Nitric Oxide, NOC-12, and S-Nitrosoglutathione Modulate the Skeletal Muscle Calcium Release Channel/Ryanodine Receptor by Different Mechanisms

AN ALLOSTERIC FUNCTION FOR O$_2$ IN S-NITROSYLAITION OF THE CHANNEL*

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The skeletal muscle Ca$^{2+}$ release channel/ryanodine receptor (RyR1) contains ~50 thiols per subunit. These thiols have been grouped according to their reactivity/ responsiveness toward NO, O$_2$, and glutathione, but the molecular mechanism enabling redox active molecules to modulate channel activity is poorly understood. In the case of NO, very low concentrations (submicromolar) activate RyR1 by S-nitrosylation of a single cysteine residue (Cys-3635), which resides within a calmodulin binding domain. S-Nitrosylation of Cys-3635 only takes place at physiological tissue O$_2$ tension (pO$_2$ i.e. ~10 mm Hg) but not at pO$_2$ ~ 150 mm Hg. Two explanations have been offered for the loss of RyR1 responsiveness to NO at ambient pO$_2$, i.e. Cys-3635 is oxidized by O$_2$ versus O$_2$ suberves an allosteric function (Eu, J. P., Sun, J. H., Xu, L., Stamler, J. S., and Meissner, G. (2000) Cell 102, 499–509). Here we report that the NO donors NOC-12 and S-nitrosoglutathione both activate RyR1 by release of NO but do so independently of pO$_2$. Moreover, NOC-12 activates the channel by S-nitrosylation of Cys-3635 and thereby reverses channel inhibition by calmodulin. In contrast, S-nitrosoglutathione activates RyR1 by oxidation and S-nitrosylation of thiols other than Cys-3635 (and calmodulin is not involved). Our results suggest that the effect of pO$_2$ on RyR1 S-nitrosylation is exerted through an allosteric mechanism.

The large, homotetrameric skeletal muscle Ca$^{2+}$ release channel/ryanodine receptor (RyR1) contains several classes of regulatory thiols. These classes are distinguished by reactivity or responsiveness to O$_2$ tension (pO$_2$) (1, 2), redox active molecules such as glutathione (3) and nitric oxide (NO) (1), transmembrane glutathione redox potential (4), and allosteric effecter molecules (Ca$^{2+}$, Mg$^{2+}$) (5). It has recently been shown that cysteine 3635, which is localized to the calmodulin (CaM) binding domain of RyR1 (6–8), confers responsiveness to NO. In contrast, the identities of the remaining regulatory thiols are not known. NO forms a covalent bond with the thiol group of Cys-3635 (i.e. S-nitrosylation) in vivo and thereby reverses the inhibitory effect of CaM on the channel (6). Full-length RyR1 channels with an alanine residue substituted for Cys-3635 are not S-nitrosylated by physiological concentrations of NO, and channel activity is unaffected by NO. S-nitrosylation of Cys-3635 only occurs at low O$_2$ tension (pO$_2$ ~ 10 mm Hg, comparable with that found in skeletal muscle in vivo) (1, 6). At this pO$_2$, 6–8 (of ~50) thiols per RyR1 subunit are actively maintained in the reduced state (1). Thus, one explanation for the failure of NO to S-nitrosylate RyR1 at ambient pO$_2$ is that Cys-3635 is oxidized. An alternative possibility is that the oxidation of pO$_2$-sensitive thiols leads to a change in channel conformation; in this state S-nitrosylation of Cys-3635 is unfavorable. Alternatively stated, O$_2$ is either serving as an oxidant (of Cys-3635) or as an allosteric effector (of Cys-3635 reactivity).

NO donors, compounds capable of donating NO and redox active forms thereof, are widely used to mimic the effects of NO synthase (9). A number of these compounds are capable of modulating RyR1 activity (1, 10–15). RyR1 contains a large number of reactive thiols (1, 2), and the action of NO donors may differ widely depending on the mechanisms and rates of NO release, the chemistry of NO group transfer, the base structure of the NO donor compound, and the reactivity of substrate thiol. In particular, members of the S-nitrosothiol (SNO) class of NO donors can modulate protein function by transnitrosylation as well as NO release (16, 17). In contrast, the NONOate class of NO donors is thought to be less susceptible to transnitrosylation chemistry (18). It is important to note, however, that NONOate compounds may directly interact with proteins through polyamine recognition sites and/or through ionic interactions.

In the present study, we examined the activation of the skeletal muscle Ca$^{2+}$ release channel by NOC-12 and GSNO, an endogenous S-nitrosothiol, and compared their effects to solutions of NO. We found that both NOC-12 and GSNO activated RyR1 independently of O$_2$ tension and that the NO scavenger, C-PTIO, blocked the effects of both. Whereas NOC-12 mediated its effects by S-nitrosylation of a single cysteine (Cys-3635), GSNO activation involved the S-nitrosylation and oxidation of multiple thiols. Moreover, Cys-3635 was not required for activation by GSNO. Thus, NO, NOC-12, and GSNO activate the prototypic redox-sensitive RyR1 channel by different mechanisms, and the effect of O$_2$ tension on S-nitrosylation by NO is best rationalized by an allosteric mechanism.

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‡ The abbreviations used are: RyR, ryanodine receptor; RyR1, skeletal muscle isoform of RyR; pO$_2$, O$_2$ tension; CaM, calmodulin; NO, nitric oxide; SNO, S-nitrosothiol; GSNO, S-nitrosoglutathione; SR, sarcoplasmic reticulum; NOC-12, N-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethanamine; WT, wild type; HEK293, human embryonic kidney 293;
EXPERIMENTAL PROCEDURES

Materials—[3H]Ryanodine was a product of PerkinElmer Life Sciences. CaM, myosin light chain kinase-derived CaM binding peptide and anti-S-nitrosothiol antibodies were from Roche Molecular Biochemicals. An ECL detection reagent kit was from Amersham Biosciences. NO gas (purity > 99%, National Welders) was scrubbed to remove O2 and nitrite by passing through an argon-purged column filled with KOH pellets and then a solution of NaOH. The concentration of NO was determined by a hemoglobin titration assay and an NO electrode (WPI Instruments) as described (1). All other chemicals were of analytical grade.

Sample Preparations—Skeletal muscle sarcoplasmic reticulum (SR) vesicles were isolated from rabbit skeletal muscle in the presence of protease inhibitors (19). The construction and expression of wild type (WT) and C3635A mutant RyR1s have been described (6). WT and C3635A RyR1s were expressed in HEK293 cells, and crude membrane fractions were prepared as described (6).

Quantification of RyR1 Free Thiols and S-Nitrosothiols—RyR1 free thiol (SH) and SNO contents were determined by the monobromobimane fluorescence method and a photolysis/chemiluminescence-based NO detection assay, respectively (1).

Electrophoresis and Detection of S-Nitrosothiol on Western Blots—All procedures were performed under non-reducing conditions (6). Membranes were incubated in 0.125 M KCl, 20 mM imidazole, pH 7.0, and 8 μM free Ca2+ for 1 h at 24°C in room air in the absence and presence of NOC-12 or GSNO. Protein samples were separated by 3–20% SDS-PAGE under non-reducing conditions and transferred to polyvinylidene difluoride membranes. The membranes were blotted with 5% nonfat milk in 0.05% Tween 20 phosphate-buffered 0.1 M saline solution at 24°C for 2 h and probed with anti-S-nitrosothiol polyclonal antibody (Calbiochem; 1:500) and secondary peroxidase-conjugated anti-rabbit IgG antibody (Calbiochem; 1:2000). Anti-S-nitrosothiol signals were detected with an ECL kit (Amersham Biosciences). After that, the membranes were re-probed with anti-RyR1 monoclonal antibody D110 (1:10) and peroxidase-conjugated anti-mouse IgG (Calbiochem; 1:2000). NO-gated anti-rabbit IgG antibody (Calbiochem; 1:2000). Anti-S-nitrosothiol signals were detected with an ECL kit (Amersham Biosciences). All procedures were performed under non-reducing conditions (6).

Release of NO by NOC-12 and GSNO—NOC-12 and GSNO (WPI Instruments) in the buffer used for [3H]ryanodine binding at 24°C in room air (pO2 ~ 150 mm Hg). The half-life time of 1 μM NO under this condition was ~10 min (1).

TABLE I

| NO Donors (Molecular Formula) | Molecular Structure | Peak Concentration of Released NO (pmol/mg protein) | Half Lifetime of Released NO (s) |
|------------------------------|--------------------|---------------------------------------------------|----------------------------------|
| NOC-12 (10 mM)               | ![Figure A](image1) | 2.6 ± 0.4 μM                                      | 6.5 ± 0.1 s                      |
| GSNO (1 mM)                  | ![Figure B](image2) | 1.4 ± 0.3 μM                                      | 2.8 ± 0.01 s                     |

The stock solutions of NO donors (10 mMl) were prepared fresh, and NO release by 0.1 mM each NO donor was recorded with a NO electrode (WPI Instruments) in the buffer used for [3H]ryanodine binding at 24°C in room air (pO2 ~ 150 mm Hg). The half-life time of 1 μM NO under this condition was ~10 min (1).

(* p < 0.05; ** p < 0.01 compared with respective control without NO or NO donor).

RESULTS

Release of NO by NOC-12 and GSNO—NOC-12 releases two NO molecules per donor (23), whereas GSNO releases only one (24). An NO electrode was used to characterize the peak concentrations and the durations of NO release under conditions employed in the [3H]ryanodine binding measurements. NOC-12 (0.1 mM) and GSNO (0.1 mM) attained peak concentrations of 2.6 ± 0.4 μM and 1.4 ± 0.3 μM (n = 3 each) with
Redox Modulation of RyR1 by NO and NO Donors

Fig. 2. Effects of CaM, NOC-12, and GSNO on \(^{3}H\)ryanodine binding of skeletal muscle SR vesicles. Skeletal muscle SR vesicles were preincubated in the presence of 100 \(\mu\)M Ca\(^{2+}\) without or with 1 \(\mu\)M CaM or 1 \(\mu\)M CaM binding peptide (CaMBP) at 24 °C for 30 min. Specific \(^{3}H\)ryanodine binding was assayed at 8 \(\mu\)M Ca\(^{2+}\) (A) or 0.3 \(\mu\)M Ca\(^{2+}\) (B) as described in Fig. 1 in the absence and presence of 0.1 mM NOC-12 or 0.2 mM GSNO in pO\(_2\) ~150 mm Hg. Data are the mean ± S.D. of three to four experiments. *, \(p<0.05\); **, \(p<0.01\) compared with controls with NO donor in each group.

Half-life times of ~6.5 and 2.8 h, respectively (Table I). In a majority of the experiments, we matched the NO peak concentrations by comparing the groups treated with 0.1 mM NOC-12 with those treated with 0.2 mM GSNO. There was no difference in peak concentrations of NO released by either donor as a function of pO\(_2\) (pO\(_2\) ~10 mm Hg versus ~150 mm Hg) (data not shown). The half-life time of NO was ~10 min.

O\(_2\) Tension-independent Modulation of RyR1 by NOC-12 and GSNO—Modulation of RyR1 by NO is O\(_2\) tension dependent; only at a pO\(_2\) comparable with that found in skeletal muscle in vivo (pO\(_2\) ~10 mm Hg) can physiological amounts of NO (sub-micromolar) S-nitrosylate and activate RyR1 (1). In Fig. 1, SR vesicles were treated with increasing concentrations of NO, NOC-12, or GSNO, and RyR1 activities were determined by \(^{3}H\)ryanodine binding at pO\(_2\) ~10 mm Hg (Fig. 1A) or at pO\(_2\) ~150 mm Hg (Fig. 1B). Ryanodine is a highly specific plant alkaloid that is widely used as a probe of channel activity because of its preferential binding to the open channel states (25, 26). As shown previously (1), only at pO\(_2\) ~10 mm Hg did NO (1–10 \(\mu\)M) cause a significant increase in \(^{3}H\)ryanodine binding (Fig. 1A). Elevated levels of NO were inhibitory at pO\(_2\) ~10 mm Hg. In striking contrast, NOC-12 and GSNO activated RyR1 channel activity at either O\(_2\) tension. Control experiments showed that NOC-12 and GSNO left to incubate for 48 h at room air (i.e. spent compounds) were without effect on RyR1 channel activity (data not shown). Under both O\(_2\) tensions, NOC-12 concentrations higher than 0.1 mM caused a slight decrease in \(^{3}H\)ryanodine binding, whereas GSNO concentrations higher than 0.2 mM further increased \(^{3}H\)ryanodine binding. Thus, in contrast to NO, both NOC-12- and GSNO-activation of RyR1 is independent of pO\(_2\) (over a wide range of NO donor concentrations).

Modulation of RyR1 by NOC-12 Is Dependent on CaM, whereas GSNO Modulation Is Not—The functional effects of S-nitrosylation of RyR1 Cys-3635 by NO are CaM-dependent (1, 6). At [Ca\(^{2+}\)] >1 \(\mu\)M, the Ca\(^{2+}\)-bound form of CaM (CaCaM) inhibits RyR1, whereas at [Ca\(^{2+}\)] <1 \(\mu\)M the Ca\(^{2+}\)-free form of CaM (apoCam) activates the receptor (27). We therefore assessed the effects of the NO donors on \(^{3}H\)ryanodine binding in the presence or absence of both the Ca\(^{2+}\)-bound form of CaM and the Ca\(^{2+}\)-free form of CaM. Sequestration of endogenous CaM with a CaM binding peptide (28) caused an increase in RyR1 channel activity over control at 8 \(\mu\)M free Ca\(^{2+}\) (Fig. 2A) and a decrease at 0.3 \(\mu\)M free Ca\(^{2+}\) (Fig. 2B). NOC-12 caused an increase in \(^{3}H\)ryanodine binding in the presence of CaM but not after CaM had been sequestered (Fig. 2, A and B). In contrast, GSNO caused an additional enhancement of RyR1 channel activity even after endogenous CaM sequestration. These results support the idea that NOC-12 controls RyR1 via the S-nitrosylation of Cys-3635, which is found in the CaM binding region of RyR1. On the other hand, redox modulation by GSNO does not appear to be dependent on S-nitrosylation or oxidation of Cys-3635. More definitive evidence for the role of Cys-3635 in the redox modulation of RyR1 is given below using a RyR1 construct with a Cys-3635 to Ala substitution.

Modulation of RyR1 Single Channel Activities by NOC-12 and GSNO—The ability of the two NO donors to activate RyR1 under ambient oxygen tension was confirmed in single channel recordings. Skeletal SR vesicles were incorporated into planar lipid bilayers, and single RyR1 channels were recorded with Cs\(^{+}\) as the current carrier. As shown in Fig. 3A, 0.1 mM NOC-12 significantly activated RyR1 channel in the presence of 2 \(\mu\)M free Ca\(^{2+}\) and 1 \(\mu\)M CaM. Similarly, 0.2 mM GSNO
activated the channel (Fig. 3B). Fig. 3C shows that the averaged channel open probability ($P_o$) of RyR1 tripled after the addition of 0.1 mM NOC-12 or 0.2 mM GSNO. Thus, both $[^3H]$ryanodine binding and single channel measurements show that under comparable conditions these two NO donors activate the RyR1 to the same extent.

**Redox-related Basis of RyR1 Modulation by NOC-12 and GSNO**—We next determined whether modulation of RyR1 by NO donors involved the formation of a single SNO per RyR1 subunit, as was shown previously for NO at pO$_2$ ~ 10 mm Hg (1). We thus determined both the free thiol and SNO content of RyR1s treated with NOC-12 or GSNO at pO$_2$ ~ 150 mm Hg. Exposure of SR vesicles to 0.1 or 1.0 mM NOC-12 increased $[^3H]$ryanodine binding to a similar extent and reduced the RyR1 thiol content by approximately 25% per RyR1 subunit, which was accounted for by the formation of 1 SNO per RyR1 subunit (Table II). The stoichiometry of 1 SNO/RyR1 subunit agreed with that obtained by exposure to 0.75 μM NO at pO$_2$ ~ 10 mm Hg (1). 0.1 mM NOC-12 optimally activated RyR1 in single channel recordings in less than 1 min (Fig. 3A).

In contrast to NOC-12, 0.2 mM GSNO activated RyR1 at ambient O$_2$ tension via the S-nitrosylation or oxidation of multiple thiols or a combination of both redox-based modifications. As shown in Table II, 0.2 mM GSNO S-nitrosylated approximately two RyR1 thiols in addition to oxidizing approximately two thiols per RyR1 subunit (loss of approximately four thiols per RyR1 subunit). At an elevated concentration (1.0 mM), GSNO further increased the level of $[^3H]$ryanodine binding and S-nitrosylated approximately three thiols and oxidized approximately four thiols (loss of approximately seven thiols/RyR1 subunit). Both oxidation and S-nitrosylation of RyR1 by GSNO (and S-nitrosylation by NOC-12) were prevented in the presence of 5 mM reduced glutathione (not shown).

We considered the possibility that GSNO S-nitrosylates RyR1 via transnitrosylation using C-PTIO, a NO scavenger, and NOC-12 as a control. NOC-12 (0.1 mM) no longer had any effect on RyR1 in the presence of 0.1 mM C-PTIO, a NO scavenger, and NOC-12 (0.1 mM) no longer had any effect on RyR1 in the presence of 0.1 mM C-PTIO, neither S-nitrosylating nor activating RyR1 (not shown). Similarly, 0.1 mM C-PTIO eliminated RyR1 S-nitrosylation and activation by 0.2 mM GSNO (not shown). These results suggest that S-nitrosylation of RyR1 by GSNO is dependent on release of NO, as is the release of NO from NOC-12. We caution, nevertheless, that C-PTIO may have other effects, including scavenging and generating additional reactive radicals.

**Cysteine 3635 Is Critical for RyR1 Modulation by NOC-12 but Not by GSNO**—The aforementioned data using SR vesicles suggest that at ambient pO$_2$ NOC-12 S-nitrosylates Cys-3635 and activates RyR1 by antagonizing the inhibitory effect of CaM. In contrast, GSNO works by a different mechanism. We tested this hypothesis using a strategy that was previously employed to demonstrate selective modification of Cys-3635 by NO (6). Full-length WT or single-site C3635A RyR1 mutant

### TABLE II

**Free thiol (SH) and S-nitrosothiol (SNO) contents of RyR1 and $[^3H]$ryanodine binding levels in the absence and presence of NOC-12 and GSNO**

| Preparations | Free thiol content (SH/RyR1 subunit) pmol/mg protein | S-nitrosothiol content (SNO/RyR1 subunit) pmol/mg protein | $[^3H]$ryanodine binding pmol/mg protein |
|--------------|----------------------------------------------------|--------------------------------------------------------|---------------------------------------|
| Normal       | 29.3 ± 1.2 (7)                                     | 0.41 ± 0.02 (6)                                        | 0.56 ± 0.05 (6)                       |
| NOC-12       | 29.1 ± 1.0 (6)                                     | 1.43 ± 0.21 (6)                                        | 0.78 ± 0.05 (3)                       |
| 1.0 mM       | 28.9 ± 0.2 (3)                                     | 1.45 ± 0.36 (3)                                        | 0.74 ± 0.08 (3)                       |
| GSNO         | 25.5 ± 0.8 (5)                                     | 2.05 ± 0.35 (4)                                        | 0.72 ± 0.03 (3)                       |
| 1.0 mM       | 22.6 ± 1.2 (5)                                     | 3.02 ± 0.24 (4)                                        | 0.83 ± 0.06 (3)                       |

*Fig. 4. Effects of NOC-12 and GSNO on WT RyR1 and C3635A RyR1 activities.* Specific $[^3H]$ryanodine binding to membrane fractions prepared from HEK293 cells expressing WT or C3635A RyR1s was determined in 5 μM free Ca$^{2+}$ medium as described in the Fig. 1 legend in the presence of indicated concentrations of NOC-12 (A) or GSNO (B) in pO$_2$ ~ 150 mm Hg. $[^3H]$Ryanodine binding data are the mean ± S.D. of three to five experiments. *p < 0.05; **p < 0.01, compared with each control (without NO donors).
Cys-3635 is one of the 6

S-nitrosylation of Cys-3635 and GSNO by S-nitrosylation and/or oxidation of an additional/alternative class of RyR1 thiols.

DISCUSSION

The massive (~2,200 kDa) ryanodine receptors contain numerous allosteric sites subserving multiple levels of control (25). It has been firmly established that all three mammalian ryanodine receptor isoforms are redox sensitive, i.e. the channels contain regulatory thiols whose oxidation or covalent modification alters their activities (1, 2, 29–32). These thiols (~50/ subunit) have been grouped according to their differential reactivities toward NO, O₂, and glutathione, which in turn may be linked to binding of allosteric effectors (1, 2). We have recently shown that NO, at low pO₂, selectively modifies Cys-3635 (6). pO₂ is dynamically linked to the redox state of a class of 6–8 thiols. However, the identities of these regulatory thiols and the mechanistic basis of the pO₂ regulation of NO binding (homotropic versus heterotropic) remain to be determined. Here we have probed this question by taking advantage of the different reactivities and properties of alternative classes of NO donors.

Cysteine 3635 is part of a predicted hydrophobic motif for S-nitrosylation (33) located within the RyR1 CaM binding domain (7, 8); NO regulation of RyR1 activity is thus CaM dependent (1, 6). We posited that the inability of NO to S-nitrosylate Cys-3635 at ambient O₂ tension is either due to Cys-3635 being oxidized (i.e. Cys-3635 is one of the 6–8 thiols) or to a change in channel conformation brought about by the oxidative posttranslational modification. As a first step to address this question, we determined the dependence of NOC-12 and GSNO on Cys-3635 and pO₂. NOC-12 and GSNO had very similar effects on RyR1 channel activity (at concentrations matched for NO release), and neither compound showed O₂ dependence (pO₂ ~10 mm Hg versus pO₂ ~150 mm Hg). However, the underlying mechanism of activation was quite different in each case. GSNO activated RyR1 via poly-S-nitrosylation and/or oxidation of RyR1 thiols. Cys-3635 and CaM were not essential for activation. These data are highly reminiscent of the effects of GSNO on cardiac muscle isoform of RyR1 (RyR2), except that O₂ and CaM dependence were not explored at that time (20). NO and NOC-12 have little effects on RyR2. In stark contrast, NOC-12 activates RyR1 via S-nitrosylation of Cys-3635, and the increases in activity (³¹Hryanodine binding in intact SR) is CaM dependent, as seen with NO. Specifically, the only modification observed by RyR1 by NOC-12 was control oxidation of a single thiol,

[^2]: J. Sun and G. Meissner, unpublished studies.
and full-length, heterogeneously expressed RyR1 with a C3635A mutation was not activated. Unlike NO, however, S-nitrosylation by NOC-12 is seen at high pO2. Thus we conclude that Cys-3635 of RyR1 is not oxidized at ambient O2 tension.

Why NO and NOC-12 mediated S-nitrosylation differ in their pO2-dependence remains unclear. NOC-12 evidently depends on released NO, because its RyR1-activating effect was inhibited by C-PTIO (a NO scavenger) and was not reproduced by the spent compound. It is unlikely that differences in half-life of NOC-12 versus NO (>6 h versus 10 min, Table I) provide an explanation, because the effect of NOC-12 in single channel recordings was seen within 1 min. Instead, we favor the idea that access of NO to the cysteine thiol is responsible for the differences. Hydrophobic domains that concentrate nitrosylating equivalents and the quaternary structure of the target site are both important determinants for S-nitrosylation (33, 34). NOC-12 may interact with the RyR1 (ionic interactions of these compounds are seen with other proteins),3 perhaps in a way that is conducive to nitrosylation irrespective of RyR conformation.

An interaction with the protein would also have the effect of increasing the effective molarity of the NONOate, thereby potentiating nitrosylation chemistry involving O2. In contrast, access of solution NO (i.e. through the protein) to Cys-3635 might be available in the low pO2 conformation but blocked at high pO2. The hydrophobic pocket where Cys-3635 resides may even serve to concentrate NO/O2 to produce nitrosylating equivalents. In this scenario, the allosteric function subserved by low pO2 is 2-fold: 1) to produce a nitrosylation-responsive conformation of the RyR1; and 2) to catalyze nitrosylation chemistry (micellar catalysis).

An intriguing finding was that NOC-12 and GSNO operate by different mechanisms. NO release from GSNO is evidently S-nitrosylation/oxidation of up to seven RyR1 thiol groups during NOC-12 oxidation) can occur independent of Cys-3635 and CaM. Thus O2, NO, and GSNO react with different classes of thiols, and the role of pO2 in RyR1 S-nitrosylation is likely mediated through allostery.

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