and glycine in the modulation of chemosensory responses in *Hydra vulgaris* (Cnidaria, Hydrozoa)

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Abstract

Phylogenetic studies suggest that GABA and glycine receptors derive, as a result of divergent evolution, from a common ancestral protoreceptor originated in a unicellular organism. This raises the possibility that members of the ligand-gated ion channels (LGIC) superfamily might be widely present in living organisms including bacteria and primitive invertebrates. High-affinity GABA receptors occur in the tissues of *Hydra vulgaris* whose pharmacological characteristics compare with those of mammalian ionotropic GABA receptors. Behavioural studies have shown that activation of these $GABA_A$-like receptors by their allosteric modulators increases the duration of response to reduced glutathione (GSH). Recently, strychnine-sensitive glycine receptors have been shown to occur in *Hydra* tissues. Activation of these glyR also results in increased duration of the response to GSH. In order to investigate the contribution of endogenous transmitters to the modulation of the feeding response, we studied the effects of exposing the polyps to brief depolarizing pulses prior to the GSH test. A severe inhibition of the response was observed following exposure to KCl or veratridine. Administration of GABA or muscimol counteracted the effects of the pulses in a dose-dependent manner. The effects of GABA or muscimol were suppressed by the $GABA_A$-specific antagonist gabazine both in pulse-untreated and treated polyps. By contrast, glycine and its agonist taurine were not able to restore the physiological duration of response in pulse-treated *Hydra*, while another glyR agonist, β-alanine, partially reduced the pulse-induced inhibition. We conclude that GABA appears to be the major inhibitory transmitter responsible for the regulation of the feeding response. Molecular studies aimed at identifying GABA receptor subunits are in progress.

Introduction

Aquatic animals rely largely on chemical senses for many biological activities ensuring survival such as food recognition and feeding, avoidance of predators, enhancement of reproduction, and recognition of suitable habitats. In the freshwater polyp *Hydra vulgaris* (Cnidaria, Hydrozoa) feeding is achieved through a complex behavioural pattern prompted by substances outflowing from wounded prey (Loomis, 1955). In the absence of prey some of these behaviours, e.g. tentacle writhing, tentacle concerting, and mouth opening, can be produced by polyps' exposure to reduced glutathione (GSH), which is the physiological stimulant of the feeding behaviour in *Hydra* as well as in other cnidarian species. Onset of response, i.e. mouth opening, occurs 1–2 min after GSH addition. Duration of the response, i.e. the time interval between mouth opening and mouth closure, is linearly related to the stimulus intensity in the low micromolar GSH concentration range. Different structural GSH analogs are able to inhibit GSH activity by preventing mouth opening. These

findings led to the hypothesis that a specific chemoreceptor mediated this response (see Lenhoff, 1974, for a review). Later studies by Venturini (1987) and Grosvenor et al. (1992) demonstrated the occurrence in *Hydra* tissues of GSH receptors that were further characterized, solubilized, and partially purified (Bellis et al., 1992, 1994).

The feeding behaviour is interrupted upon GSH removal, but the mechanisms by which termination of the response is achieved are still poorly understood. Lenhoff's suggestion (1974) that mouth closure simply derived from receptor desensitization, based on his detailed analysis of the kinetics of the response, was challenged by later studies. Grosvenor et al. (1996) found that wounded prey release other substances, besides GSH, that are able to shorten response duration by competitively inhibiting GSH binding. Our group previously reported that two inhibitory amino acid transmitters, GABA and glycine, modulate the feeding behaviour by prolonging duration of the response to GSH. Furthermore, glycine reduces duration of the response when all strychnine-sensitive binding sites are blocked by strychnine; this effect is mimicked by D-serine, a glycine agonist at the glycine-binding site of vertebrate glutamate NMDA receptors.

The action of these amino acids is mediated by receptors occurring in *Hydra* tissues whose biochemical and pharmacological characteristics compare with those of ionotropic mammalian GABA and glycine receptors (Concas et al., 1998; Pierobon et al., 2001). The inhibitory effect on response duration exerted by NMDA coagonists suggests that glutamate receptors also may be involved in the modulation of effector responses, besides the direct action of glutamate as a competitive GSH antagonist at the GSH receptor (Lenhoff, 1961). However, other studies (Bellis et al., 1991; Grosvenor et al., 1992) found that glutamate, which prevents GSH-induced mouth opening *in vivo*, did not displace the specific binding of radiolabeled GSH, and must therefore block the feeding response by a mechanism other than competitive inhibition. An increasing body of evidence thus supports the hypothesis that in *Hydra* duration of the response to GSH stimulation is finely tuned by GABA and glutamate with opposite actions, while glycine has a dual role. Since times of mouth opening after GSH administration are not affected, but only times of mouth closure vary because of treatment, the observed effects may well be the result of a coordinated interplay of different target cells (neurons?) activated by the GSH transduction pathway.

Materials and methods

Duration of the GSH response can be experimentally measured by recording times of mouth opening and closure for each animal, thus making the GSH test a reliable quantitative assay for studies of behavioural physiology. In order to investigate the contribution of endogenous GABA or glycine to the modulation of the response to GSH, we studied the effects of exposing the polyps to brief (15–20 s) depolarizing pulses prior to GSH administration. Animals were treated with 1 ml of 56 mM KCl or 1 μM veratridine (VTD), quickly washed with physiological solution buffered with 1 mM Tris–HCl (pH 7.4), and then assayed by the GSH test immediately, i.e. within 60 s after the pulse. The test was initiated by adding 1 ml of buffered physiological solution containing either GSH (1–10 μM) or GSH plus GABA, its agonists and antagonists, or glycine and its agonists at different concentrations. In all experiments three to five groups of animals were treated with GSH only and served as control. Details of methods and data analysis are described elsewhere (Pierobon et al., 2001). GSH, GABA, muscimol, pentobarbital, glycine, taurine, and β-alanine were obtained from Sigma (Milan, Italy). Gabazine, VTD, and tetrodotoxin (TTX) were obtained from Research Biochemicals (Milan, Italy).

Results and discussion

Effects of depolarization on the feeding response

A severe inhibition of the response was obtained in polyps exposed to 56 mM KCl for 15 s, quickly washed, and tested with 1–10 μM GSH. The duration of the response was significantly reduced starting at 4 μM GSH ($-18 \pm 1.2\%$); the maximal decrease was observed at 10 μM GSH ($-38.7 \pm 2.4\%$). Administration of the plant alkaloid VTD (1 μM), either by pulses or bath

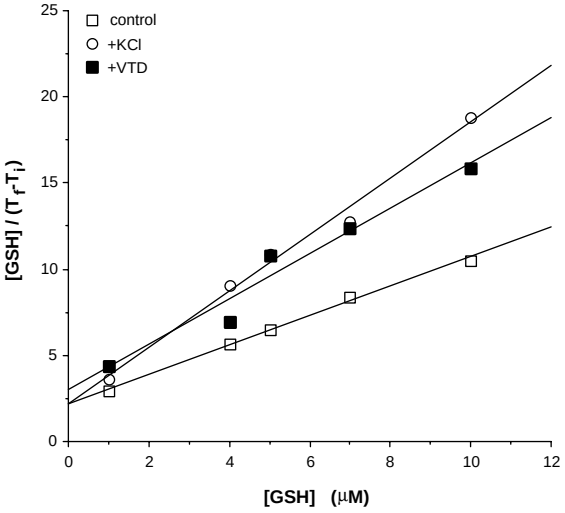

Figure 1. Linear regression curves of the response to different GSH concentrations (control) after 56 mM KCl or 1 μM veratridine pulses. Curves are obtained by a modified Lineweaver–Burk equation, where [GSH] represents the stimulus concentration and (T_f–T_i) is the time measured at the corresponding GSH dose.

applied, also produced a significant decrease in duration of the response (−36.7 ± 2.1% at 10 μM GSH) (Fig. 1). The reduced duration of response depended solely on anticipated times of mouth closure, while mouth opening was not affected by treatment (Table 1). In order to test the reversibility of the inhibition, in a different set of experiments the GSH test was performed at various times after the pulse. The effects of the pulse gradually attenuated starting 30 min after the KCl pulse. A complete recovery of response duration was observed 2 h after the pulse. Similar results were obtained in polyps exposed to VTD pulses (Fig. 2).

VTD or high KCl doses are commonly used in studies of transmitter release from neuronal preparations. Since in our experiments the entire animal underwent treatment, all excitable cells or cellular structures were simultaneously stimulated; therefore, the observed effects may well represent the composite result of a variety of cellular activities. Nonetheless, time to onset of mouth opening was not modified, suggesting that the binding ability of GSH receptors was not affected. Furthermore, duration of the response was still proportional to GSH concentration (Fig. 1). The observed decrease in response duration could then depend either on a reduced efficiency of the GSH signalling pathway or on a reduced ability of effector cells to respond to the stimulus (ion channel inactivation, onset of refractory periods, receptor desensitization, depletion of stored neurotransmitters). In the latter hypothesis, the GSH stimulus would reach its target cells after these have been activated by one or more transmitters released by the depolarizing pulse, thus being only partially effective. Alternatively, depletion of transmitter stores produced by the pulse may reduce availability of transmitters normally involved in the regulation of the effector response to the GSH stimulus. The time course of effect reversal is consistent with this model. The lack of an effect of pulses on the mechanisms of muscle contraction/

Table 1. Times of mouth opening and closing after GSH administration

Drug	T_i	T_f	T_f–T_i
Solvent	36″ ± 12″	21′58″ ± 1′13″	21′22″ ± 1′14″
56 mM KCl pulse	36″ ± 8″	13′10″ ± 1′31″	12′34″ ± 1′27″
1 μM veratridine	36″ ± 9″	13′35″ ± 1′14″	12′59″ ± 1′16″
100 μM GABA	33″ ± 13″	27′33″ ± 2′04″	27′00″ ± 2′08″
100 μM muscimol	40″ ± 10″	26′46″ ± 1′42″	26′06″ ± 1′41″
10 μM pentobarbital	36″ ± 8″	26′33″ ± 1′16″	25′57″ ± 1′16″
10 μM gabazine	44″ ± 14″	14′49″ ± 1′14″	14′05″ ± 1′21″

Times of response (minutes and seconds) after 10 μM GSH administration (T_0). Data are the means ± SD of individual times recorded in 8–10 separate experiments for each drug. 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 the control (ANOVA).

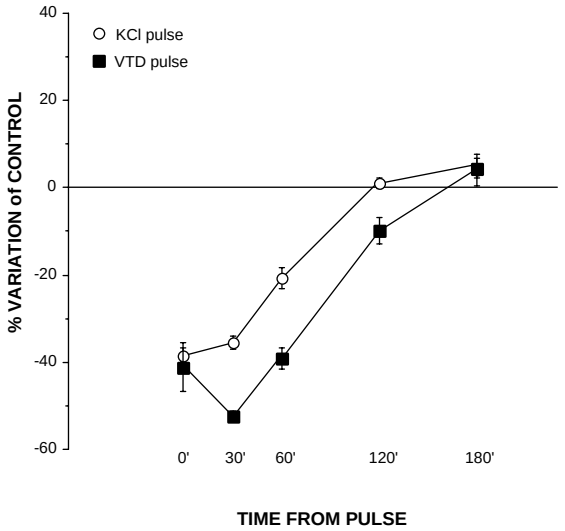

Figure 2. Recovery of times of response to 10 μM GSH at different times after KCl or VTD pulses. Data are expressed as a percentage of the control (10 μM GSH) value and are the means ± SEM from three to five separate experiments.

relaxation presiding over mouth opening (Campbell, 1987), though intriguing, is beyond the purpose of the present investigation and cannot be properly addressed here.

Effects of GABA, its agonists and antagonists

1 or 10 μM GABA, i.e. ineffective doses in KCl-untreated animals, counteracted the decrease in response duration in animals tested immediately after the KCl pulse. 100 μM GABA produced a significant enhancement of the GSH response that compared with the increase observed in KCl-untreated polyps (Table 2). The $GABA_AR$ agonist muscimol also prolonged response duration in a 10–100 μM concentration range. However, it was able to reverse KCl effects only at 100 μM but it failed to produce enhancement of the response (Table 2). Similar results were obtained in VTD-treated animals (Fig. 3). The increase in response duration induced by GABA and muscimol was suppressed by the simultaneous administration of the specific $GABA_AR$ antagonist, gabazine, in a dose-dependent manner (Table 3). Gabazine *per se* significantly reduced response duration in a 1–10 μM concentration range but did not produce a further inhibition of the response in KCl-treated polyps (Fig. 4).

The general anesthetic pentobarbital (100 nM–100 μM), a positive allosteric modulator of mammalian $GABA_A$ receptors, also produced a sig-

Table 2. Effects of GABA, glycine and agonists, alone or in combination with KCl pulses, on the duration of the response to GSH

Drug	Concentration (μM)	Response duration (% control)	
		Drug	56 mM KCl pulse + drug
Solvent		100.0 ± 4.4	61.3 ± 2.1*
GABA	1	105.3 ± 0.5	100.4 ± 4.5†
GABA	10	107.7 ± 3.2	103.3 ± 1.8†
GABA	100	127.4 ± 2.9*	123.3 ± 4.8†
Muscimol	1	100.2 ± 4.2	68.6 ± 3.0*
Muscimol	10	118.4 ± 3.2*	76.1 ± 3.7*
Muscimol	100	123.1 ± 3.1*	103.9 ± 2.6†
Pentobarbital	1	116.9 ± 3.1	75.5 ± 7.0*
Pentobarbital	10	122.4 ± 1.9*	75.9 ± 2.3*
Pentobarbital	100	120.9 ± 6.2*	57.0 ± 6.4*
Baclofen	100	107.6 ± 2.3	64.2 ± 1.5*
Glycine	100	124.9 ± 3.4*	63.5 ± 4.4*
Taurine	100	126.5 ± 3.0*	68.1 ± 1.8*
β-alanine	100	125.0 ± 2.3*	83.5 ± 1.5†

Data are expressed as a percentage of the control (10 μM GSH) value and are means ± SEM from five to seven separate experiments for each drug.
*$p < 0.001$ versus control; $^\dagger p < 0.001$ versus KCl-treated control group (ANOVA followed by Scheffé's test).

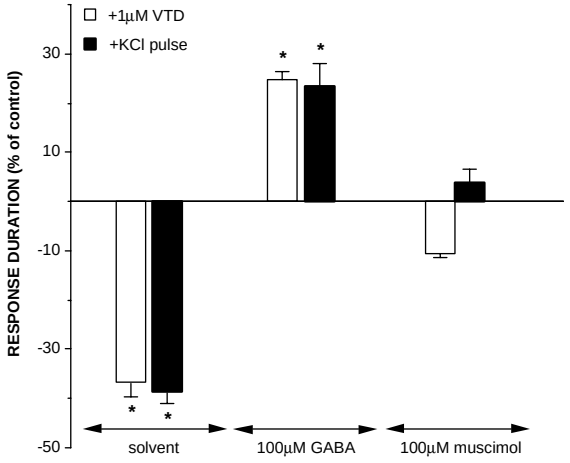

Figure 3. Effects of 1 μM VTD or 56 mM KCl on the duration of response to 10 μM GSH, in the presence or absence of 100 μM GABA or muscimol. Bars represent percent variations of control and are the means ± SEM from eight separate experiments (GABA) and from six separate experiments (muscimol). *$p < 0.001$ versus control.

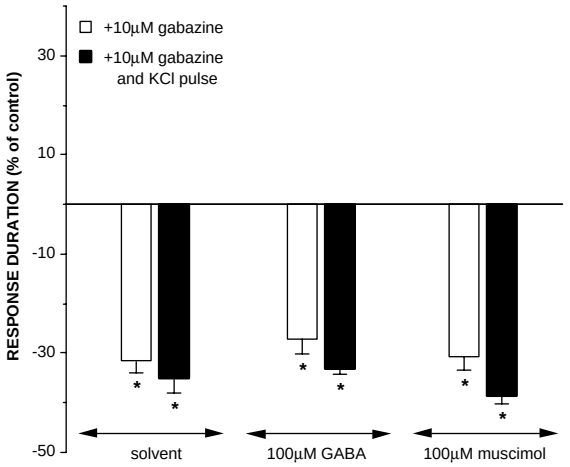

Figure 4. Effects of 10 μM gabazine on the duration of response to 10 μM GSH in KCl-untreated and KCl-treated animals, in the presence or absence of 100 μM GABA or muscimol. Bars represent percent variations of control and are the means ± SEM from eight separate experiments (GABA) and from six separate experiments (muscimol). Differences between KCl-untreated and treated groups are not significant. *$p < 0.001$ versus control.

nificant increase in response duration in KCl-untreated animals; the maximal increase was obtained by 10 μM pentobarbital (Table 2). Furthermore, co-administration of 100 nM pentobarbital and 1 μM GABA produced a significant increase in duration of the response to 10 μM GSH (+33.2 ± 5.1%). These effects were abolished by the simultaneous administration of gabazine (Table 3). Pentobarbital (1–100 μM) was completely ineffective in KCl-treated polyps (Table 2), while it counteracted the effects of bath applied VTD in a dose-dependent manner (Fig. 5). When polyps were treated with VTD pulses, only high (100 μM) pentobarbital doses were able to suppress the pulse-induced decrease of response (Fig. 5). The results obtained by GABA agonists and antagonists in this behavioural study are in good agreement with the results of previous binding experiments (Concas et al., 1998). Taken together these data also suggest a role for endogenous GABA in regulation of the feeding response. In fact, GABA, even at low concentrations, was able to restore physiological times of response in animals treated immediately after the depolarizing pulse. Muscimol mimicked the effects of GABA, though with lower efficacy. Since *Hydra* GABAR have a high affinity for the agonist muscimol (Pierobon et al., 1995), this finding could be explained with the hypothesis of a relative heterogeneity of GABAR populations. The effects

Table 3. Dose–response effects of gabazine, alone or in combination with GABA and agonists, on the duration of the response to GSH

Gabazine (μM)	Drug			
	Solvent	GABA (100 μM)	Muscimol (100 μM)	Pentobarbital (10 μM)
0.1	102.9 ± 4.4	134.3 ± 2.5*	128.6 ± 3.1*	127.7 ± 2.9*
1	95.0 ± 1.8	101.6 ± 3.8†	97.3 ± 2.4†	96.9 ± 2.7†
5	85.5 ± 2.2	88.7 ± 1.6†	88.9 ± 0.8†	66.7 ± 3.3†
10	68.4 ± 2.0*	72.9 ± 3.1†	69.3 ± 2.7†	60.2 ± 2.3†

Data are expressed as a percentage of the control (10 μM GSH) value and are means ± SEM from three to five separate experiments for each drug.
*$p < 0.001$ versus control; $^{\dagger}p < 0.001$ versus respective solvent-treated group (ANOVA followed by Scheffè).

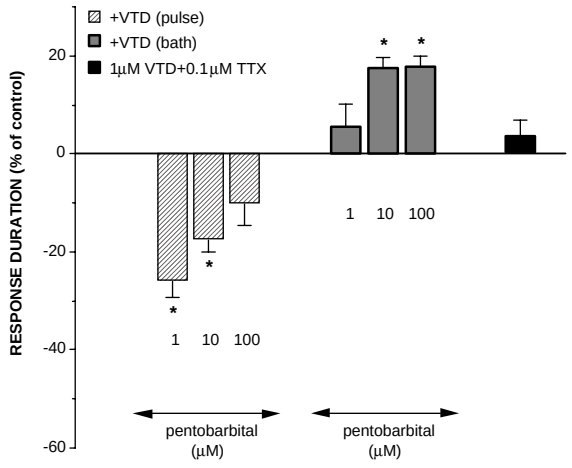

Figure 5. Effects of 1, 10, and 100 μM pentobarbital on duration of the response to 10 μM GSH after 1 μM VTD pulses or co-administration with 1 μM VTD. The effects of 1 μM VTD (see Fig. 3) are suppressed by 100 nM TTX; the difference is significant ($p < 0.001$). Bars represent percent variations of control and are the means ± SEM from four to six separate experiments. *$p < 0.001$ versus control.

of GABA or muscimol were suppressed by the specific $GABA_AR$ antagonist gabazine, indicating a receptor-mediated action. Pentobarbital, which potentiates [^{3}H]GABA binding *in vitro* (+78% at 300 μM pentobarbital), was only effective *in vivo* when animals were not subjected to KCl pulses. This result lends further support to the hypothesis that endogenous GABA may no longer be available as a consequence of the pulse, thus preventing pentobarbital potentiation.

By contrast, low pentobarbital levels were sufficient to restore or to increase response duration when co-administered with VTD, while high pentobarbital concentrations were required to attenuate the inhibitory effects of VTD pulses. In other organisms pentobarbital, besides potentiating GABA binding, is antagonistic to VTD, possibly by exerting a blocking action on voltage-gated, TTX-sensitive Na^+ channels, which are the primary target of a number of liposoluble neurotoxins (Wang & Wang, 2003). In our experiments the ability of pentobarbital to reverse VTD effects was not quite dependent on pentobarbital concentration, but rather on the timing of VTD administration. This finding is consistent with the hypothesis that the VTD-induced decrease of response could depend on the opening of a voltage-gated Na^+ channel. In fact, co-administration of 100 nM TTX and 1 μM VTD restored physiological times of response to 1–10 μM GSH (Fig. 5). Accordingly, pentobarbital would be able to antagonize the action of simultaneously applied VTD by competing onto a common target, while it would be substantially ineffective when VTD was pre-administered.

Finally the specific $GABA_BR$ agonist baclofen failed to modify the response either in untreated or in KCl-treated animals (Table 2). Instead, baclofen increases the probability of discharge of stimulated desmonemes (Kass-Simon & Scappaticci, 2004). In an electrophysiological study (Kass-Simon et al., 2003) bicuculline was found to suppress the decrease in frequencies of ectodermal and endodermal Contraction Pulses produced by GABA. In previous biochemical studies we have shown that [^{3}H]GABA binding is completely displaced by muscimol or gabazine, but not by bicuculline or baclofen, and it is potentiated by $GABA_AR$ positive allosteric modulators such as neurosteroids, general anesthetics and benzodiazepines. The evidence emerging from the bulk of these results supports the view that different GABA receptor populations are involved in the regulation of coordinated behaviours in *Hydra*, with different roles and specific pharmacological properties. Molecular studies aimed at identifying GABA receptor subunits are in progress.

Effects of glycine, taurine and β-alanine

Administration of 100 μM glycine or the glyR agonist taurine significantly increases duration of the response to GSH (Pierobon et al., 2001). However, when polyps were exposed to depolarizing pulses, both amino acids failed to restore physiological times of response (Table 2). In the presence of β-alanine, another glycine receptor agonist, a different result was obtained: duration of the response was significantly longer than that obtained after the KCl pulse, though still shorter than the control (−16.5 ± 1.5%). This partial recovery of response duration was suppressed by simultaneous administration of 10 μM gabazine (−34.7 ± 4.6%). The results obtained by β-alanine could be explained with the hypothesis of a modified molecular structure of *Hydra* glycine receptor subunits (Schmieden et al., 1993). Alternatively,

β-alanine is also known to act as a high affinity inhibitor of GAT-2 and GAT-3 GABA transporters (Cherubini & Conti, 2001). The suppression of glycine or taurine-induced enhancement of the response following depolarization could be tentatively explained with the hypothesis that modulation of the response to GSH by ionotropic glyR may require activation of GABAergic cells. It is interesting to note that co-administration with 1 μM gabazine suppressed the enhancement of response induced by either glycine or taurine in polyps not subjected to depolarization (Fig. 6). In biochemical experiments neither gabazine nor picrotoxin were found to displace [^{3}H]strychnine binding (−18 and −14% respectively in a 10^{-6}–10^{-4} M antagonist concentration range), thus excluding a direct action of $GABA_A$ antagonists on strychnine-sensitive *Hydra* glyR. By contrast, the action of GABA was not modified by simultaneous administration of 1 μM strychnine, the specific ionotropic glyR antagonist (Fig. 6).

Conclusions

Taken together, these results indicate that: (1) When the animal is exposed to KCl or veratridine pulses, it retains the ability to perceive the GSH stimulus and to react appropriately. However, the efficiency of the response is significantly reduced. (2) GABA and its agonist muscimol are able to restore or to enhance duration of the response in stressed animals, while pentobarbital is effective only in KCl-untreated polyps. (3) Suppression of the effects of GABA and its agonists by gabazine indicates that these effects are exerted through $GABA_A$ receptors. Furthermore, the effects of gabazine are not synergistic with those of KCl treatment. (4) Glycine and its agonist taurine do not affect the KCl-induced decrease of the response. Gabazine suppresses the effects of glycine and agonists in KCl-untreated polyps; this inhibition of glycine activity is not receptor-mediated, based on the results of [^{3}H]strychnine binding experiments. We conclude that GABA appears to be the major inhibitory transmitter responsible for the regulation of the feeding response, and that GABA and glycine act by different pathways.

The results obtained by the present approach, even though quite indirect, may begin to disclose the complexity of regulatory mechanisms underlying termination of the response to chemical stimuli in *Hydra*. Future studies on the cellular localization and regional distribution of these receptors will help to describe the circuitry involved and to understand the respective roles of inhibitory amino acid transmitters.

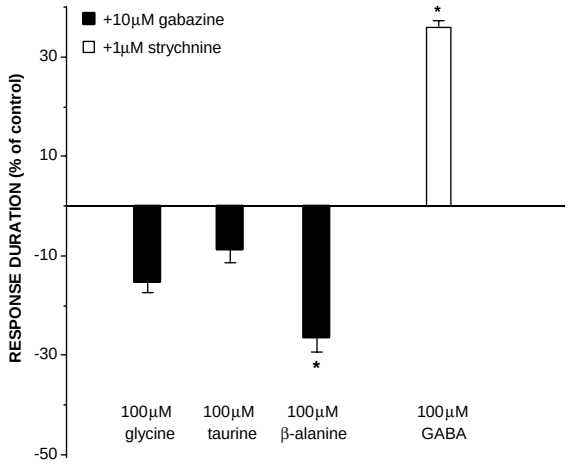

Figure 6. Inhibition by 1 μM gabazine of the enhancement of the response to 10 μM GSH produced by 100 μM glycine, taurine, or β-alanine. Co-administration of 100 μM GABA and 1 μM strychnine does not affect enhancement of the response. Bars represent percent variations of control and are the means ± SEM from four (glycine, taurine) to six (β-alanine) separate experiments. *$p < 0.001$ versus control.

Acknowledgments

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