JPET Fast Forward. Published on February 8, 2005 as DOI: 10.1124/jpet.104.077891

Sulfone Metabolite of Fipronil Blocks GABA- and Glutamate-activated Chloride Channels in Mammalian and Insect Neurons

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Running title: Fipronil sulfone on ligand-gated chloride channels

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Number of text pages: 39
Number of tables: 2
Number of figures: 9
Number of references: 31

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ABBREVIATIONS: BIDN, 3,3-bis(trifluoromethyl)bicyclo[2,2,1]heptane-2,2-dicarbonitrile; DRG, dorsal root ganglion; [^{3}H]EBOB, 4'-ethynyl-4-n-[2,3-$^3H_2$]propylbicycloorthobenzoate; GABA, γ-aminobutyric acid; GluCl, glutamate-activated chloride channel; IC_{50}, concentration to block 50% channels

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ABSTRACT

Fipronil sulfone, a major metabolite of fipronil in both insects and mammals, binds strongly to GABA receptors and is thought to play a significant role in poisoning by fipronil. To better understand the mechanism of selective insecticidal action of fipronil, we examined the effects of its sulfone metabolite on GABA- and glutamate-activated chloride channels (GluCls) in cockroach thoracic ganglion neurons and on $GABA_A$ receptors in rat dorsal root ganglion neurons using the whole-cell patch clamp technique. Fipronil sulfone blocked both desensitizing and non-desensitizing GluCls in the cockroach. Activation was required for block and unblock of desensitizing GluCls. In contrast, activation was not pre-requisite for block and unblock of non-desensitizing channels. After repetitive activation of the receptors, the IC_{50} of fipronil sulfone to block the desensitizing GluCls was reduced from 350 nM to 25 nM, and that for blocking non-desensitizing GluCls was reduced from 31.2 to 8.8 nM. This use-dependent block may be explained by its slow unbinding rate. In cockroach and rat neurons, fipronil sulfone blocked GABA receptors in both activated and resting states with IC_{50} values ranging from 20 to 70 nM. In conclusion, whereas fipronil sulfone is a potent inhibitor of cockroach GABA receptors, desensitizing and non-desensitizing GluCls and rat $GABA_A$ receptors, its selective toxicity in insects over mammals appears to be associated with its potent blocking action on both desensitizing and non-desensitizing GluCls, which are lacking in mammals.

Fipronil, a highly effective phenylpyrazole insecticide, has become widely used for control of a wide range of crop, public hygiene, amenity and veterinary pests since its introduction to the market in 1993 (Moffat, 1993; Tingle et al., 2003). The mechanism of insecticidal action of fipronil was thought to be due to inhibition of GABA-activated chloride channels (Casida, 1993; Cole et al., 1993; Gant et al., 1998). More recently, the glutamate-activated chloride channels (GluCls) that exist specifically in invertebrates but not in mammals are recognized as another crucial target of fipronil (Horozok et al., 2001; Ikeda et al., 2003; Zhao et al., 2004b).

Under normal use conditions, fipronil can be degraded mainly to fipronil sulfone via biotic/abiotic oxidation and to a desulfinyl photoproduct via photolysis (EPA, 1996; Bobe et al., 1998; Fenet et al., 2001; Ngim et al., 2000; Tingle et al., 2003). Fipronil sulfone is rapidly formed through cytochrome P_{450} NADPH-dependent oxidation in human recombinant CYP3A4 system and in human liver microsomes *in vitro*. The metabolite was also found in the brain and liver of mice treated intraperitoneally with fipronil (Caboni et al., 2003). In European corn borer (*Ostrinia nubilalis* Hubner), fipronil sulfone was formed through the microsomal cytochrome P_{450} oxidation system in the midgut *in vivo* and *in vitro* (Durham et al., 2002). The biological conversion of fipronil to fipronil sulfone was completely blocked with piperonyl butoxide, a cytochrome P_{450} monooxygenase inhibitor, *in vivo* and *in vitro* (Caboni et al., 2003; Hainzl et al., 1998). This inhibitor has been used *in vivo* to test whether or not the metabolites of fipronil are active as insecticides.

It is still unclear whether the conversion of fipronil to fipronil sulfone represents detoxication in mammals and insects. When pretreated with piperonyl butoxide, the toxicity of fipronil increased in mice (Caboni et al., 2003; Hainzl et al., 1998) and houseflies (Cole et al., 1993; Hainzl and Casida, 1996) a result indicating that conversion to fipronil sulfone is a

detoxifying process. However, in some other studies, when pretreated with piperonyl butoxide, the toxicity of fipronil mildly decreased in German cockroaches (Valles et al., 1997) and western corn rootworms (Scharf and Siegfried, 1999), indicating that conversion to fipronil sulfone is not a detoxifying process. Consistent with this notion are the observations that the toxicity of fipronil sulfone to freshwater invertebrates, freshwater fish and birds was indeed higher than that of the parent compound (EPA, 1996; Schlenk et al., 2001; Tingle et al., 2003). Other studies showed that the toxicological and neurological effects of fipronil sulfone were comparable to those of fipronil in mammals and insects (Cole et al., 1993; Scharf and Siegfried, 1999). One way to resolve these conflicting results is to directly examine the effects of fipronil sulfone on the target sites of action.

The mechanism of selective toxicity of fipronil sulfone in insects and mammals is not well understood. Sulfone metabolite showed a similar potency to its parent compound in inhibiting the 4'-ethynyl-4-n-[2,3-$^3H_2$]propylbicycloorthobenzoate ([^{3}H]EBOB) binding to housefly and fruit fly GABA receptors, but had a higher potency than fipronil in mammalian $GABA_A$ receptors (Hainzl et al, 1998). Thus, based on their binding specificity for GABA receptors, fipronil sulfone is more toxic than fipronil in the vertebrate. Besides GABA-activated chloride channels, glutamate-activated chloride channels (GluCls) have recently been found to be very sensitive to fipronil blocking action (Raymond et al., 2000; Horoszok et al., 2001; Ikeda et al., 2003; Zhao et al., 2004b). However, no functional studies have been performed for the actions of fipronil sulfone on insect GABA receptors and GluCls. To further explore the mechanism of action of fipronil sulfone and its selective toxicity, the present study was undertaken to compare the effects of fipronil sulfone on chloride channels activated by cockroach GABA- and glutamate receptors and by mammalian $GABA_A$ receptors using the whole-cell patch clamp technique. Our

results indicate that fipronil sulfone is a potent inhibitor of cockroach GABA receptors, GluCls and rat $GABA_A$ receptors and that its selective toxicity in insects over mammals is associated with its potent action on multiple insect target sites.

Materials and Methods

Rat Dorsal Root Ganglion Neurons. The dorsal root ganglia (DRG) were dissected from the lumbosacral region of newborn Sprague-Dawley rats (2-5 day old) under halothane anesthesia, and were immediately placed into ice-cold, Ca^{2+}/Mg^{2+}-free phosphate-buffered saline solution (PBS) supplemented with 6 g/liter glucose. The ganglia were then digested in Ca^{2+}/Mg^{2+}-free PBS containing 2.5 mg/ml trypsin (type XI, Sigma-Aldrich, St. Louis, MO) for 20 min at 37°C. Digestion was terminated by washing with Dulbecco's modified Eagle's medium (DMEM) containing 0.1 mg/ml fetal bovine serum and 0.08 mg/ml gentamycin. The ganglia were then dissociated by repeated trituration using a fire-polished Pasteur pipette in 2 ml DMEM. The dissociated cells were placed onto coverslips coated with poly-L-lysine hydrobromide (M.W. > 30,000, 0.1 mg/ml, Sigma-Aldrich). Neurons were maintained in DMEM containing fetal bovine serum and gentamycin in a 90% air and 10% CO_2 atmosphere controlled at 37°C. The neurons were ready for experiments 3-4 hours later and used in one day.

Cockroach Neurons. Adult cockroaches (*Periplaneta americana*) were purchased from Carolina Biological Supply Company (Burlington, NC) and were maintained at room temperature (22-24ºC) with free access to water and food. Isolation of neurons from thoracic ganglia was performed at room temperature using enzymatic digestion and mechanical dissociation as described previously (Zhao et al., 2003). Briefly, a cockroach was immobilized with pins dorsal side up on a dissection dish coated with wax. The dorsal cuticle, gut, and some muscles were removed to gain access to the ventral nerve cord. Three thoracic ganglia were carefully dissected and placed in cockroach saline solution containing 200 mM NaCl, 3.1 mM KCl, 4 mM $MgCl_2$, 20 mM D-glucose, and 10 mM HEPES-acid, with pH adjusted to 7.3 with 1 mM NaOH. After removing the remaining nerve fibers and nerve sheets, the ganglia were

incubated for 40 min at room temperature in the saline solution containing collagenase (type A, 0.5 mg/ml, Boehringer Mannheim, Germany) and hyaluronidase (type I-S, 1 mg/ml, Sigma-Aldrich). The ganglia were then rinsed twice in saline solution supplemented with 5 mM $CaCl_2$ and 5% (v/v) fetal calf serum, and were mechanically dissociated by gentle trituration through a fire-polished Pasteur pipette. The dissociated neurons, suspended in the supplemented saline solution, were allowed to settle on glass cover slips coated with poly-L-lysine. The neurons were ready for experiments 3-4 hours later and used in one day.

Recording Solutions. The internal solution for rat DRG neurons contained 140 mM CsCl, 5 mM EGTA , 1 mM $MgCl_2$ and 10 mM HEPES. The external solution contained 136 mM choline chloride, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES. The pH of internal and external solutions was adjusted to 7.3 with Tris base, and the osmolarity of internal and external solutions was adjusted to 290-300 mOsm with sucrose.

The internal solution for measurements of GABA- and glutamate-activated chloride channel currents from cockroach neurons contained 15 mM NaCl, 170 mM KCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM phosphocreatine diTris, 20 mM HEPES and 3 mM ATP-Mg^{2+}. The pH was adjusted to 7.4 with KOH, and the osmolarity was 420 mOsm. The external solution contained 167 mM NaCl, 3.1 mM KCl, 33 mM D-gluconic acid, 5 mM $CaCl_2$, 4 mM $MgCl_2$ and 10 mM HEPES-acid. The pH was adjusted to 7.4 with NaOH, and the osmolarity was 420 mOsm.

Current Recordings. Membrane currents in response to agonists were recorded from rat and cockroach neurons using the whole-cell patch clamp technique at room temperature. The agonists, GABA or glutamate, were applied at 100 μM which was near or slightly above their EC_{50} values to activate the respective receptors. Neurons were continuously perfused with the

respective external solution. Pipette electrodes were made from 1.5-mm (o.d.) borosilicate glass capillary tubes, and had a resistance of 1-2 MΩ when filled with the internal solution. The membrane potential was clamped at –60 mV unless otherwise stated. The recording of whole-cell currents began at least 10 min following membrane rupture so that the intracellular milieu was adequately equilibrated with the pipette solution. Currents were recorded with an Axopatch-200A amplifier (Axon Instruments, Union City, CA) interfaced to a PC computer and controlled by ClampEX 6.0.4 program. The currents were digitalized at 5 kHz and filtered at 2 kHz, stored by a PC-based data acquisition system, and analyzed off-line using Clampfit program.

Drug Application. Two drug application methods, bath perfusion and U-tube application, were used separately or in combination in the present study. A U-tube was used for rapid application of external solution containing agonist (GABA or glutamate) alone or with test compounds. External solution containing agonist alone or with test compounds was fed through the tube by gravity from a container located above the bath. Closure of a computer-operated solenoid valve in the outlet side of the tube allowed the U-tube solution to flow out of the hole located near the cell. Another valve controlling a suction tube with an opening on the other side of the cell was also opened, allowing the test solution to be sucked away quickly. The opening and closing of two solenoid valves were operated by ClampEX 6.0.4. With this method, the external solution surrounding the cell could be completely exchanged with a test solution in 30 ms.

Chemicals. Agonist (GABA or glutamate) was first dissolved in de-ionized water as stock solution and then diluted with the external solution immediately before experiment. Fipronil sulfone was first dissolved in dimethylsulfoxide to make stock solutions and stored in freezer in glass vials. The fipronil sulfone stock solution was diluted to various concentrations with the

external solution immediately before each experiment. The concentration of dimethylsulfoxide in test solutions was 0.1% (v/v) and did not cause any noticeable effects on GABA- or glutamate-activated chloride channel currents in DRG and cockroach neurons.

Data Analysis. Whole-cell currents were initially analyzed with Clampfit 6.0.4 program to measure the current amplitude and decay kinetics. The statistical analysis and the nonlinear regression analysis were carried out using Sigmaplot software (SPSS, version 8.0, Chicago, IL). The dose-response relationship for test chemicals to modulate the GABA or glutamate response was evaluated by fitting the data to Hill equation: $Y = Y_{max}IC_{50}^{h}/(C^{h} + IC_{50}^{h})$, where Y is the response in the percentage of control, Y_{max} the maximum response, C the test compound concentration, IC_{50} the concentration of test compound to inhibit the currents 50%, and *h* the Hill coefficient. Data, where appropriate, were expressed as the mean ± S.E.M.

Results

Differential Responses of Desensitizing and Non-Desensitizing Components of GluCl Currents to Fipronil Sulfone. The majority of GluCl currents evoked by glutamate in cockroach neurons comprised variable fractions of desensitizing and non-desensitizing currents (Zhao et al., 2004a). Fig. 1A depicts these mixed type currents evoked by a 5-s applications of 100 μM glutamate every 60 s at a holding potential of –60 mV. The actions of fipronil sulfone on non-desensitizing currents were examined mainly on the mixed type GluCl currents because it was difficult to find neurons that generated non-desensitizing currents only. At least 10 min following rupture of the membrane and after several stable control recordings were obtained, fipronil sulfone at 100 nM was applied via bath and U-tube perfusion. Both desensitizing component (peak current) and non-desensitizing components (measured at a 5-s time point during the glutamate pulse) were decreased gradually during application of glutamate and fipronil sulfone. However, it took a longer time for the desensitizing component to reach a steady-state block than the non-desensitizing component. Taking advantage of different decaying kinetics, the peak amplitude of the desensitizing current could be parsed from the total current by subtracting out the non-desensitizing component. The degrees of steady-state inhibition of currents by 10 and 100 nM fipronil sulfone were 62.0 ± 1.5% (n = 6) and 92.2 ± 4.5% (n = 7), respectively, for the non-desensitizing component, and 44.0 ± 5.2% (n = 6) and 79.8 ± 6.4% (n = 7), respectively, for the desensitizing component (Fig. 1B and C). From these results, the IC_{50} for fipronil sulfone against the desensitizing current was calculated to be 25 ± 2 nM (n=4), and the IC_{50} against the non-desensitizing current was calculated to be 8.5 ± 0.4 nM (n = 4).

Block of Desensitizing GluCl Current by Fipronil Sulfone. To study fipronil sulfone block of the desensitizing GluCl current, fipronil sulfone at a concentration of 1 μM was co-

applied for 30 s with 100 μM glutamate via a U-tube at an interval of 3 min. The peak amplitude of current was gradually decreased in a use-dependent manner to 97.7 ± 3.5%, 65.2 ± 2.0%, 35.0 ± 2.2% and 25.9 ± 4.4% (n = 4) of the control following four consecutive co-applications of fipronil sulfone (Fig. 2A and B). Meanwhile, the decay phase of current was gradually accelerated from the control time constant of 575 ± 28 ms (n = 4) to 205 ± 23 ms (n = 4) at the 4^{th} co-application of fipronil sulfone. After washing with insecticide-free external solutions for 10 min in the absence of repetitive glutamate stimulations, the decay time constant increased slightly but no recovery of current amplitude was seen (Fig. 2A and B). However, recovery of the current amplitude and decay time constant was accelerated by activation of the receptors by glutamate. The results suggested that block and unblock of the desensitizing currents by fipronil sulfone required the activation or opening of GluCls.

Due to the rarity of finding neurons with a pure desensitizing current, a full dose-response relationship for fipronil sulfone was not established. However, using the co-application protocol, the IC_{50} of fipronil sulfone was estimated to be 350 nM, a value smaller than that of 730 nM measured for fipronil (Ikeda et al., 2001), and much weaker than when fipronil was applied in the bath as well (Fig. 1).

Block of Non-Desensitizing GluCl Current by Fipronil Sulfone. To study fipronil sulfone block of the non-desensitizing GluCl current, the same protocols as those for the desensitizing GluCl current were used to generate the mixed desensitizing and non-desensitizing currents (Fig. 3). Since the non-desensitizing currents were more sensitive to fipronil sulfone than the desensitizing currents (Fig. 1B and C), a low concentration of fipronil sulfone was used. Fipronil sulfone at 10 nM was co-applied for 30 s with 100 μM glutamate to the neuron at an interval of 3 min following stable control recordings (Fig. 3A). Fipronil sulfone suppressed the

non-desensitizing current in a use-dependent manner. The steady-state current amplitude measured at a 30-s time point during the glutamate pulse gradually decreased to 31.9 ± 7.4% (n = 4) of the control after five co-applications of fipronil sulfone and glutamate while the desensitizing component remained almost unchanged (Fig. 3A and B). After washing the neuron with insecticide-free external solutions for 10 min without glutamate application, the non-desensitizing currents recovered to 69.1 ± 3.2% (n = 4) of the control. The current further recovered with prolonged washout. The results suggested that a large proportion of the non-desensitizing receptors were able to recover from fipronil sulfone block without receptor activation.

Differences in Fipronil Sulfone Block of GluCls. The use-dependent block of the desensitizing and non-desensitizing currents by fipronil sulfone suggests that the activated receptor has a higher affinity for the sulfone. To determine whether fipronil sulfone blocks GluCls in the activated or resting state, two protocols were used. One protocol involved repetitive activations of GluCls while the neuron was exposed to the drug, and in the second protocol GluCls were not activated during 10 min of drug exposure. In the first protocol, fipronil sulfone at a concentration of 100 nM was applied via bath perfusion for 10 min, during which fipronil sulfone was also co-applied with glutamate via U-tube repetitively at an interval of 60 s (Fig. 4A). Both the desensitizing and non-desensitizing components of currents were suppressed gradually. Since the desensitizing component was completely desensitized during 3-s application of glutamate (Zhao et al., 2004a), the current amplitude at the 3-s time point from the beginning of glutamate pulse was taken as a measure of the non-desensitizing current. The amplitudes of peak current and current measured at 3-s time point were suppressed to 10.2 ± 4.4% (n = 5) and 3.0 ± 1.2% (n = 5) of the control, respectively, at the end of 10-min fipronil sulfone perfusion.

As seen in Fig. 1, the reduction of the peak of the desensitizing current by simultaneous bath and U-tube application of fipronil sulfone is much more pronounced than that obtained after the U-tube application alone (see Fig. 2).

The result of experiments using the second protocol is shown in Fig. 4B. Without receptor activation during 10-min bath perfusion of 100 nM fipronil sulfone, the amplitudes of the peak current and the current measured at 3-s during glutamate pulse were decreased to 96.9 ± 6.9% (n = 7) and 75.4 ± 7.0% (n = 7) of the control, respectively. The reduction in peak current was not significant ($p > 0.05$), while the reduction in current measured at 3 s was significant ($p < 0.05$). The latter reduction is much smaller than the reduction observed in the activated receptor as subsequent repetitive U-tube co-applications of glutamate and fipronil sulfone reduced the desensitizing currents to 5.2 ± 1.3% of the control (n = 6, data not shown). These results suggested that the activation of receptors is pre-requisite for the block of the desensitizing current while it significantly enhanced the blocking action of fipronil sulfone on non-desensitizing GluCl currents.

Kinetic and Steady-State Analysis of Fipronil Sulfone Suppression of Activated Non-Desensitizing GluCls. To examine the fipronil sulfone block of activated non-desensitizing GluCls in more detail, fipronil sulfone at various concentrations was co-applied with 100 μM glutamate for 30 s at an interval of 3 min. Co-applications of fipronil sulfone caused use- and dose-dependent decreases in current amplitude (Fig. 5A). At the end of the 1^{st}, 2^{nd} and 3^{rd} co-applications, fipronil sulfone suppressed the non-desensitizing currents with IC_{50}s of 31.2 ± 4.1 nM, 12.3 ± 1.4 nM and 8.8 ± 1.1 nM (n = 3-5), respectively (Fig. 5B). The IC_{50} of 8.8 nM probably represents the affinity of fipronil sulfone for the activated receptors.

The time-dependent reduction of the non-desensitizing current was assumed to be due mainly to fipronil sulfone block of the activated receptor. To estimate the rate of fipronil sulfone block of the activated receptor, currents were fitted with a single exponential function. The time constant for the decay of non-desensitizing current could be determined from neurons generating the mixed current 3 s after its onset, as the desensitizing current, which decayed with a time constant of 400 ms, was essentially over by then. The non-desensitizing current decayed faster with an increase in fipronil sulfone concentration. Since there was a slow decay in the control current in the absence of test drug, the current traces obtained with co-applications of fipronil sulfone were normalized to the corresponding control in each cell to determine the drug-induced decay. The slow time constant was determined by fitting the decay of normalized current with a single exponential function. The time constants were 44.6 ± 5.30 s, 34.0 ± 7.0 s, 22.2 ± 0.9 s, 10.6 ± 1.9 s and 7.2 ± 0.5 s (n = 3-8), respectively, for the currents recorded with the first co-applications of fipronil sulfone at 10, 30, 100 and 300 nM. The reciprocals of the time constants are plotted as a function of fipronil sulfone concentration in Fig. 5C, and linear regression fit to the data gave the binding rate constant of $3.1\times10^{5} \pm 2.0\times10^{4}$ $M^{-1}s^{-1}$, the unbinding rate constant of $1.9\times10^{-2} \pm 3.1\times10^{-3}$ s^{-1}, and a calculated dissociation constant of 31 nM. The results indicate that the binding constant of fipronil sulfone is similar to that of fipronil, but the unbinding rate constant is one-third that of fipronil.

Actions of Fipronil Sulfone on Insect GABA Receptors. GABA receptors of insects and mammals were also blocked by fipronil sulfone as indicated by binding studies (Hainzl et al., 1998). To further investigate the actions of fipronil sulfone on the function of insect GABA receptors, a whole-cell patch clamp study was conducted using cockroach neurons. Currents were evoked by 1-s applications of 100 μM GABA at an interval of 1 min (Figs. 6A and B).

After several stable control recordings were obtained, fipronil sulfone at a concentration of 10 nM or 100 nM was applied through bath perfusion and U-tube. The current gradually decreased during a 10-min fipronil sulfone treatment in a use- and concentration-dependent manner (Fig. 6C). The GABA-induced currents were suppressed by 23.4 ± 4.6% (n = 5) and 95.3 ± 0.8% (n = 5) by fipronil sulfone at 10 nM and 100 nM, respectively. In another protocol in which GABA stimulations were not applied during a 10-min bath perfusion of 100 nM fipronil sulfone, the GABA currents were suppressed by 83.3 ± 3.8% (n = 6, data not shown). Thus, like fipronil, fipronil sulfone is capable of inhibiting both the resting and activated GABA receptors.

The blocking action of fipronil sulfone on the activated state of GABA receptors was also determined with a 30-s co-application of GABA and test drug via U-tube. Following having recorded several stable control currents, fipronil sulfone at various concentrations was co-applied with 100 μM GABA (Fig. 7A). The steady-state current amplitude measured at 30-s time point of GABA application was suppressed by 6.4 ± 1.8% (n = 4), 22.4 ± 5.6% (n = 4), 64.9 ± 5.6% (n = 7), 75.7 ± 2.7% (n = 4) and 87.7 ± 4.2% (n = 5) after the first co-application of fipronil sulfone at 10, 30, 100, 300 and 1000 nM, respectively. The concentration-response relationships for fipronil sulfone block established for the first, second and third co-applications of fipronil sulfone were fitted by a sigmoid curve with IC_{50}s of 53.7 ± 8.0 nM, 26.6 ± 4.9 nM and 15.4 ± 2.1 nM and Hill coefficients of 1.6, 1.4 and 1.3, respectively (Fig. 7B). The results indicated that fipronil sulfone was a potent blocker of the cockroach GABA receptor in the activated state and that repetitive activation of the receptors enhanced the block.

The kinetic analysis of the blocking action of fipronil sulfone on the activated cockroach GABA receptors was carried out on the acceleration of current decay in the presence of various

concentrations of fipronil sulfone (Fig. 7C). The fit to the data gave the blocking rate constant of 7.3×10^{4} $M^{-1}s^{-1}$, the unblocking rate constant of 3.2×10^{-2} s^{-1}.

Actions of Fipronil Sulfone on Mammalian $GABA_A$ Receptors. Differences in GABA receptor sensitivity to fipronil blocking action appear to be one of the major determinants in fipronil selective toxicity to insects relative to the vertebrates (Hainzl et al., 1998). In electrophysiological studies, insect GABA receptors were shown to have a higher sensitivity than mammalian $GABA_A$ receptors to the blocking action of fipronil (Ikeda et al., 2001; Zhao et al., 2003). In a study of the displacement of [^{3}H]EBOB binding (Hainzl et al., 1998), fipronil bound to insect GABA receptors with an affinity at least 100-fold higher than it bound to mammalian GABA receptors. Fipronil sulfone was less selective, but was nevertheless 20-fold more active against insect as opposed to mammalian GABA receptors, as measured by [^{3}H]EBOB binding. To further examine whether the sensitivity of GABA receptors to fipronil sulfone plays a role in selective toxicity in insects and mammals, the effect of fipronil sulfone on rat DRG neuron $GABA_A$ receptors was studied. Figure 8A depicts the currents evoked by 2-s applications of 100 μM GABA at an interval of 30 s. After several stable control recordings were obtained, fipronil sulfone at 100 nM was applied via both bath perfusion and U-tube. The peak amplitudes of currents were gradually decreased to 41.9% of the control and were maintained at a steady-state level (Fig. 8A and B). The currents gradually recovered during 10-min washout with drug-free external solutions. The $GABA_A$ receptors were almost completely blocked by a higher concentration of fipronil sulfone at 1000 nM (Fig. 8A and B). The dose-response relationship for fipronil sulfone block of the rat $GABA_A$ receptors is shown in Fig. 8C, and the data were fitted with an IC_{50} of 70.2 ± 3.9 nM (n = 4-5). To determine the relative contribution of resting state block and activated state block to the steady-state block obtained in Fig. 8, two

protocols as described earlier (Fig. 4) were used. Currents were induced by 1-s applications of 100 μM GABA at an interval of 30 s. With repetitive activation of $GABA_A$ receptors during 10-min bath perfusion of 1000 nM fipronil sulfone, GABA currents were suppressed by 95.3 ± 2.6% (n = 5). Without receptor activation by GABA, currents were suppressed by 78.6 ± 2.3% (n = 5). The difference between the two suppressions is statistically significant ($p < 0.01$). The results indicated that $GABA_A$ receptors were blocked by fipronil sulfone in both resting and activated states, and that the activated state of $GABA_A$ receptors was slightly more sensitive than the resting state to the blocking action of fipronil sulfone. The resting state block, however, contributes mostly to the steady-state block observed with the protocol in Fig. 8.

To analyze the blocking action of fipronil sulfone on the activated state of rat $GABA_A$ receptors, GABA was applied for 30 s to evoke currents at an interval of 3 min. Fipronil sulfone at various concentrations ranging from 1 nM to 3000 nM was co-applied with 100 μM GABA after recording several stable control currents. The decrease in the current amplitude measured at the 30-s time point from the beginning of GABA pulse application was taken as a measure of fipronil sulfone block of the activated state. The steady-state current amplitude decreased in a concentration-dependent manner with co-applications of fipronil sulfone (Fig. 9A). The concentration-response relationship was fitted by a sigmoid curve with an IC_{50} of 46.6 nM (n = 4-7) (Fig. 9B). Following the second 30-s application of GABA and fipronil sulfone, the IC_{50} was reduced to 20 nM. Thus, mammalian $GABA_A$ receptors showed similar sensitivities to insect GABA receptors to the blocking action of fipronil sulfone.

The kinetic analysis of the fipronil sulfone block of the $GABA_A$ receptor-mediated currents in DRG neurons was complex because the control current decayed with a two-exponential time course (Table 1). The control current activated by 100 μM GABA decayed with a fast time

constant of 1.67 ± 0.13 s and a slow time constant of 13.8 ± 0.92 s (n = 16). The fast time constant was reduced to around 1 s by fipronil sulfone in a concentration-independent manner whereas the slow time constant was decreased by fipronil sulfone in a concentration-dependent manner. We interpreted the acceleration of the non-desensitizing component of current decay as being due to the blocking action of fipronil sulfone on the activated receptor. As such, the reciprocal values of the slow time constants were linearly related to the concentrations of fipronil sulfone (Fig. 9C). The regression analysis of the data gave a blocking rate constant of 4.3×10^{4} $M^{-1}s^{-1}$ and an unblocking rate constant of 8.4×10^{-2} s^{-1}. Thus, the unblocking rate constant of fipronil sulfone is similar to that of fipronil, whereas the blocking rate constant of fipronil sulfone is almost 7-fold larger than that of fipronil.

Discussion

In both mammals and insects espoused to fipronil, much of the insecticide is oxidized to the sulfone (Hainzl et al., 1998) so that the toxic effects of fipronil are in fact mediated to a large extent by the sulfone. Accordingly, it is important to study the sulfone on the known targets of its parent compound. The present study has demonstrated that fipronil sulfone potently blocked the cockroach glutamate- and GABA-activated chloride channels in a dose-dependent manner. It also blocked the rat GABA-activated chloride channels at concentrations similar to those for cockroach GABA-activated chloride channels.

Cockroach GluCls. Inhibitory glutamate-activated receptor chloride channels are a member of the ligand-gated anion channel superfamily that is found in invertebrates but not in vertebrates (Cleland, 1996; Raymond and Sattelle, 2002). GluCl gene sequences have been successfully cloned in *Caenorhabditis elegans* (Cully et al., 1994), *Drosophila melanogaster* (Cully et al., 1996), and *Haemonchus contortus* (Forrester et al., 1999). Although the GluCls in cockroaches have not been cloned yet, the functional study using the whole-cell patch clamp technique revealed the existence of two pharmacologically distinct GluCls in dorsal unpaired median neurons of the cockroach, with the two GluCls differing in their sensitivity to the blocking action of picrotoxin and 3,3-bis(trifluoromethyl)bicyclo[2,2,1]heptane-2,2-dicarbonitrile (BIDN) (Raymond et al., 2000). More recently, two GluCls were further characterized kinetically as desensitizing and non-desensitizing chloride currents in unidentified thoracic ganglion neurons of American cockroaches (Ikeda et al., 2003; Zhao et al., 2004a). Both types of GluCls were activated by glutamate and ibotenic acid, an analog of glutamate, whereas the non-desensitizing GluCls were much more sensitive than the desensitizing GluCls to the blocking action of picrotoxinin and dieldrin (Ikeda et al., 2003; Zhao et al., 2004a).

Fipronil inhibited both desensitizing and non-desensitizing GluCls in cockroach neurons but with different potencies. The steady-state inhibition of the desensitizing currents by repetitive co-applications of 1000 nM fipronil was 43% (Zhao et al., 2004b), whereas the inhibition caused by its sulfone metabolite at 1000 nM was 90 % (present study). The estimated IC_{50} value for fipronil sulfone to block the desensitizing current was 25 nM, which is 30 times less than that for fipronil. These results indicate that fipronil sulfone is much more potent than fipronil to block the desensitizing currents. The difference in the potency to block the non-desensitizing GluCls between fipronil sulfone and fipronil is rather small, their IC_{50} values were 31.2 nM and 27.0 nM (Zhao et al., 2004b), respectively, for fipronil sulfone and fipronil, as measured with a single co-application with glutamate. The potency was increased following subsequent activation of the receptors, as reflected in the decrease in the IC_{50} value to 8.8 and 11.0 nM, respectively, for fipronil sulfone and fipronil, after the third application.

Similar to fipronil (Zhao et al., 2004b), use-dependent fipronil sulfone block of the desensitizing and non-desensitizing GluCls required receptor activation. Recovery of the desensitizing current from fipronil sulfone block required channel opening, whereas recovery of the non-desensitizing current from block was independent of channel opening. Kinetic analysis revealed that the blocking rate constant (3.1×10^{5} $M^{-1}s^{-1}$) for fipronil sulfone interaction with the activated receptor is equal to that for fipronil (3.0×10^{5} $M^{-1}s^{-1}$), whereas the unblocking rate constant (1.9×10^{-2} s^{-1}) for fipronil sulfone is 3-fold lower than that for fipronil (5.8×10^{-2} s^{-1}). Thus, the slower unbinding rate of fipronil sulfone might explain its higher potency and stronger use-dependent block of non-desensitizing GluCls as compared to fipronil. The potent blocking action of fipronil sulfone on both desensitizing and non-desensitizing currents indicates that both GluCls are crucial targets of fipronil metabolite.

JPET Fast Forward. Published on February 8, 2005 as DOI: 10.1124/jpet.104.077891
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Cockroach GABA Receptors. Cockroach GABA receptors are another member of the ligand-gated chloride channel superfamily and are distinctly different from mammalian $GABA_A$ receptors in kinetics and pharmacological properties (Lees et al., 1987; Sattelle et al., 1991). Although picrotoxin blocks both GABA receptors, bicuculline, which blocks the mammalian $GABA_A$ receptor, does not block the insect GABA receptor (Buckingham et al., 1994). Cockroach GABA receptors were also blocked by fipronil sulfone. In binding studies with insect GABA receptor, fipronil sulfone and fipronil were found to be equi-potent in blocking [^{3}H]EBOB binding with IC_{50}s varying from 4 to 9 nM (Hainzl et al., 1998). They exerted a similar blocking action on the GABA-activated currents as well. For example, the cockroach resting GABA-gated chloride channels were suppressed by 9.7% and 88.7%, respectively, by bath perfusion of fipronil at 10 nM and 100 nM (Zhao et al., 2003). In the present study, 23.4% and 95.3% suppressions were observed by fipronil sulfone at concentrations of 10 nM and 100 nM, respectively. Thus, the blocking action of fipronil sulfone on activated GABA receptors was comparable to that of fipronil with IC_{50}s of 53.0 nM and 35 nM, respectively, after a single co-application with glutamate. These results suggest that fipronil sulfone is as effective as fipronil in blocking the insect GABA receptors.

Mammalian $GABA_A$ Receptors. Mammalian $GABA_A$ receptors were also reversibly blocked by fipronil sulfone in a dose-dependent manner, with an IC_{50} of 70.2 nM for the receptors at the resting state and with an IC_{50} of 46.6 nM for the receptors at the activated state. Our previous study showed that fipronil blocks the resting and activated $GABA_A$ receptors with an IC_{50} of 1600 nM (Ikeda et al., 2001). Thus, fipronil sulfone is at least 20-times more potent than fipronil to block mammalian $GABA_A$ receptors. The different sensitivities of mammalian $GABA_A$ receptors to fipronil and its sulfone metabolite were also supported by the [^{3}H]EBOB

binding study, which showed that fipronil sulfone was 6-fold more potent than fipronil in vertebrate brain membrane preparations with an IC_{50} of 175 nM and 1103 nM, respectively (Hainzl et al., 1998). The kinetic analysis of block of the activated $GABA_A$ receptors showed that while fipronil and fipronil sulfone did not differ in the unbinding rate constant, the binding rate constant of fipronil sulfone was almost 7-fold larger than that of fipronil. Therefore, the higher potency of fipronil sulfone as compared to fipronil in blocking the GABA-activated chloride channels appears to be related to its higher binding rate constant for the $GABA_A$ receptor.

Relations to the Selective Toxicities. The action of fipronil is made complex by the facts that the actions of two active molecules at multiple receptors need to be considered. Table 2 summarizes the fipronil sulfone potency data obtained in this paper with patch clamp, with literature data for fipronil itself, and with [^{3}H]-EBOB binding data. Furthermore, the potency was measured by patch clamp with two different protocols and both are listed here. Fipronil sulfone blocked the cockroach GABA receptor by co-application with a first-pulse IC_{50} of 53.7 nM, whereas fipronil blocked it with a first-pulse IC_{50} of 35 nM. By the third pulse the IC_{50} of the sulfone was reduced to 15 nM, and with more pulses it would approach an even lower steady-state value, comparable to the EBOB result. It would likewise be expected that the IC_{50} of fipronil itself would decrease with repetitive pulses, but the data are not available. The non-desensitizing GluCl was also similarly sensitive to both the parent and the sulfone, and slightly more sensitive than the GABA receptor. More importantly, fipronil sulfone is 30 times more potent than fipronil to block the desensitizing GluCl, with an IC_{50} of 25 nM when applied in the bath and U-tube.

Fipronil sulfone was significantly more potent than fipronil itself against rat DRG GABA receptors, as it also was in the [^{3}H]-EBOB binding study of Hainzl et al. (1998). In this respect, the finding that the sulfone and parent are equitoxic to mice (Hainzl et al. (1998), even when pretreatment with piperonyl butoxide prevents metabolism of fipronil to the sulfone, remains surprising.

Taking these and our previous results together and with the assumption that these three receptors have critical roles in the insect CNS, we can conclude that fipronil sulfone has three highly sensitive target sites in insects, while fipronil itself has two. If these target sites are coded by distinct genes, it should greatly slow the development of target-based resistance.

Acknowledgments

We thank Julia Irizarry for secretarial assistance.

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Footnote

This study was supported by an NIH grant NS14143.

Figure Legends

Fig. 1. Differential blocking actions of fipronil sulfone on the desensitizing and non-desensitizing GluCls in cockroach neurons. A, sample traces of chloride currents with a desensitizing and a non-desensitizing component were evoked by 5-s applications of 100 μM glutamate at an interval of 60 s at –60 mV holding potential. Fipronil sulfone at 100 nM was applied via bath perfusion and U-tube. B and C, time courses of blocking action of 10 nM and 100 nM fipronil sulfone on the desensitizing (filled circles) and non-desensitizing (open circles) components (n = 6 for B, and n = 7 for C).

Fig. 2. Block and unblock of desensitizing GluCls by 1000 nM fipronil sulfone in cockroach neurons. Desensitizing currents were evoked by 100 μM glutamate applied for 30 s at an interval of 3 min at –60 mV holding potential. A, the peak current was gradually suppressed during repetitive co-applications of fipronil sulfone with glutamate (left panel). The neuron was then washed with drug-free external solutions for 10 min during which period no glutamate was applied. The recovery of the GluCls occurred during repetitive applications of glutamate in continuing washout (right panel). B, The time course of block and unblock of desensitizing GluCls by fipronil sulfone in 4 cells. The peak currents were suppressed to 29.0 ± 4.3% of the control after 4 consecutive co-applications of fipronil sulfone. The GluCls did not recover after 10-min washout without activation of the receptors by glutamate, but recovered after repetitive activations of GluCls by glutamate.

Fig. 3. Block and unblock of non-desensitizing GluCls by fipronil sulfone. The current with desensitizing and non-desensitizing components was evoked by 100 μM glutamate applied for 30

s at an interval of 3 min in cockroach neurons. A, fipronil sulfone at a concentration of 10 nM was co-applied with glutamate. The amplitude of non-desensitizing component of current decreased gradually and reached a steady state after 4 co-applications of fipronil sulfone, while the desensitizing component remained almost constant. The neuron was then washed with drug-free external solutions for 10 min during which period no glutamate was applied to activate the receptors. The recovery of the non-desensitizing currents occurred by repetitive applications of glutamate during continuing washout. The non-desensitizing component partially recovered after 10-min washout without glutamate activation and recovered almost completely after additional 3 applications of glutamate. B, The time course of fipronil sulfone block and unblock of non-desensitizing GluCls in 4 cells. The steady-state current amplitudes measured at 30-s time point during glutamate pulse decreased to 31.9 ± 7.4% of the control after 5 consecutive co-applications of fipronil sulfone and glutamate. The GluCls recovered to 69.1 ± 3.2% of the control after 10-min washout without activation of the receptors by glutamate. Further recovery was obtained with repetitive activations of GluCls by glutamate.

Fig. 4. Use-dependent block of GluCls by fipronil sulfone in cockroach neurons. Two protocols were used: A, GluCl currents with a desensitizing and a non-desensitizing component were induced by 5-s applications of 100 μM glutamate at an interval of 60 s. Fipronil sulfone at 100 nM was bath-perfused and repetitively co-applied with glutamate for 10 min. B, similar protocol to A but no glutamate was applied during 10-min bath perfusion of 100 nM fipronil sulfone. A test pulse of glutamate was applied to determine the degree of block. After 10-min bath perfusion of 100 nM fipronil sulfone, the non-desensitizing current amplitude was almost completely suppressed in protocol A, whereas it was suppressed only partially in protocol B. Thus,

activation of the receptors significantly increases the blocking action of fipronil on the non-desensitizing component.

Fig. 5. Kinetic analysis of fipronil sulfone block of the activated non-desensitizing GluCls in cockroach neurons. A, glutamate (100 μM) was applied for 30 s to induce currents (control), with co-application of various concentrations of fipronil sulfone at an interval of 3 min. B, the dose-response relationships for fipronil sulfone block of the activated non-desensitizing GluCls. The current amplitude was measured at the end of 30-s co-application during the 1^{st}, 2^{nd}, and 3^{rd} pulses of fipronil sulfone and glutamate. The fit to the dose-response data gave IC_{50}s/Hill coefficients of 31.2 ± 4.1 nM/0.9 ± 0.1 after the first (circles), 12.3 ± 1.4 nM/1.7 ± 0.4 after the second (triangles), and 8.8 ± 1.1 nM/2.2 ± 0.9 after the third (squares) applications (n = 4-5). C, the slow decay phase of the currents was normalized to the control and was fitted with a single exponential function to obtain the time constant of fipronil sulfone block. The reciprocal of the time constant ($1/\tau$) is plotted as a function of fipronil concentration to calculate the blocking and unblocking rate constants for fipronil sulfone interaction with the activated receptor. Data points are best fitted to the solid line according to the equation $1/\tau = k_{+1}$[Fipronil sulfone] + k_{-1}, where k_{+1} and k_{-1} are the blocking and unblocking rate constants, respectively, and [Fipronil sulfone] is the fipronil sulfone concentration. The correlation coefficient of 0.995 gives a significance level of $p < 0.05$. $k_{+1} = 3.1\times10^{5}$ $M^{-1}s^{-1}$; $k_{-1} = 1.9\times10^{-2}$ s^{-1}; the calculated K_d = 61.7 nM (n = 4-8).

Fig. 6. Fipronil sulfone block of cockroach GABA receptors. Chloride currents were evoked by 1-s applications of 100 μM GABA at a holding potential of –60 mV. Fipronil sulfone at concentrations of 10 nM and 100 nM was applied via bath perfusion and U-tube at an interval of

60 s. A, control current sample and current recorded at the end of 10-min bath perfusion of 10 nM fipronil sulfone. B, similar to A but with 100 nM fipronil sulfone. C, time course of the blocking action of fipronil sulfone. The currents were normalized to the respective control to calculate the blocking percentages. Each data point was the average value obtained from 5 cells. The peak current was decreased to 76.6 ± 4.6% (open circles, n = 5) of the control by 10 nM and to 4.7 ± 0.8% (filled circles, n = 5) by 100 nM fipronil sulfone after 10-min bath perfusion.

Fig. 7. The blocking action of fipronil sulfone on the activated cockroach GABA receptors. A, currents evoked by 100 μM GABA applied for 30 s every 3 min at a holding potential of –60 mV without and with co-application of various concentrations of fipronil sulfone. B, dose-response relationships of fipronil sulfone block of the activated GABA receptors. The blocking percentages were calculated from the steady-state current amplitude measured at the 30-s time point of GABA pulses. The dose-response curves for the first, second and third co-applications of fipronil sulfone were fitted by the sigmoid curves with IC_{50}s of 53.7 ± 8.0 nM, 26.6 ± 4.9 nM and 15.4 ± 2.1 nM, and Hill coefficients of 1.6, 1.4 and 1.3, respectively (n = 4-7). C, the slow decay phase of the GABA currents was normalized to the control and was fitted with a single exponential function to obtain the time constant of fipronil sulfone block. The reciprocal of the time constant ($1/\tau$) is plotted as a function of fipronil concentration to calculate the blocking and unblocking rate constants for fipronil sulfone interaction with the activated receptor. Data points are best fitted to the solid line with the blocking and unblocking rate constants of 7.3×10^{4} $M^{-1}s^{-1}$ and 3.2×10^{-2} s^{-1}, respectively. The calculated dissociate constant (Kd) was 440 nM (n = 4-7).

Fig. 8. Fipronil sulfone block of rat DRG $GABA_A$ receptors. A, GABA currents evoked by 2-s applications of 100 μM GABA were recorded before and during bath perfusion and U-tube co-application of fipronil sulfone at 100 nM and 1000 nM. B, time course of the blocking action of fipronil sulfone. The currents were normalized to the control. The peak amplitude of the currents decreased to 41.9% of the control after bath perfusion and co-application of 100 nM fipronil sulfone, and completely recovered after 10-min washout with drug-free solution. When 1000 nM fipronil sulfone was applied after washout, the GABA current decreased to 5.0% of the control. C, dose-response relationship of the blocking action of fipronil sulfone. Each data point was the average value obtained at a steady state from separate cells (n = 4-5). The dose-response relationship was fitted with an IC_{50} of 70.2 ± 3.9 nM and a Hill coefficient of 0.4.

Fig. 9. The blocking action of fipronil sulfone on the activated rat DRG $GABA_A$ receptors. A, currents evoked by 100 μM GABA applied for 30 s every 3 min at a holding potential of –60 mV without and with co-application of various concentrations of fipronil sulfone. B, dose-response relationships of fipronil sulfone block of the activated $GABA_A$ receptors. The blocking percentages were calculated from the steady-state current amplitude measured at a 30-s time point of GABA pulses. The dose-response relationship was fitted by a sigmoid curve with an IC_{50} of 46.6 nM and a Hill coefficient of 0.6 (n = 4-7). C, the decay phase of currents was fitted with two-exponential function to obtain a fast and a slow time constants of fipronil sulfone block. The reciprocal of the slow time constant ($1/\tau$) is plotted as a function of fipronil concentration to calculate the blocking and unblocking rate constants for fipronil sulfone interaction with the activated receptor. Data points are best fitted to the solid line with blocking

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and unblocking rate constants of 4.3×10^{4} $M^{-1}s^{-1}$ and 1.9×10^{-2} s^{-1}, respectively. The calculated K_d = 1930 nM (n = 3-16).

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TABLE 1

Kinetic analysis of the effects of fipronil sulfone on the $GABA_A$ receptor-mediated currents in rat DRG neurons

Fipronil sulfone	n	GABA current decay (Mean ± S.E.M.)	
		τ_{fast}	τ_{slow}
nM		*s*	*s*
0	16	1.67 ± 0.13	18.81 ± 0.92
10	4	1.21 ± 0.16	12.82 ± 1.11
30	5	0.93 ± 0.06	11.44 ± 0.64
100	5	1.12 ± 0.19	11.75 ± 0.85
300	6	0.96 ± 0.10	9.76 ± 0.85
1000	3	0.97 ± 0.12	8.70 ± 1.62
3000	4	1.06 ± 0.22	4.95 ± 0.68

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TABLE 2

Summary of the blocking actions of fipronil sulfone and its parent compound fipronil on cockroach GluCls, GABA receptors and rat $GABA_A$ receptors.

	Cockroach GluCl-D[a]	Cockroach GluCl-N[b]	Cockroach GABA-Rs	Rat DRG $GABA_A$Rs
Fipronil sulfone				
IC_{50} (*nM*): U[c]	350	31.2, 12.3, 8.8[d]	53.7, 26.6, 15.4[d]	46.6, 20[d]
B/U[e]	25	8.5	20	70.2
EBOB[f]			9 (insect mean)	175 (vertebrate mean)
Fipronil				
IC_{50} (*nM*): U	750	27, 17.3, 11.9[g]	35	1610[h]
B/U	801[i]	10[i]	27.6[g]	n/a
EBOB[f]			7.0 (insect mean)	1103(vertebrate mean)

[a] Desensitizing GluCl; [b] Non-desensitizing GluCl; [c] Measured by U-tube co-application; [d] Data for the 1st, 2nd and 3rd pulses; [e] Bath and U-tube application; [f] data from Hainzl et al. (1998); g Zhao et al. (2003); [h] Ikeda et al. (2001); [i] Zhao et al. (2004b).

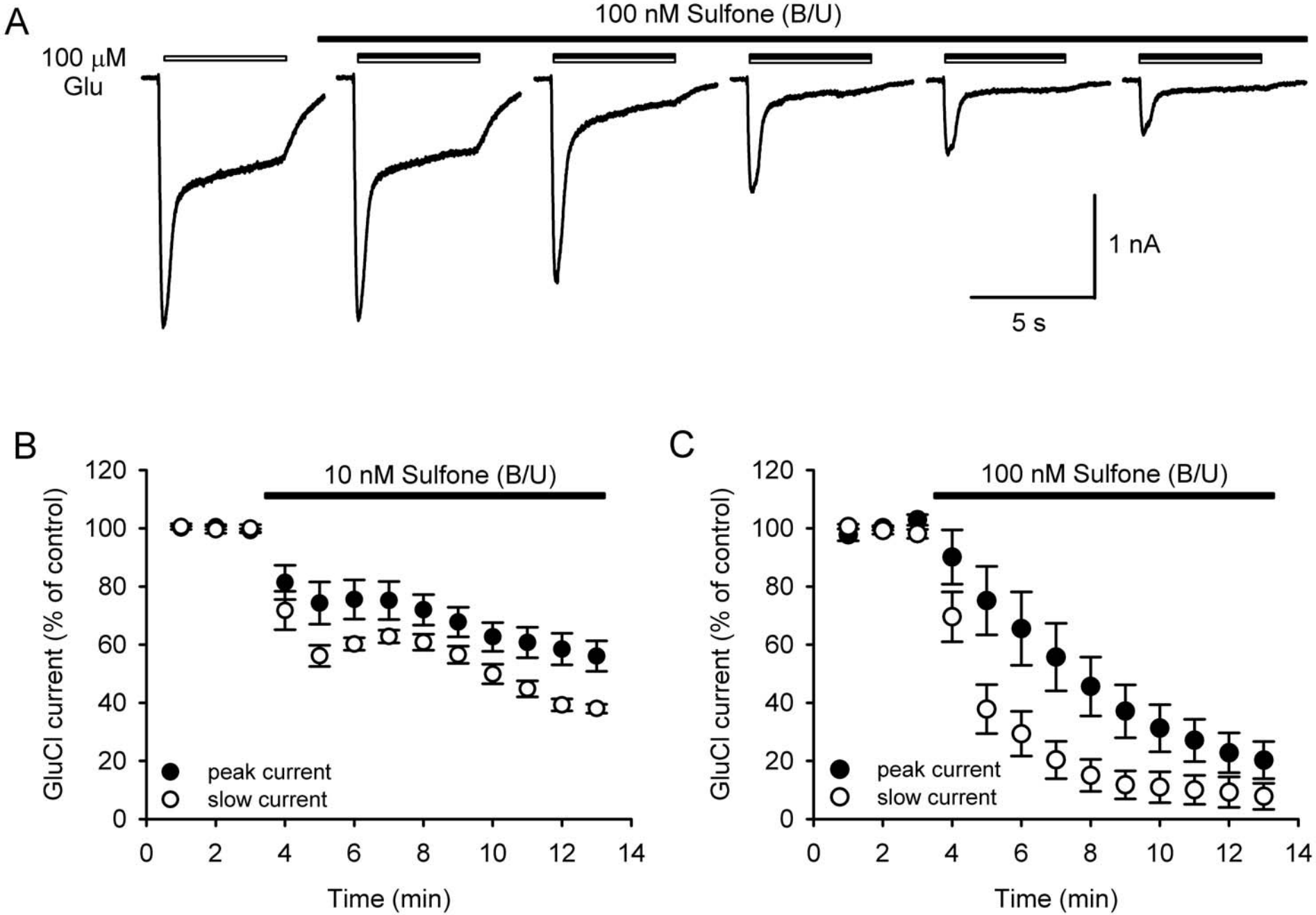

Fig. 1.

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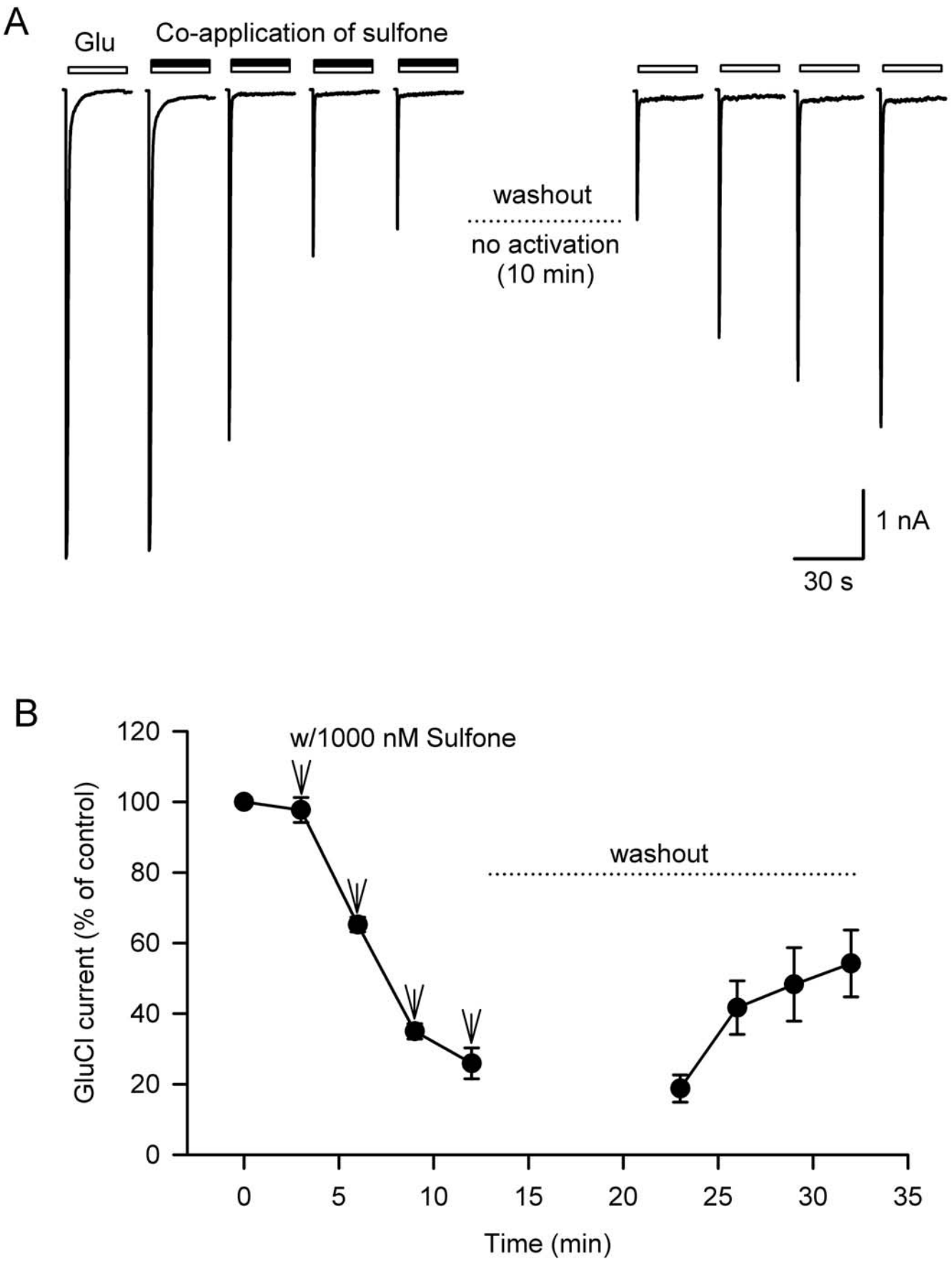

Fig. 2.

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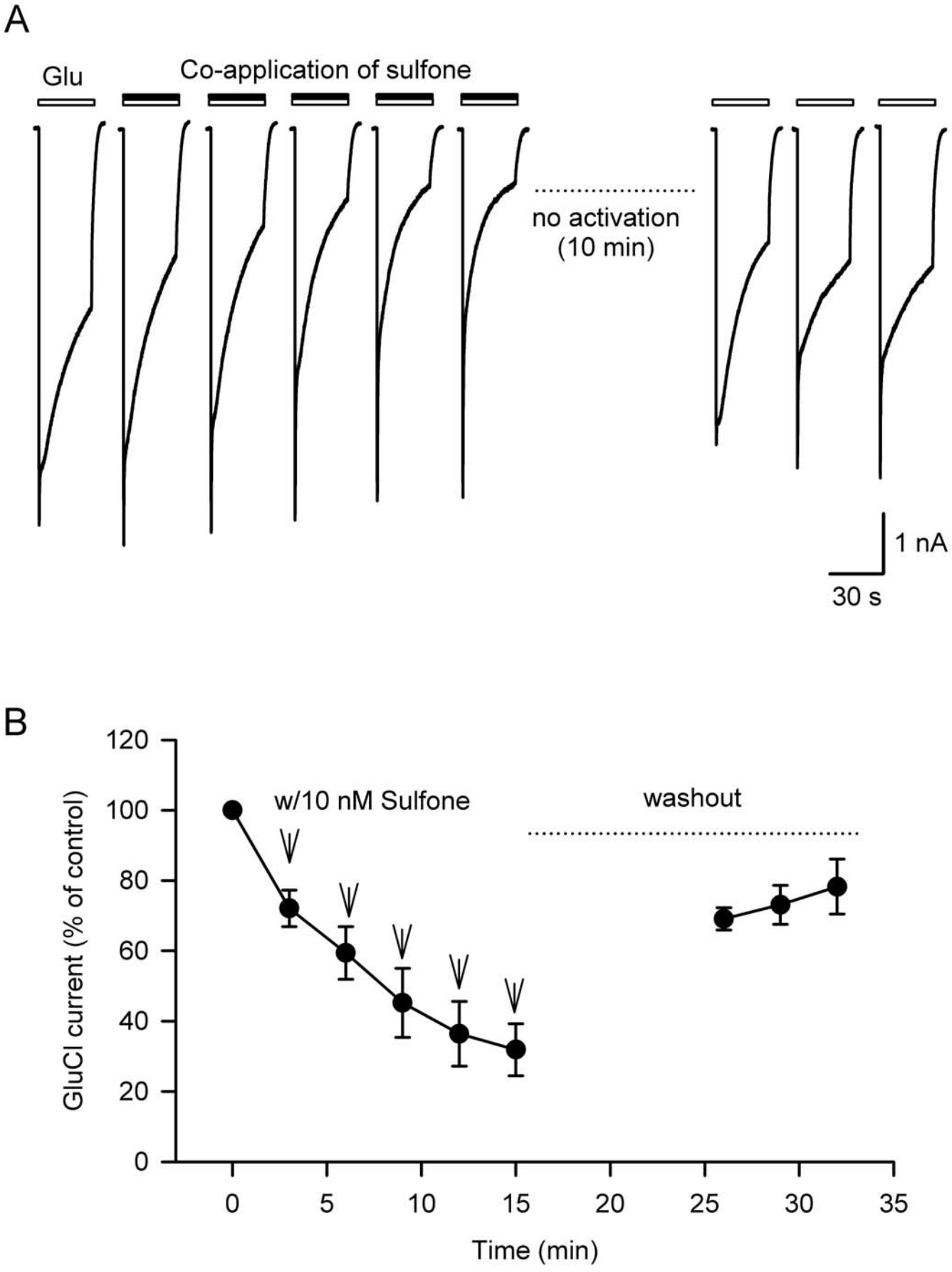

Fig. 3.

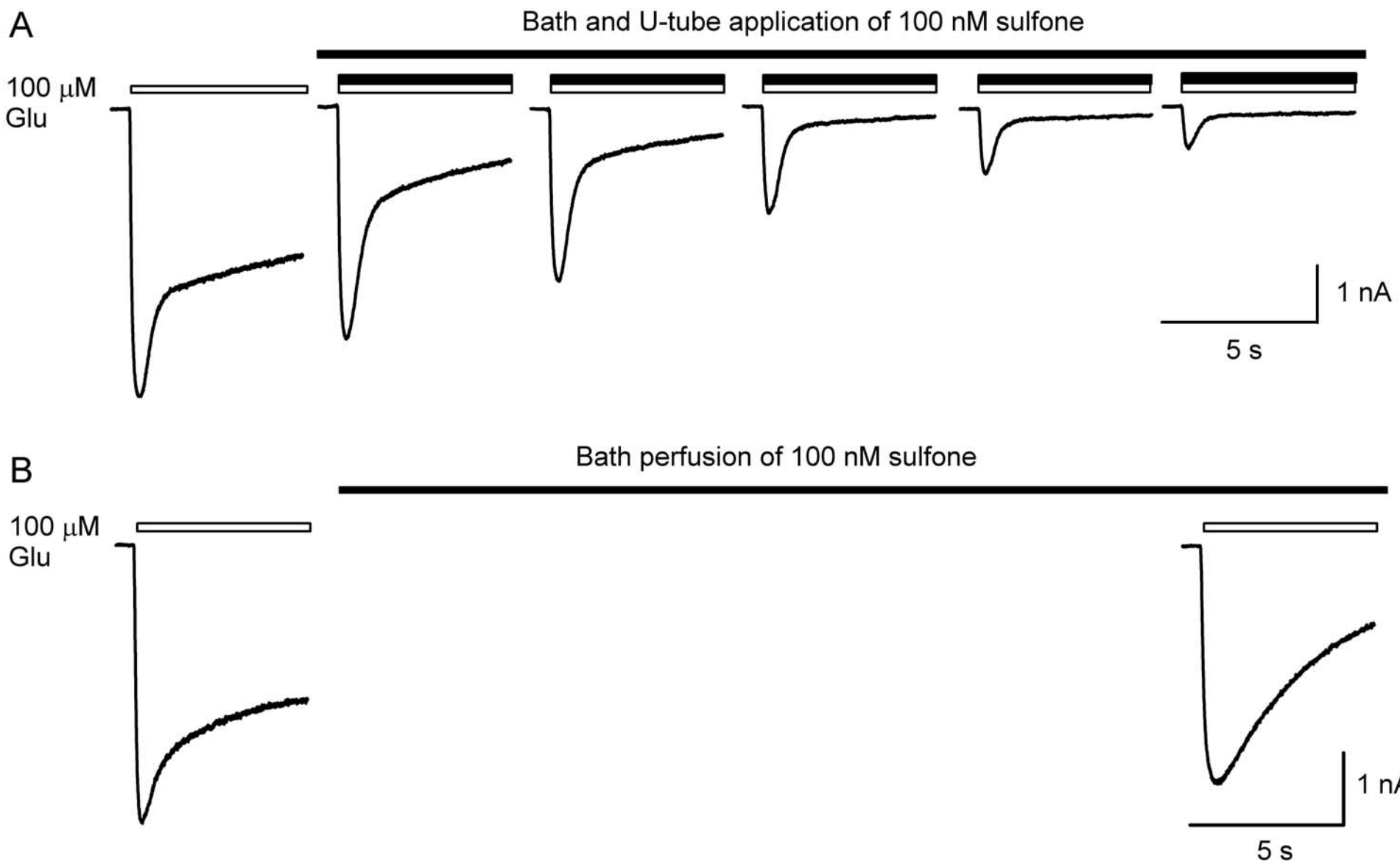

Fig. 4.

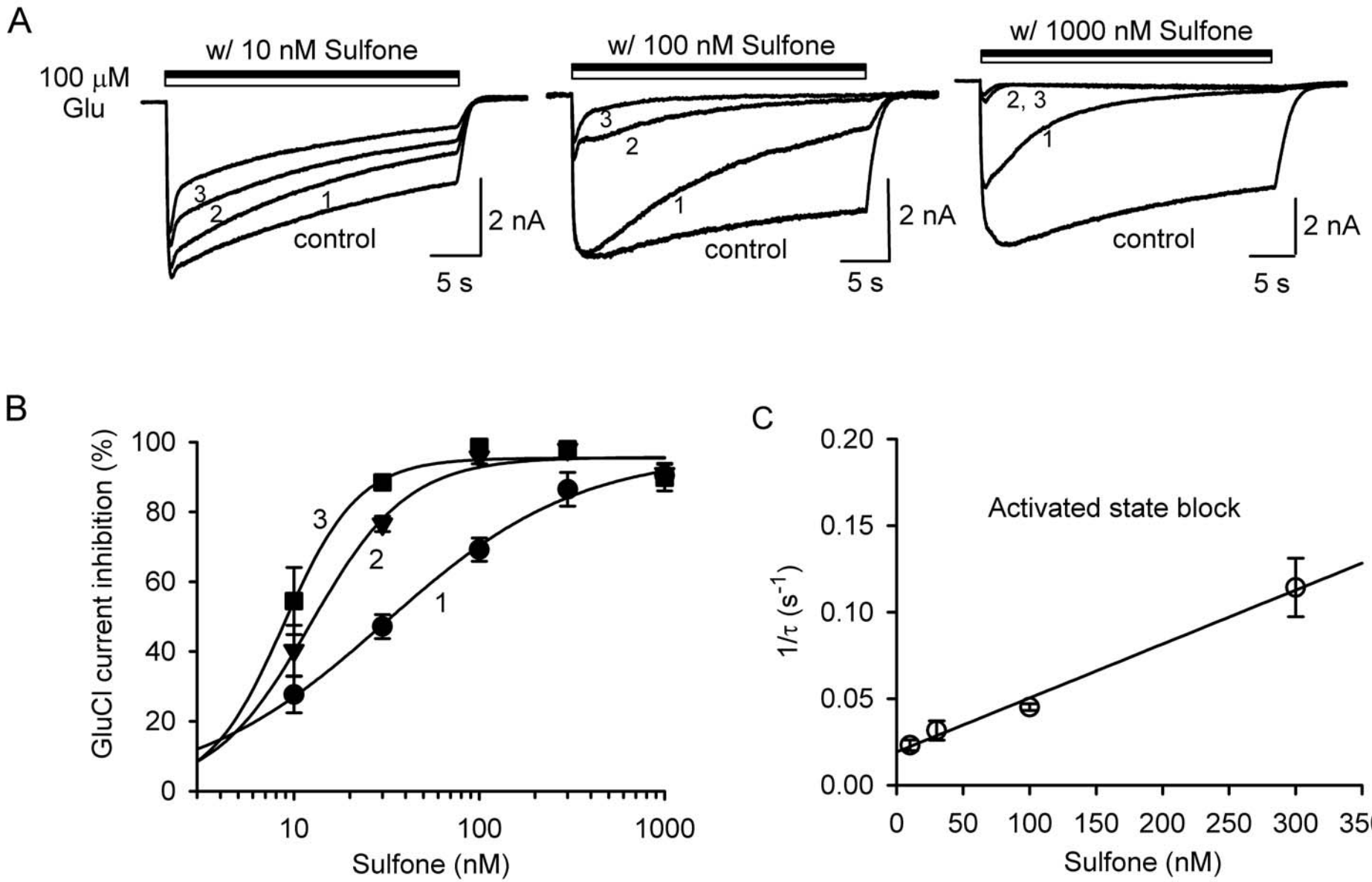

Fig. 5.

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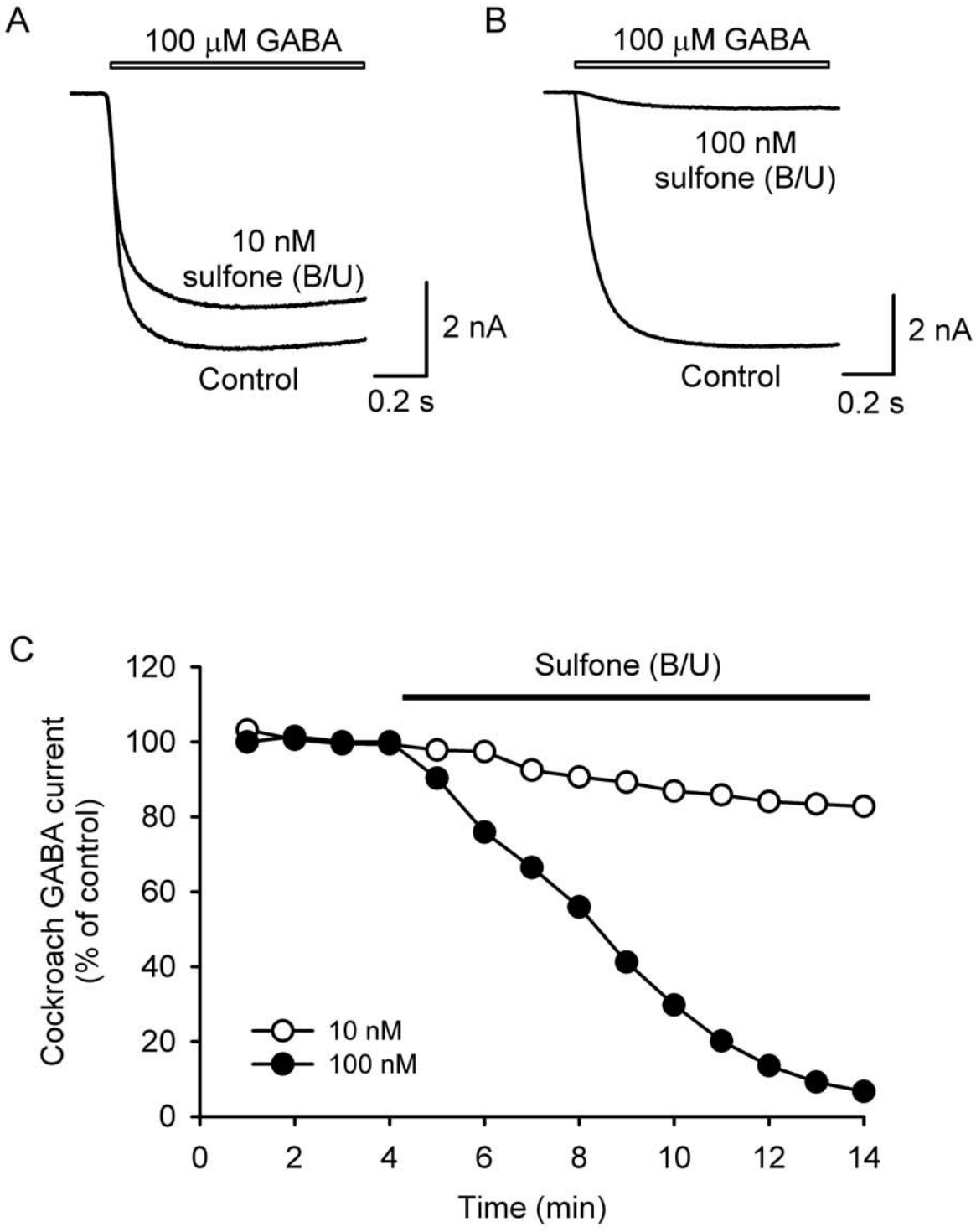

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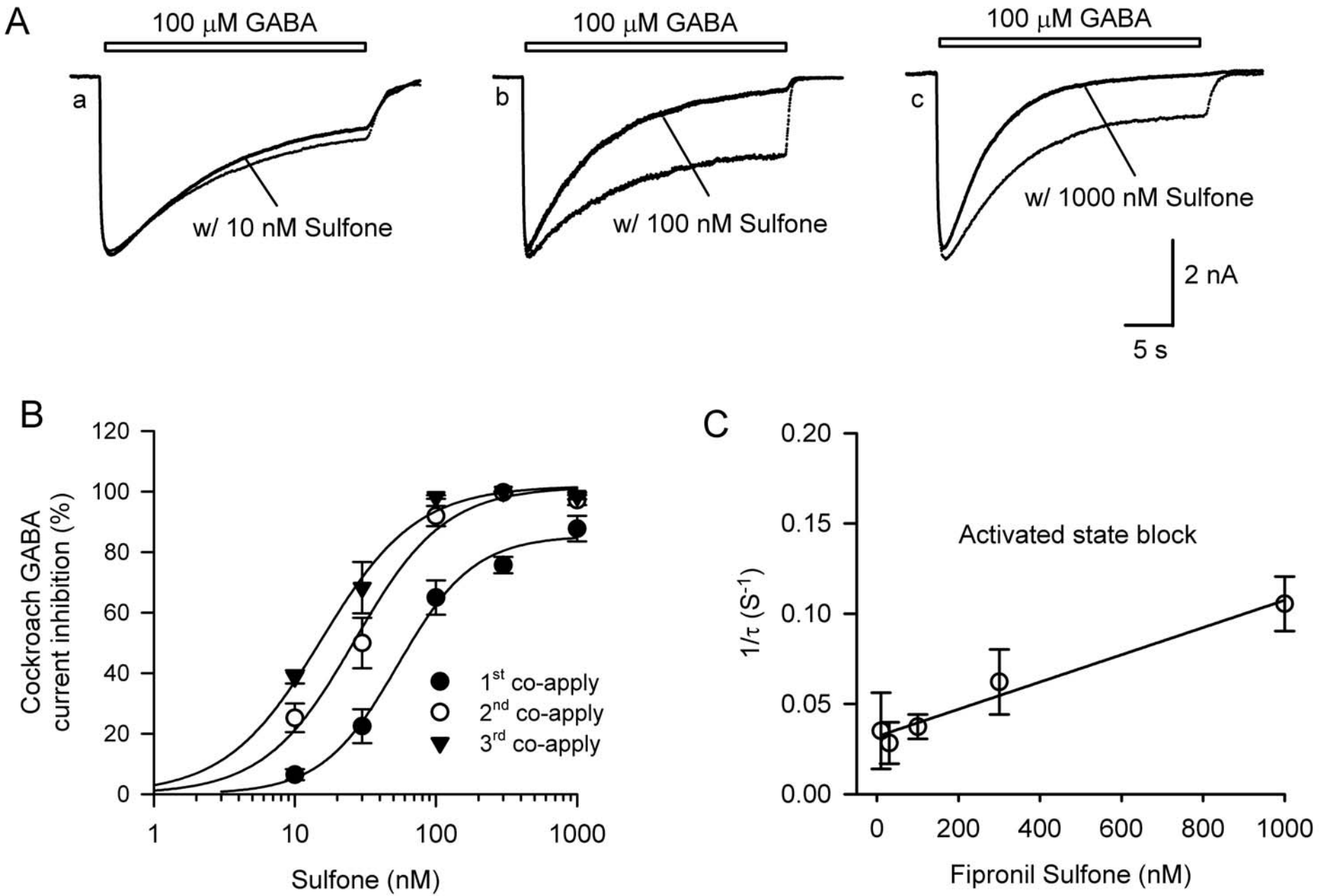

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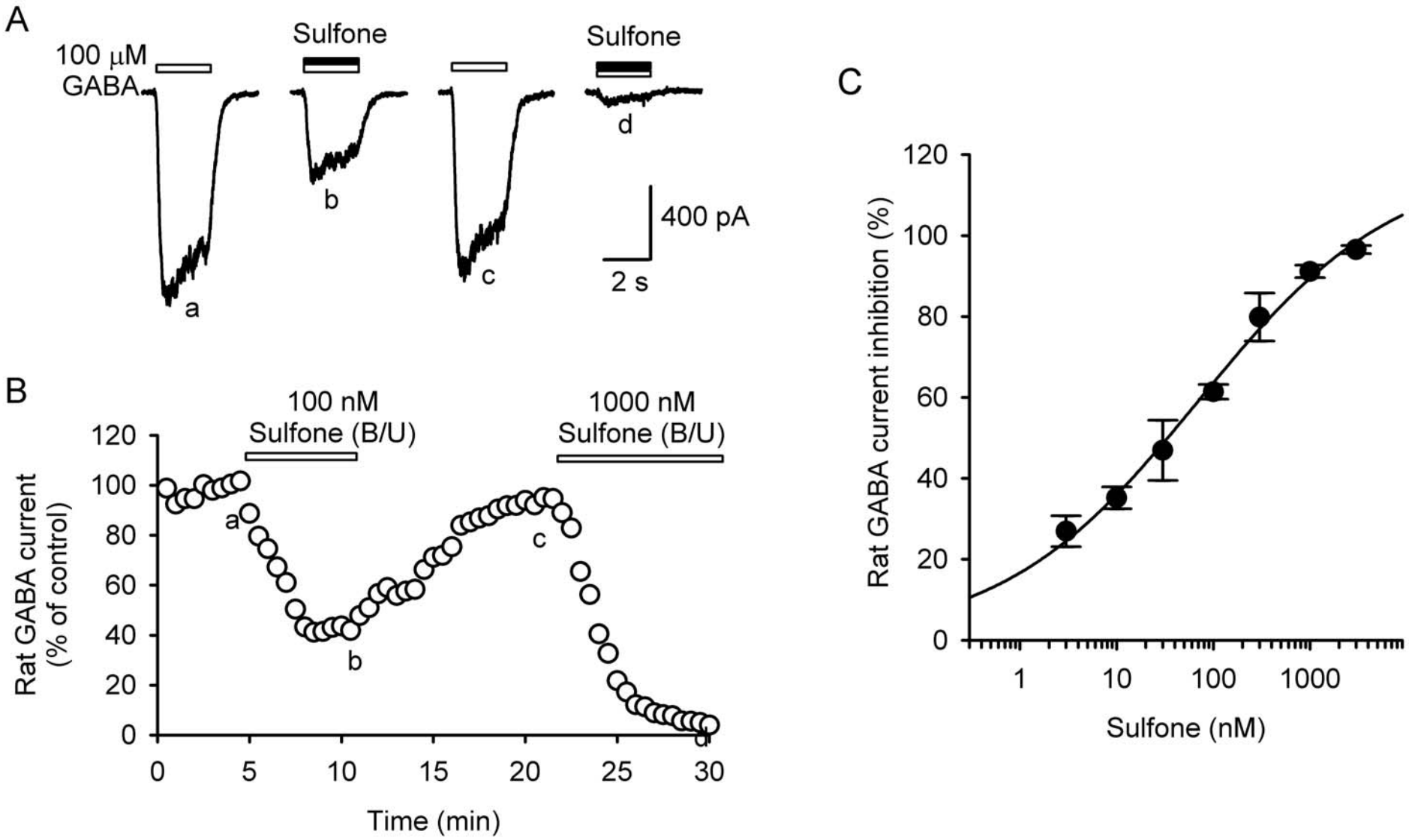

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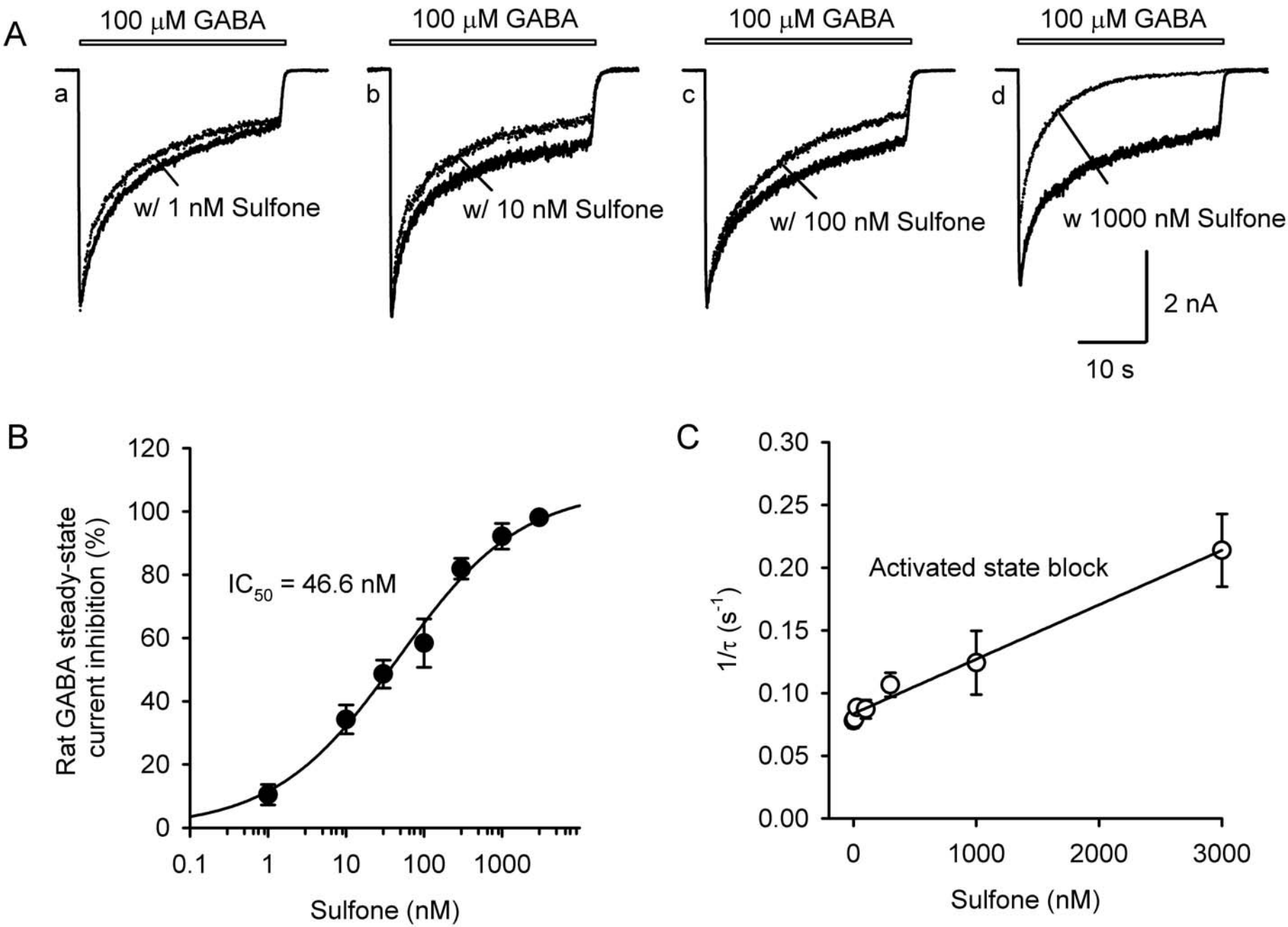

Fig. 9.