Membrane hyperpolarization and depolarization of rat thymocytes by azoxystrobin, a post harvest fungicide

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Highlights

• Azoxystrobin hyperpolarizes the membranes of rat thymocytes.

• The hyperpolarization is due to activation of Ca^{2+}-dependent K^+ channels.

• Azoxystrobin depolarizes the membranes in the presence of quinine.

• These effects of azoxystrobin are probably harmful for lymphocytes.

Abstract

Azoxystrobin, a broad-spectrum fungicide, has been increasingly used in the agricultural industry. In Japan in 2018, azoxystrobin at five times the normal limit was detected in a shipment of Australian barley that had been used in food products. Therefore, the effects of azoxystrobin need to be carefully examined to predict potential adverse reactions in humans. In this study, the effects of azoxystrobin on the membrane potential and intracellular Ca^{2+} levels of thymocytes have been photochemically examined using flow cytometry. Azoxystrobin hyperpolarized plasma membrane potential. This hyperpolarization appeared to be due to the activation of Ca^{2+}-dependent K^+ channels, as both the removal of extracellular Ca^{2+} and addition of charybdotoxin attenuated the observed hyperpolarization. In the presence of quinine, an anti-malarial drug that blocks Ca^{2+}-dependent K^+ channels, azoxystrobin depolarized the membranes instead. Azoxystrobin increased intracellular Ca^{2+} levels in a concentration-dependent manner through the influx of extracellular Ca^{2+} and intracellular release of Ca^{2+}, as confirmed by reduction in azoxystrobin-induced response in the absence of extracellular Ca^{2+}. It appears likely that azoxystrobin at micromolar concentrations modifies membrane ion permeability in thymocytes. Since changes in membrane potential and intracellular Ca^{2+} levels occur during typical physiological lymphocyte responses, azoxystrobin may disturb lymphocyte function.

Keywords: azoxystrobin; lymphocytes: membrane potential; intracellular calcium

1. Introduction

Azoxystrobin is a broad-spectrum fungicide that has seen increased use in the agricultural industry every year since it was first introduced in 1998 [1]. This agent blocks electron transfer in mitochondrial respiration, resulting in inhibition of ATP production and, ultimately, oxidative stress in fungi [2,3]. Azoxystrobin is also toxic to both vertebrates and invertebrates in freshwater and marine systems [4]. Due to the potential for contamination, toxicological studies on azoxystrobin in non-target organisms (including humans and other mammals) are warranted [5,6]. To our knowledge, however, there is no existing data demonstrating toxicity of azoxystrobin in humans at environmental levels. In Japan in 2018, azoxystrobin at five times the normal limit was detected in a shipment of Australian barley [7]. Barley from the same batch had already been used in food products, which the food company voluntarily recalled. This event further shows that the effects of azoxystrobin must be carefully examined to predict possible adverse effects in humans and wild mammals.

In this study, the effect of azoxystrobin on the membrane potential of thymocytes (lymphocytes) has been examined through flow cytometry using appropriate fluorescent probes. Non-excitable cells are not typically considered a target for azoxystrobin but changes in membrane potential also occur in non-excitable cells, including lymphocytes and hepatocytes, during normal physiological behavior and may also be affected [8–10]. Furthermore, the principal determinants of membrane potential are transmembrane ion gradient and membrane ion permeability. These integral factors are maintained by cellular energy metabolism and specific membrane-bound ion channels, such as K^+ channels. Inhibition of K^+ channels in lymphocytes results in their malfunction [11–13]. Compounds that affect membrane potentials (such as azoxystrobin) may affect some lymphocyte functions, resulting in adverse effects on the immune system. Furthermore, the thymus is very active in pre-adolescent and neonatal periods. Due to concerns about the adverse effects of azoxystrobin on the health of children, any information concerning changes in thymocytes caused by azoxystrobin may be clinically important.

2. Materials and methods

2.1. Chemicals

Azoxystrobin (standard purity 99.8 %) was purchased from Wako Pure Chemicals (Tokyo, Japan). Propidium iodide (PI), *bis*(1,3-dibutylbarbituric acid)trimethine oxonol (oxonol), and Fluo-3-AM were obtained from Invitrogen (Eugene, OR, USA) or Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid disodium salt (EDTA; Dojindo Molecular Technologies, Inc.) was used as a chelator for extracellular divalent metal cations such as Ca^{2+} and Zn^{2+}. *N*,*N*,*N*',*N*'-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN; Dojindo Molecular Technologies, Inc.) was used for chelation of intracellular Zn^{2+}. All other reagents were obtained from Wako Pure Chemicals (Tokyo, Japan).

2.2. Cell preparation

Experiments using rats were carried out under approval (T29-52) from the committee of Tokushima University (Tokushima, Japan) for animal experiments. The thymus glands of male Wistar rats (6–8 week-old) were triturated in chilled Tyrode's solution (2–4 °C) to dissociate thymic lymphocytes (thymocytes). The solution containing thymocytes (cell suspension) was stored at 36–37 °C for 1 h at least before use in experiments. It is noted that the cell suspension contained small amounts of zinc (216.9 ± 14.4 nM) from the cell preparation (composition salts in Tyrode's solution and organs) [14].

Thymocytes were used in this chemical cytotoxicity experiment because living cells with intact membranes are easily dissociated from organs without enzymatic treatment. However, during prolonged incubation thymocytes may spontaneously undergo apoptosis. Thus, all experiments were conducted within 6–7 h after organ dissection to avoid significant spontaneous apoptosis. Since cell shrinkage is one of the parameters detected during early stages of apoptosis [15], forward scatter was used to assess cell size. The forward scatter and cell viability in control cells were unchanged during the experiment, confirming that apoptosis had not occurred. It is also

noted that the information in this study is limited in the aspect of cytotoxic actions of azoxystrobin because rat thymocytes are not a right model to address human immune T cell functions.

Azoxystrobin (0.3–100 mM in 2 μL dimethyl sulfoxide) was added to the suspension (1998 μL) to prepare bath concentrations of 0.3–100 μM and the cells were treated with azoxystrobin for 1 h. Dimethyl sulfoxide (up to 0.3 %) did not affect cell viability and fluorescent cellular parameters measured in this study. A sample (100 μL) from each cell suspension was analyzed by flow cytometry. Data acquisition from 2500 or 3000 cells took approximately 10–15 s. Cellular parameters monitored using 2500 cells were similar to those estimated using 10000 cells. Thus, 2500 or 3000 cells were deemed sufficient to study the cellular effects of azoxystrobin.

2.3. Measurements of cellular parameters with fluorescent probes

Fluorescence was analyzed using a flow cytometer and software package (CytoACE-150; JASCO, Tokyo, Japan). Azoxystrobin at concentrations used in this study exhibited no fluorescence under the experimental conditions described here. PI (5 μM) was added to the suspension to allow assessment of cell lethality by measuring the percentage population of cells exhibiting PI fluorescence. Measurement of plasma membrane potentials was performed using 1 μM oxonol. In order to monitor the change in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$), cells were incubated with 1 μM Fluo-3-AM [16] for 50–60 min before the start of measurement. Fluo-3 fluorescence was monitored in cells in the presence of 10 μM TPEN, as a chelating agent, to avoid the contribution of Zn^{2+} to Fluo-3 fluorescence. Azoxystrobin-induced changes in oxonol and Fluo-3 fluorescence were studied only in living cells with intact membranes that did not exhibit PI fluorescence. The cells with intact membranes retained Fluo-3 molecules inside the cells. The excitation wavelength of all probes (PI, Fluo-3, and oxonol) was 488 nm, and emissions were detected at 530 ± 20 nm for oxonol and Fluo-3, and at 600 ± 20 nm for PI.

2.4. Statistical analysis and presentation

The data obtained in these experiments were statistically analyzed using Tukey's multivariate method. *P*-values of < 0.05 indicated significant difference between cell groups.

Values are expressed as mean and standard deviation of 4–8 samples. Each experimental series was conducted in triplicate unless stated otherwise.

3. Results

3.1. Changes in oxonol fluorescence induced by azoxystrobin

The measured oxonol fluorescence of rat thymocytes was attenuated when the cells were treated with 30 μM azoxystrobin for 1 h (Figure 1A). The histogram of oxonol fluorescence was observed to shift to a lower intensity, indicating membrane hyperpolarization. Figure 1B summarizes the hyperpolarization and cytotoxicity observed in thymocytes exposed to a range of azoxystrobin concentrations. The threshold concentration of azoxystrobin for the induction of hyperpolarization was approximately 1–3 μM when administered to cells for 1 h (Figure 1B). Increase in azoxystrobin concentration (up to 100 μM) further decreased the intensity of oxonol fluorescence in a concentration-dependent manner (Figure 1B). When the cells were incubated with 100 μM azoxystrobin for 1 h, the percentage of cells exhibiting propidium fluorescence (presumably dead cells and/or cells with compromised membranes) increased slightly, but significantly (Figure 1B). Peak hyperpolarization by azoxystrobin at non-cytotoxic levels was observed at a concentration of 30 μM.

(Figure 1 near here)

3.2. Mechanism of azoxystrobin-induced changes in oxonol fluorescence

In order to determine the pathways through which azoxystrobin induces membrane hyperpolarization, additional fluorescence experiments were also conducted with a series of additives (Figures 2–4). Membrane hyperpolarization usually occurs when the K^+ channels open, resulting in an increase in membrane K^+ permeability. The membranes of rat thymocytes express Ca^{2+}-activated K^+ channels [17]. To ascertain the role of Ca^{2+} on azoxystrobin-mediated hyperpolarization, the Oxonol fluorescence experiment was repeated under extracellular Ca^{2+}-free conditions. The effects of azoxystrobin on membrane polarization were tested under

extracellular Ca^{2+}-free conditions. As shown in Figure 2, the base level of oxonol fluorescence slightly increased under extracellular Ca^{2+}-free conditions, but treatment of cells with 30 μM azoxystrobin for 1 h had no significant effect on oxonol fluorescence in the absence of extracellular Ca^{2+}. There was a significant decrease in the intensity of oxonol fluorescence in the cells treated with azoxystrobin in the presence of extracellular Ca^{2+} compared to those in the extracellular Ca^{2+}-free environment (Figure 2). From these results, it can be assumed that extracellular Ca^{2+} is a key component of azoxystrobin-induced hyperpolarization.

To further determine the role of Ca^{2+}-dependent K^+ channels in this process, the effect of 30 μM azoxystrobin was tested in the presence of quinine possessing an inhibitory action on Ca^{2+}-dependent K^+ channels [18]. Treatment of cells with 300 μM quinine alone did not significantly change the intensity of oxonol fluorescence. However, treatment with 30–300 μM quinine in the presence of 30 μM azoxystrobin significantly increased the intensity of oxonol fluorescence in a concentration-dependent manner, indicating depolarization instead of hyperpolarization (Figure 3). It is surprising that the combination of azoxystrobin and quinine induces strong depolarization in lymphocytes.

Further experiments were conducted to elucidate the involvement of Ca^{2+}-dependent K^+ channels, examining the effect of 30 μM azoxystrobin in the presence of 300 nM charybdotoxin (CTX; Figure 4), an alternative blocker of Ca^{2+}-dependent K^+ channels [19]. The attenuation of oxonol fluorescence by 30 μM azoxystrobin in the presence of charybdotoxin was significantly reduced compared to that observed in its absence, suggesting that azoxystrobin-induced hyperpolarization was partly inhibited by charybdotoxin. Thus, charybdotoxin-sensitive K^+ channels, presumably Ca^{2+}-dependent K^+ channels, may contribute to the azoxystrobin-induced hyperpolarization.

(Figures 2, 3, and 4 near here)

3.3. Changes in Fluo-3 fluorescence induced by azoxystrobin

Fluo-3 fluorescence is a useful tool for measuring intracellular levels of Ca^{2+}. Fluo-3

fluorescence is sensitive not only to Ca^{2+}, but also to Zn^{2+} [16], which could potentially lead to interference when monitoring changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels. To avoid this interference, 10 μM TPEN (a chelator of intracellular Zn^{2+}) was added to the cell suspension in this series of Fluo-3 fluorescence experiments (Figure 5). As shown in Figure 5A, treatment of cells with 30 μM azoxystrobin shifted the histogram of Fluo-3 fluorescence to a higher intensity compared to the control. The threshold concentration of azoxystrobin for the increase of Fluo-3 fluorescence was less than 3 μM in the presence of TPEN (Figure 5B). Azoxystrobin at 3–30 μM produced concentration-dependent augmentation of Fluo-3 fluorescence, indicating azoxystrobin-induced increase in $[Ca^{2+}]_i$ (Figure 5B). Removal of extracellular Ca^{2+} greatly reduced the augmentation of Fluo-3 fluorescence by 30 μM azoxystrobin, although augmentation was still observed (Figure 5C). It is likely that this azoxystrobin-induced augmentation is due to extracellular Ca^{2+} influx and intracellular Ca^{2+} release.

(Figure 5 near here)

The combination of azoxystrobin and quinine caused significant depolarization (Figure 3). Therefore, a further increase in $[Ca^{2+}]_i$ by the combination was reminiscent. As shown in Figure 6, the simultaneous treatment of cells with 30 μM azoxystrobin and 300 μM quinine for 1 h further augmented Fluo-3 fluorescence, indicating further increase in $[Ca^{2+}]_i$.

(Figure 6 near here)

4. Discussion

4.1. Mechanism and possible risk

Agents that increase intracellular Ca^{2+} cause membrane hyperpolarization in cells possessing Ca^{2+}-activated K^+ channels. This also seems to be the case with azoxystrobin. Concentrations of azoxystrobin required to elevate $[Ca^{2+}]_i$ in thymocytes are similar to those that induce hyperpolarization. The attenuation of oxonol fluorescence (indicative of hyperpolarization) by azoxystrobin was reduced in the absence of extracellular Ca^{2+} or in the

presence of the Ca^{2+}-dependent K^+ channel blocker charybdotoxin. However, the combination of azoxystrobin and the alternative blocker quinine caused augmentation of oxonol fluorescence (indicative instead of depolarization). Quinine alone did not change oxonol fluorescence, indicating that quinine exerts no action on membrane potential. As depolarization can be caused by the specific or non-specific increase in membrane permeability of cations, the combination of azoxystrobin and quinine may be causing depolarization by increasing membrane permeability. If azoxystrobin induces depolarization in the presence of other medical agents, this would result in potent cytotoxic action on excitable cells. The combination may further increase the $[Ca^{2+}]_i$ in excitable cells expressing voltage-dependent Ca^{2+} channels, resulting in cell death or injury [19–22]. Quinine is a cytotoxic substance [23-25]. Therefore, it may be not surprising that the combination of quinine and azoxystrobin caused significant depolarization. The combination further increased the $[Ca^{2+}]_i$, suggesting further increase in membrane permeability by the combination. At present, the mechanism of Ca^{2+} influx by azoxystrobin is not elucidated. It will be necessary to identify Ca^{2+} permeable channels by electrophysiological techniques.

4.2. Implications

The use of azoxystrobin, a broad-spectrum fungicide, increases every year [1]. In Japan, 2.5 mg/kg of azoxystrobin was detected in a shipment of Australian barley, which had already been used in food products [7]. In the present study, the threshold concentration of azoxystrobin required to induce changes in cellular parameters was found to be 1–3 μM under *in vitro* experimental conditions. This concentration is approximately equivalent to 0.4–1.2 mg/kg (azoxystrobin molecular weight: 403.388 g/mol). Although the pharmacokinetics of azoxystrobin in humans and the amount of azoxystrobin present in food products originating from the contaminated barley shipment are unknown, it is difficult to imagine that blood levels of azoxystrobin would reach 3 μM (1.2 mg/L) as this would require absorption of a significant proportion of the 2.5 mg/kg found in the raw material. The pharmacokinetics of oral azoxystrobin was studied in rats [26], and it was revealed that more than 85% of the administered azoxystrobin

gets excreted unchanged within 48 h in feces (up to 80%) and urine (< 17%). Therefore, the oral absorption of azoxystrobin is low, and it also undergoes extensive metabolism. After extrapolating these data to human, it can be predicted that the blood concentration of azoxystrobin may not get too high after intake of foods containing azoxystrobin. Hence, an adverse effect from azoxystrobin in human may be unlikely. However, there is a possibility that azoxystrobin induces strong membrane depolarization when in the presence of medical agents. Thus, further information on azoxystrobin cytotoxicity and safety, particularly during drug-drug interactions, is needed.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgements

The Japan Society for the Promotion of Science (Tokyo, Japan) provided the Grant-in-Aids for Scientific Research (C26340039) for this study.

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Figure legends

Figure 1. Azoxystrobin-induced change in Oxonol fluorescence. (A) Azoxystrobin-induced shift of Oxonol fluorescence histogram. Histograms were constructed using the fluorescence of 2500 thymocytes measured 1 h after the start of drug application. Asterisks (*) indicate the histogram obtained from cells treated with 30 μM azoxystrobin. (B) Concentration-dependent changes in cell lethality (percentage population of cells exhibiting propidium fluorescence) and mean intensity of Oxonol fluorescence in cells treated with 0.3–100 μM azoxystrobin. The column and bar indicate mean and standard deviation, respectively (n = 4). Asterisks (**) indicate significant differences ($P < 0.01$) between the control group (CONTROL) and groups of cells treated with azoxystrobin (AZOXYSTROBIN).

Figure 2. Azoxystrobin-induced changes in Oxonol fluorescence in the absence (upper pair) and presence (lower pair) of extracellular Ca^{2+} (2 mM $CaCl_2$). The column and bar indicate mean and standard deviation, respectively (n = 4). Asterisks (**) indicate significant differences ($P < 0.01$) between the control group (CONTROL) and groups of cells treated with 30 μM azoxystrobin (AZOXYSTROBIN). Pounds (##) indicate a significant difference ($P < 0.01$) between the groups of cells treated with azoxystrobin in the absence and presence of extracellular Ca^{2+}.

Figure 3. Changes in Oxonol fluorescence induced by 30 μM azoxystrobin in the absence and presence of quinine (30–300 μM). The column and bar indicate mean and standard deviation, respectively (n = 4). Asterisks (*, **) indicate significant differences ($P < 0.05$, $P < 0.01$) between the control group (CONTROL) and the test groups. Pounds (##) indicate significant difference ($P < 0.01$) between the groups of cells treated with azoxystrobin in the absence and presence of quinine.

Figure 4. Changes in Oxonol fluorescence induced by 30 μM azoxystrobin in the absence (upper pair) and presence (lower pair) of 300 nM charybdotoxin (CTX). The column and bar indicate mean and standard deviation, respectively (n = 4). Asterisks (**) indicate significant differences ($P < 0.01$) between the control group (CONTROL) and the group of cells treated with CTX. Pounds (##) indicate significant difference ($P < 0.01$) between the groups of cells treated with azoxystrobin in the absence and presence of CTX.

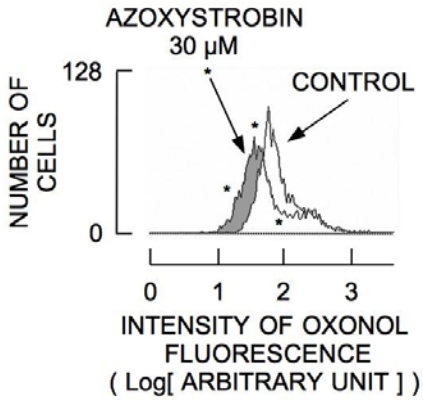
Figure 5. Azoxystrobin-induced changes in Fluo-3 fluorescence. (A) Azoxystrobin-induced shift of Fluo-3 fluorescence histogram. Histograms were constructed using the fluorescence of 2500 thymocytes measured 1 h after the start of drug application. Asterisks (*) indicate the histogram obtained from cells treated with 30 μM azoxystrobin. (B) Concentration-dependent changes in mean intensity of Fluo-3 fluorescence in cells treated with 0.3–30 μM azoxystrobin. The column and bar indicate mean and standard deviation, respectively (n = 4). Asterisks (*, **) indicate significant differences ($P < 0.05$, $P < 0.01$) between the control group (CONTROL) and groups of cells treated with azoxystrobin (AZOXYSTROBIN). (C) Azoxystrobin-induced changes in Fluo-3 fluorescence in the absence (upper pair) and presence (lower pair) of extracellular Ca^{2+} (2 mM $CaCl_2$). The column and bar indicate mean and standard deviation, respectively (n = 4). Asterisks (**) indicate significant differences ($P < 0.01$) between the control group (CONTROL) and groups of cells treated with 30 μM azoxystrobin (AZOXYSTROBIN). Pounds (##) indicate significant difference ($P < 0.01$) between the groups of cells treated with azoxystrobin in the absence and presence of extracellular Ca^{2+}.

Figure 6. Changes in Fluo-3 fluorescence induced by 30 μM azoxystrobin in the absence and presence of 300 μM quinine. The column and bar indicate mean and standard deviation, respectively (n = 3). Asterisks (*, **) indicate significant differences ($P < 0.05$, $P < 0.01$) between the control group (CONTROL) and the test groups. Pounds (##) indicate significant difference (P

< 0.01) between the groups of cells treated with azoxystrobin in the absence and presence of quinine.

Figure 1

(A)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30 μM
CONTROL
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0
1
2
3
INTENSITY OF OXONOL FLUORESCENCE
(Log[ARBITRARY UNIT])

(B)

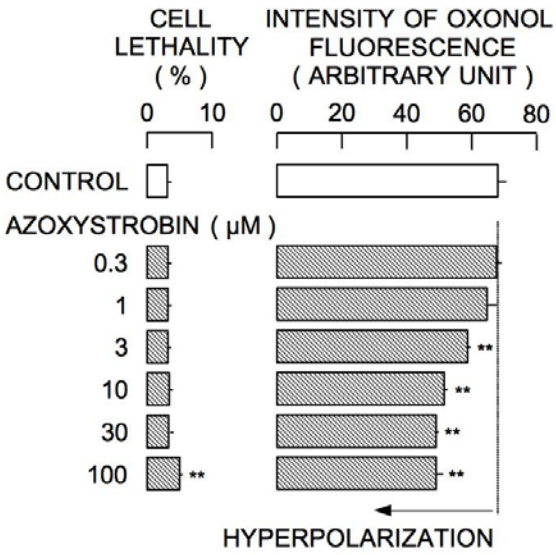
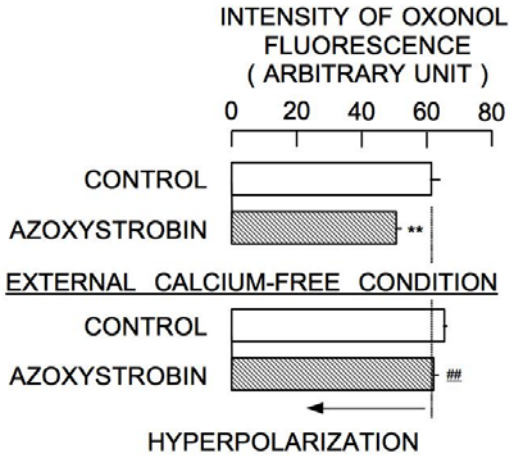

CELL LETHALITY (%)
INTENSITY OF OXONOL FLUORESCENCE (ARBITRARY UNIT)
0
10
0
20
40
60
80
CONTROL
AZOXYSTROBIN (μM)
0.3
1
3
10
30
100
**
HYPERPOLARIZATION

Figure 2


INTENSITY OF OXONOL FLUORESCENCE (ARBITRARY UNIT)
0
20
40
60
80
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AZOXYSTROBIN
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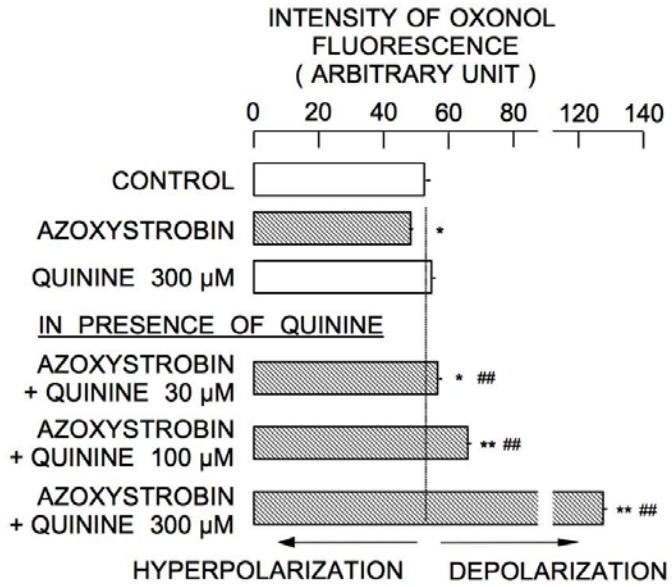
EXTERNAL CALCIUM-FREE CONDITION
CONTROL
AZOXYSTROBIN
##
HYPERPOLARIZATION

Figure 3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60
80
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140
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*
QUININE 300 µM
IN PRESENCE OF QUININE
AZOXYSTROBIN
+ QUININE 30 µM
* ##
AZOXYSTROBIN
+ QUININE 100 µM
** ##
AZOXYSTROBIN
+ QUININE 300 µM
** ##
HYPERPOLARIZATION
DEPOLARIZATION

Figure 4

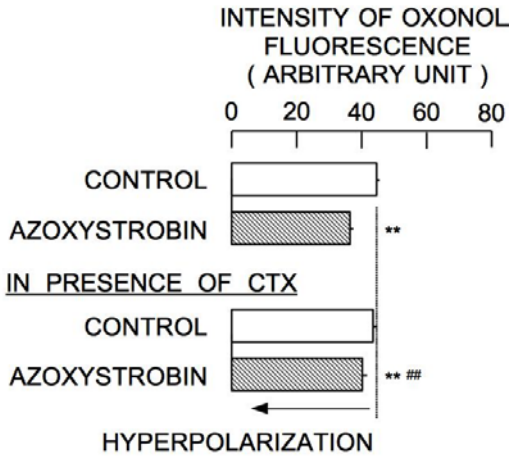
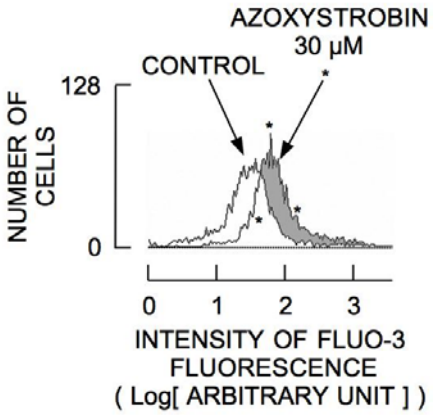
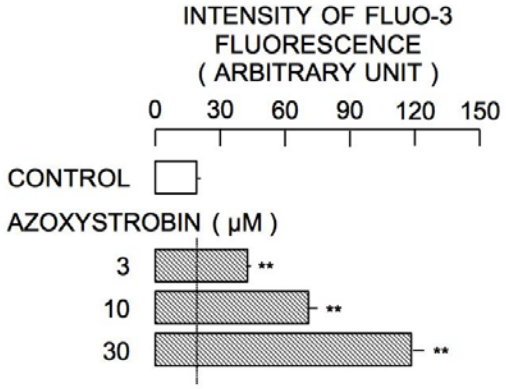

INTENSITY OF OXONOL
FLUORESCENCE
(ARBITRARY UNIT)
0
20
40
60
80
CONTROL
AZOXYSTROBIN
**
IN PRESENCE OF CTX
CONTROL
AZOXYSTROBIN
** ##
HYPERPOLARIZATION

Figure 5

(A)


AZOXYSTROBIN
30 μM
CONTROL
128
0
NUMBER OF CELLS
0 1 2 3
INTENSITY OF FLUO-3
FLUORESCENCE
(Log[ARBITRARY UNIT])

(B)


INTENSITY OF FLUO-3
FLUORESCENCE
(ARBITRARY UNIT)
0 30 60 90 120 150
CONTROL
AZOXYSTROBIN (μM)
3
10
30
**
**
**

(C)

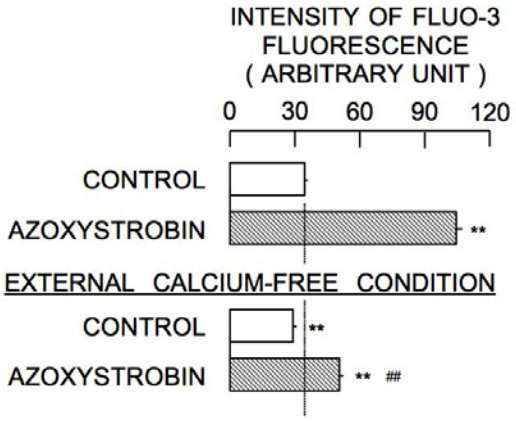
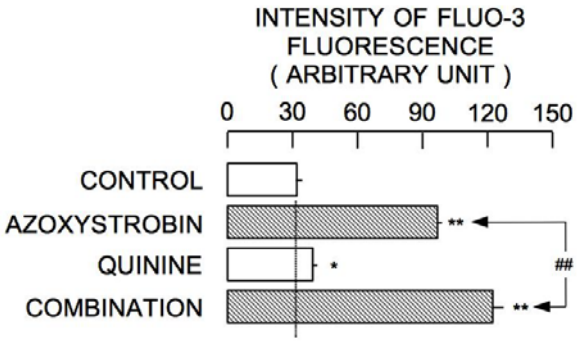

INTENSITY OF FLUO-3
FLUORESCENCE
(ARBITRARY UNIT)
0 30 60 90 120
CONTROL
AZOXYSTROBIN
**
EXTERNAL CALCIUM-FREE CONDITION
CONTROL
**
AZOXYSTROBIN
** ##

Figure 6


INTENSITY OF FLUO-3
FLUORESCENCE
(ARBITRARY UNIT)
0
30
60
90
120
150
CONTROL
AZOXYSTROBIN
QUININE
COMBINATION
**
*
**
##