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Abstract: Plants are commonly subjected to several environmental stresses that lead to an overproduction of reactive oxygen species (ROS). As plants accumulate proline in response to stress conditions, some authors have proposed that proline could act as a non-enzymatic antioxidant against ROS. One type of ROS aimed to be quenched by proline is singlet oxygen (1O2)—molecular oxygen in its lowest energy electronically excited state—constitutively generated in oxygenic, photosynthetic organisms. In this study we clearly prove that proline cannot quench 1O2 in aqueous buffer, giving rise to a rethinking about the antioxidant role of proline against 1O2.

Highlights

- Pro was proposed to be a quencher of $^1O_2$, but a direct prove is still remaining
- Direct detection of $^1O_2$ emission shows that Pro is an inefficient quencher of $^1O_2$
- Pro accumulation in plants does not prevent the oxidative damage induced by $^1O_2$

Proline does not quench singlet oxygen: Evidence to reconsider its protective role in plants

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Abstract

Plants are commonly subjected to several environmental stresses that lead to an overproduction of reactive oxygen species (ROS). As plants accumulate proline in response to stress conditions, some authors have proposed that proline could act as a non-enzymatic antioxidant against ROS. One type of ROS aimed to be quenched by proline is singlet oxygen ($^1O_2$)—molecular oxygen in its lowest energy electronically excited state—constitutively generated in oxygenic, photosynthetic organisms. In this study we clearly prove that proline cannot quench $^1O_2$ in aqueous buffer, giving rise to a rethinking about the antioxidant role of proline against $^1O_2$.

Keywords

Antioxidant; scavenger; ROS; environmental stress; proline accumulation

Abbreviations

CAT, catalase; EPR, electron paramagnetic resonance; FFA, furfuryl alcohol; LED, light-emitting diode; NaPB, 20 mM sodium phosphate pH 7.2; MB, methylene blue; Pro, proline; ROS, reactive oxygen species; SOD, superoxide dismutase; TEMP, 2,2,6,6-tetramethylpiperidine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

1. Introduction

Plants are subjected to environmental stresses that bring together an overproduction of reactive oxygen species (ROS) responsible for cellular oxidative damage and (programmed) cell death [1]. Plants accumulate proline (Pro) in response to water stress conditions [2] and different hypotheses have been put forward to explain the role of Pro under this type of stress and others that also result in Pro accumulation. However, none of these hypotheses has been incontrovertibly demonstrated [3, 4]. One hypothesis is that Pro could non-enzymatically react with ROS and prevent essential molecules from (photo)oxidative damage [5-7]. Sminorff and Cumbes evidenced that Pro was an effective hydroxyl radical ($^{\bullet}OH$) scavenger and a rate constant of $4.8x10^{8}$ $M^{-1}{\cdot}s^{-1}$ for this reaction was determined [8]. In addition, Matysik and co-workers (2002) [9] proposed a role of singlet oxygen ($^1O_2$) scavenger for Pro in plants, but the rate constant for the reaction of Pro with $^1O_2$ is still unknown. The $^1O_2$ is easily generated with UV radiation or visible light in the presence of photosensitizers *in vitro* (or chlorophylls in green tissues of plants) and its deactivation may occur by phosphorescence emission or radiationless processes including electronic-to-vibrational energy transfer, charge transfer and electronic energy transfer [10]. Alia and co-workers (2001) proposed that Pro could deactivate $^1O_2$ by physical quenching or by forming products such as superoxide radical ($O_2^{\bullet-}$) or peroxide anion $[O–O]^{2–}$. Since then, the protective role of Pro against $^1O_2$ has been widely considered by the scientific community. Nevertheless, the study by Alia and co-workers (2001) was performed in ethanol and did not unambiguously elucidate if Pro could act as a physical or chemical quencher. If Pro reacted with $^1O_2$ producing $O_2^{\bullet-}$ or $[O–O]^{2–}$, Pro should not be regarded as an efficient non-enzymatic antioxidant in plants because its reaction with $^1O_2$ yielded other types of ROS and, additionally, Pro would be rapidly depleted. However, if Pro could physically quench $^1O_2$, it would not be consumed nor would it yield any additional ROS.

Direct detection of the phosphorescence emission of $^1O_2$ at 1270 nm is nowadays the only technique that allows researchers to establish unambiguously whether $^1O_2$ is produced in their system under study or whether the presence of additional compounds can affect its phosphorescence quantum yield or temporal profile. Because the quantum yield of $^1O_2$ emission in an aqueous or biological system is as low as 10^{-6}–10^{-7} and its lifetime rather short, from few microseconds to few hundreds nanoseconds [10, 11], researchers engaged in $^1O_2$ detection use indirect methods to monitor $^1O_2$ production and quenching instead. In these latter cases, $^1O_2$ production and quenching is followed by monitoring the accumulation of a new product that reacts with $^1O_2$ or the consumption of molecular oxygen with spectrophotometric, spectrofluorometric, polarographic or EPR spin trapping techniques [7, 12-14]. The combination of direct and indirect methods can be useful for a better understanding of this issue, particularly when other ROS can come into play.

In our attempt to elucidate the bimolecular rate constants for the quenching of $^1O_2$ by Pro in neutral aqueous solutions, we reach the conclusion that Pro is, in fact, an inefficient $^1O_2$ scavenger.

2. Results and discussion

2.1 Direct detection of $^1O_2$

The temporal profile of the phosphorescence emission by $^1O_2$ at 1270 nm produced by riboflavin in the presence and absence of Pro under oxygen atmosphere is depicted in Figure 1. The observed kinetic traces were fit well to the biexponential function $I(t) = a(k_1-k_2)^{-1}[\exp(-k_2t) - \exp(-k_1t)]$, where $a > 0$, and the larger and smaller of the two rate constants determine the rise and decay of the emission signal respectively; here k_1 is identified with the larger rate constant and *vice versa*. Before the analysis of the rate constants in 20 mM sodium phosphate pH 7.2

(NaPB), experiments with NaN_3 or under a stream of N_2 were performed to unambiguously correlate the origin of the kinetic traces with $^1O_2$ emission (data not shown). The temporal profile of $^1O_2$ emission showed that $\tau_1 \equiv 1/k_1 = 0.7$ μs. A value that agrees with the expected value under oxygen atmosphere [11], where the concentration of dissolved oxygen is found to be ~3.2 times larger [15, 16]. The decay time constant $\tau_2 \equiv 1/k_2$ was slightly larger in 20 mM NaPB (4.5 μs) than that previously determined in pure water (3.7 μs) [15]. When Pro was added up to a concentration of 150 mM, mimicking the average natural accumulation of Pro in aqueous compartments of plant cells in response to adverse environmental stresses, neither the intensity of the $^1O_2$ emission nor the rise and decay constants changed within experimental error (Figure 1), indicating that first Pro does not affect triplet-triplet energy transfer from riboflavin to molecular oxygen and second Pro does not efficiently deactivate $^1O_2$. Similar conclusions were reached when lumiflavin or toluidine blue in 20 mM NaPB or ethanol were used as $^1O_2$ photosensitizers instead of riboflavin (data not shown).

2.2 Oxygen consumption

The putative reaction of Pro with $^1O_2$ was also investigated indirectly in a Clark-type electrode, keeping a nearly constant concentration of $^1O_2$ while exciting methylene blue (MB) with a red light-emitting diode (LED) source. In this experiment, the formation and deactivation of $^1O_2$ and, presumably, other types of ROS were investigated. Figure 2 shows that the addition of 100 mM Pro did not bring together any consumption of oxygen after the dark-to-light shift, indicating that $^1O_2$ photosensitized by MB is simply deactivated by H_2O molecules. In contrast, the addition of furfuryl alcohol (FFA)—a well-known chemical quencher of $^1O_2$ [17]—at a concentration of 0.4 µM consumed oxygen with a rate of 2.0 $\mu M \cdot s^{-1}$. The rate of oxygen uptake by FFA decreased to ~40% of its initial value when a physical quencher such as NaN_3 at a concentration of 1 mM was present. In contrast, the combined addition of 100 mM Pro and 0.4 µM FFA did not affect the oxygen uptake by FFA, confirming that Pro is both an inefficient physical and chemical quencher of $^1O_2$. Increasing the lifetime of $^1O_2$ by replacing H_2O by D_2O (*i.e.*, 20 mM potassium phosphate pD 7.2) did not enhance the quenching properties of Pro (data not shown). To establish unambiguously that the oxygen consumption observed in the Clark-type electrode was due to $^1O_2$ photosensitization, but not to the production of $O_2^{\bullet-}$ or H_2O_2, further experiments were performed in the presence of enzymatic antioxidants (*i.e.*, superoxide dismutase, SOD, and catalase, CAT). Figure 2 shows that the addition of 500 $U \cdot mL^{-1}$ of SOD or CAT did not induce any change in the photoinduced rate of oxygen uptake, even when 100 mM Pro was present in the medium, indicating that Pro does not produce $O_2^{\bullet-}$ or $[O–O]^{2–}$ either.

2.3 Deactivation by electronic-to-vibrational energy transfer

The results presented above show that the encounter of Pro with $^1O_2$ does not follow any process where an exciplex with partial charge transfer character becomes deactivated by intersystem crossing (physical quenching) or chemical reaction. To explore whether Pro could compete with H_2O for the deactivation of $^1O_2$ by electronic-to-vibrational energy transfer, the energy of the highest frequency mode vibration of Pro was compared with that of H_2O in liquid state. The water molecules in liquid state have stretch vibrations in the 3100–3600 cm^{-1} range and this vibration band has associated a rate constant for $^1O_2$ deactivation of ~2900 $M^{-1} \cdot s^{-1}$ [10]. The highest frequency mode vibration (ν_{NH}) of Pro in water is 3057 cm^{-1} [18] and has a rate constant for $^1O_2$ deactivation of ~1530 $M^{-1} \cdot s^{-1}$ [10], approximately half of the value for the stretching of water. These values show that Pro cannot efficiently compete with H_2O for $^1O_2$ deactivation through electronic-to-vibrational energy transfer in

(cellular) aqueous media, based simply on the above values for the rate constants and the remarkable difference in concentration between the solute and the solvent.

2.4 Effect of Pro on TEMPO accumulation

The lines of evidence presented above prove that Pro cannot quench $^1O_2$. To better understand why our results differed from those observed by Alia and co-workers (2001), $^1O_2$ production by toluidine blue was followed by electron paramagnetic resonance (EPR) spectroscopy under continuous illumination of the sample in the EPR cavity. The oxidation product of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) grew continuously with the course of time in the absence of Pro; however, TEMPO accumulated slower in the first instance and turned down after several minutes in the presence of 25 mM Pro (Figure 3). Additionally, the bandwidth of the EPR signal became narrower. This suggests that Pro do not prevent the oxidation of 2,2,6,6-tetramethylpiperidine (TEMP) by $^1O_2$, but the increase of TEMPO, indicating that the spin adduct with $^1O_2$ undergoes a change.

2.5 Possible role of Pro in plants under stress

Pro accumulates in cytosol and chloroplast stroma [19]; where isoforms of the Δ-1-pyrroline-5-carboxylate synthetase enzyme are localized [20]. Taking into account our results, together with the fact that Pro accumulates in aqueous compartments of cells, but not in thylakoid membranes, where $^1O_2$ is constitutively produced by the photosystem II and largely damages lipids and membrane proteins under photoinhibition before diffusing into the surrounding medium, we propose that the Pro does not play any significant role in the scavenging of $^1O_2$ in plants. Several studies have concluded that oxidative damage is lower when Pro accumulates in plants under stress [21], but this protective effect must then be related to the scavenging of other ROS, for example $^{\bullet}OH$ [6], but not $^1O_2$. In addition, Pro can play other protective roles; it can act as a compatible osmolyte [22, 23] or the Pro synthesis and catabolism can play essential role in redox balance [24]. Probably, both Pro itself and Pro synthesis and catabolism play combined functions in the stress adaptation of plants.

Conclusions

In brief, we determine that Pro does not quench $^1O_2$ either chemically or physically using methods that include the direct detection of the $^1O_2$ emission, oxygen consumption in a Clark-type electrode or EPR spin trapping. Moreover, stretching vibrations of Pro do not exceed the water vibrations and so the presence of Pro in the medium does not increase the ability to quench $^1O_2$ by electronic-to-vibrational energy transfer. On the basis of these facts, we conclude that Pro accumulation does not play any significant role in the scavenging of $^1O_2$ in plants under stress and that other roles in osmoprotection, $^{\bullet}OH$ scavenging or contribution in redox homeostasis should be considered.

3 Methods

4.1 Time-Resolved $^1O_2$ measurements

Time-resolved emission of $^1O_2$ at 1270 nm was studied in 20 mM NaPB under oxygen atmosphere at room temperature. Further details about the experimental setup have already been described in sufficient detail in Arellano et al. (2007) and Li et al. (2012). An absorbance of 0.9 at 445 nm for riboflavin was used to photosensitize $^1O_2$ in the assay buffer. The concentration of Pro ranged from 0 to 150 mM.

4.2 Oxygen Consumption

Oxygen uptake by $^1O_2$ scavengers was measured polarographically using a Chlorolab 2 system (Hansatech Instruments, England) at 20°C. Samples were buffered at pH 7.2 with 20 mM NaPB and contained MB with an

absorbance of 0.15 at 665 nm to photosensitize $^1O_2$. When needed, 0.4 µM FFA or 500 U·mL^{-1} of SOD or CAT were added to scavenge $^1O_2$, $O_2^{\bullet-}$ or H_2O_2. All samples were incubated in the dark for 1 min before the red LED source was switched on. The light irradiance in the electrode chamber was 2 mE·m^{-2}s^{-1}.

4.3 $^1O_2$ detection by spin trapping EPR

A reaction mixture containing 1mM toluidine blue and 10 mM TEMP was continuously irradiated with a 50 W Tungsten lamp with an optical fiber connected to the cavity (0.3 mW·cm^{-2}) and the EPR signal of TEMPO monitored at different intervals, in the absence or presence of 25 mM Pro. EPR measurements were done using a JEOL free radical monitor machine (JES-FR30) with a cylindrical cavity (TE011 mode) working in the 9.1–9.5 GHz range. The receiver gain of the EPR instrument was 200, modulation width 0.2 mT and power 4 mW. Experiments were done at room temperature and in ethanol as a solvent.

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Legends to figures

Figure 1. Temporal profile of $^1O_2$ phosphorescence in the presence and absence of Pro. $^1O_2$ was photosensitized by riboflavin in 20 mM NaPB with no further additions (black traces) and 90 mM Pro (red traces) under oxygen atmosphere. The absorbance of riboflavin was 0.9 at 445 nm. The phosphorescence emission was measured at 1270 nm. The number of averaged scans was 128. The residuals are also shown for both experiments.

Figure 2. Oxygen uptake in the Clark-type electrode chamber using MB as $^1O_2$ photosensitizer, and Pro and FFA as $^1O_2$ quenchers. Experiments were carried out in 20 mM NaPB. FFA at a concentration of 0.4 µM was present in all the experiments except when indicated. The absorbance of MB was 0.15 at 665 nm. S&C represents 500 $U \cdot mL^{-1}$ of SOD and CAT. Arrows with on and off indicate when the red LED source was turned on and off respectively. * SD = ± 0.06.

Figure 3. Effect of Pro on TEMPO accumulation. The EPR signal was monitored in the absence of Pro **(**Black squares and solid line) and 25 mM Pro (red circles and dotted line). A reaction mixture containing 1mM toluidine blue and 10 mM TEMP was continuously irradiated with a 50 W Tungsten lamp with an optical fiber

connected to the cavity (0.3 $mW \cdot cm^{-2}$) and the EPR signal of TEMPO monitored at different intervals. The inset shows the normalized EPR signal in the presence and absence of Pro for the comparison of the EPR bandwidth.

Figure 1

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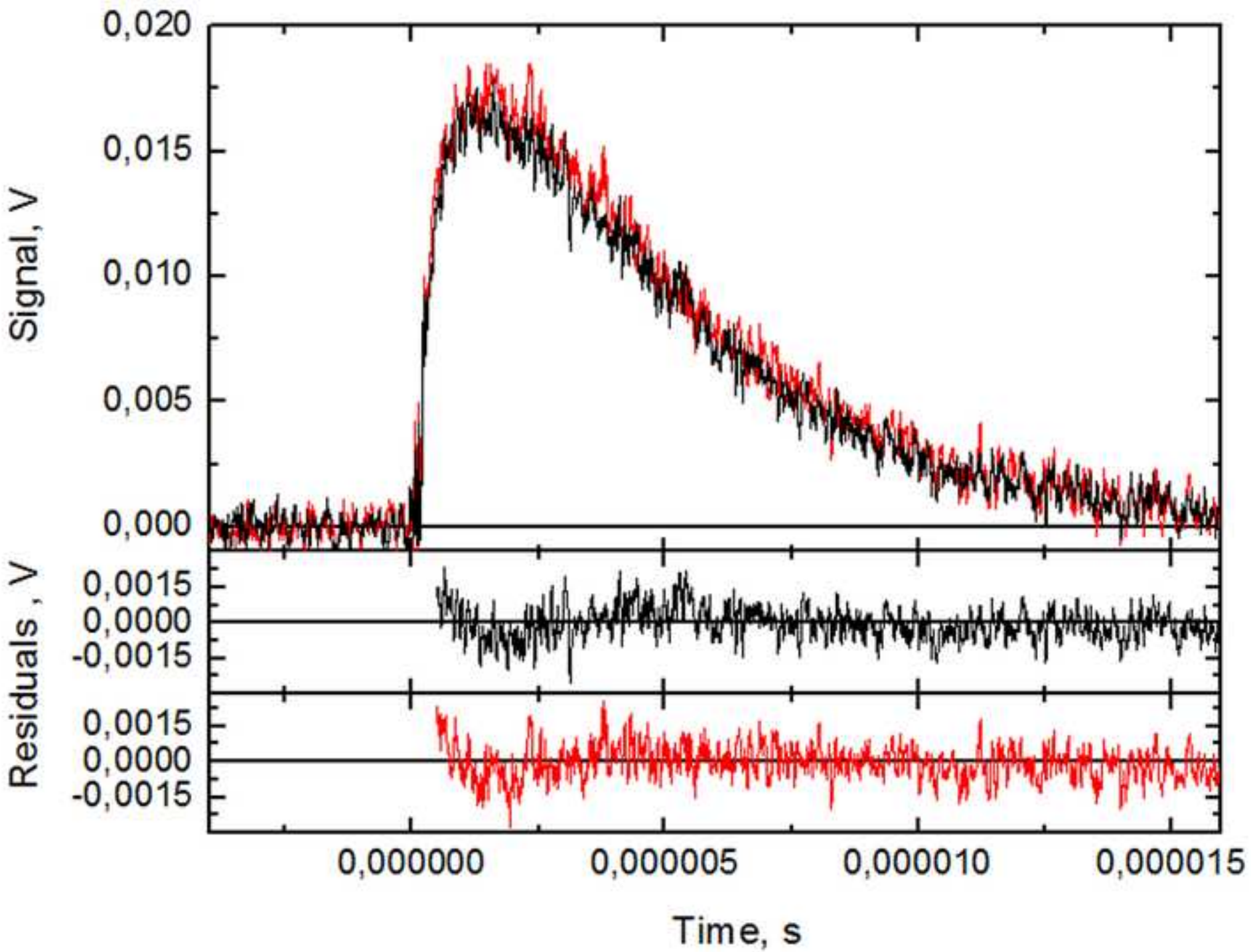

Figure 2

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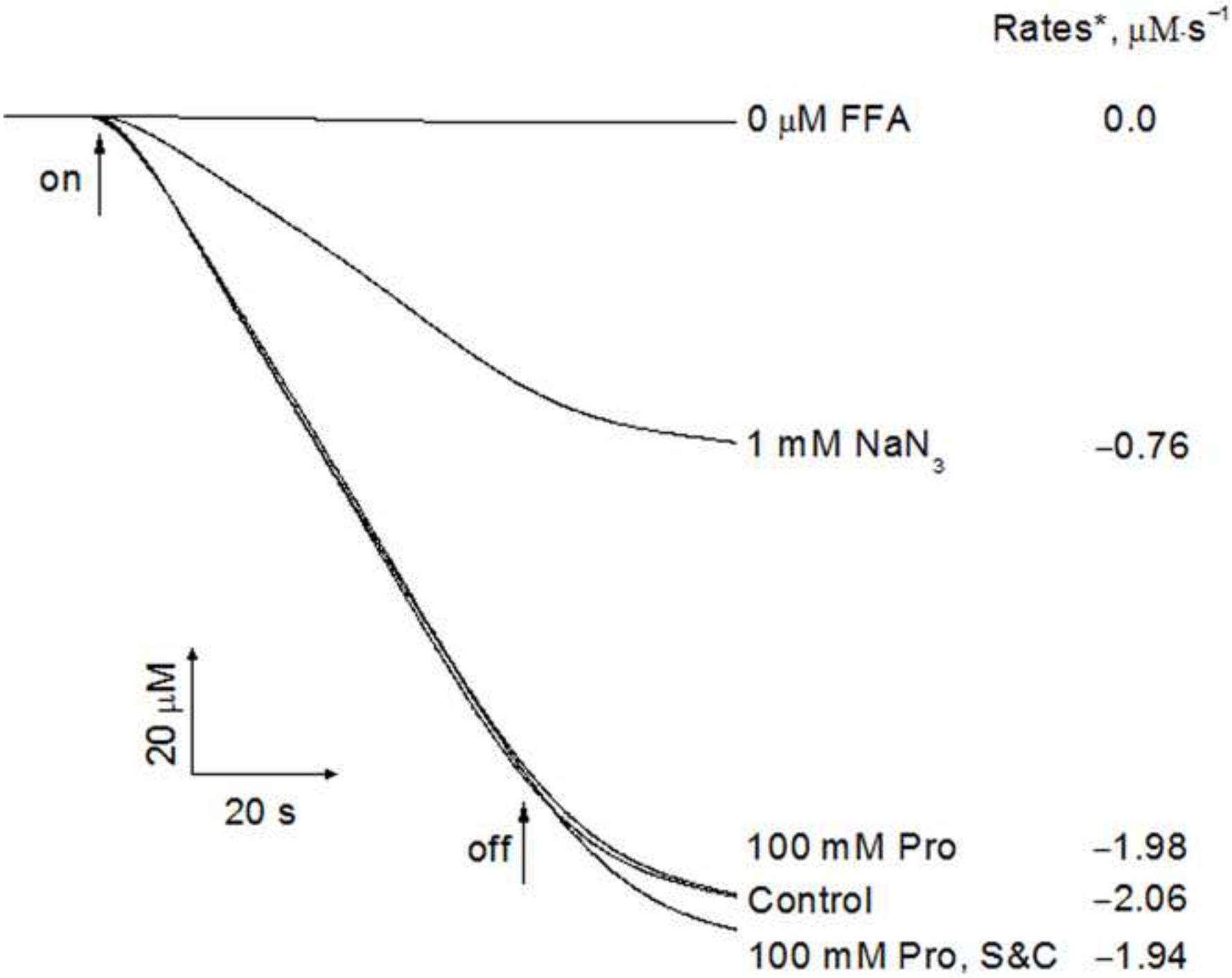

Figure 3

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