How Does Calcium-Dependent Intracellular Regulation of Voltage-Dependent Sodium Current Increase the Sensitivity to the Oxadiazine Insecticide Indoxacarb Metabolite Decarbomethoxylated JW062 (DCJW) in Insect Pacemaker Neurons?

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ABSTRACT
Decarbomethoxylated JW062 (DCJW), the active component of the oxadiazine insecticide (S)-methyl 7-chloro-2,5-dihydro-2-[[[methoxycarbonyl]4-(trifluoromethoxy)phenyl] amino]carbonyl] indeno[1,2-e][1,3,4]oxadiazine-4(3H)-carboxylate (DPX-JW062) (indoxacarb), was tested on 2 inward voltage-dependent sodium currents (named INa1 and INa2) expressed in short-term cultured dorsal unpaired median neurons of the cockroach Periplaneta americana. Under whole-cell voltage-clamp conditions, application of DCJW resulted in a biphasic dose-dependent inhibition of the global sodium current amplitude illustrating the differing sensitivity of sodium channels to DCJW. INa2 was less sensitive to DCJW [half-maximal inhibitory concentration (IC50) = 1.6 μM] compared with INa1 (IC50 = 1.7 nM). Although a previous study demonstrated that INa1 was regulated by the cAMP/protein kinase A cascade, we showed that INa2 was mainly regulated in an opposite way by the activation of calcium-calmodulin-dependent protein kinase II (CaM-kinase II). Furthermore, we demonstrated that activation of CaM-kinase II by intracellular calcium via the calcium-calmodulin complex affected the sensitivity of INa2 channels to DCJW. By increasing the intracellular calcium concentration and/or using 1,2-bis(o-aminoophenoxo)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) (a calcium chelator), N-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) (a calmodulin inhibitor), cyclosporine A (a PP2B inhibitor), and 1-[N-O-bis(5-isouquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazin (KN-62) (a CaM-kinase II inhibitor), we revealed that activation of CaM-kinase II was involved in the modulation of the voltage dependence of steady-state inactivation and that the CaM-kinase II pathway activated by elevation of the intracellular calcium concentration might render INa2 channels approximately 3000-fold more sensitive to DCJW. These results indicated that manipulating specific intracellular signaling pathways involved in the regulation of sodium channels might have fundamental consequences for the sensitivity of insects to insecticides. This finding reveals an exciting research area that could lead to improvement in the efficiency of insecticides.

Voltage-dependent sodium channels are critical elements of electrical activity in excitable cells and thus are important targets of diverse neuroactive compounds, including pyrethroids and pyrazoline-type insecticides (Dong, 2007; Soderlund, 2008; Silver et al., 2009). Synthetic pyrethroid insecticides, which are derived from natural compounds (pyrethrins), have been used for more than 40 years and account for 25% of the worldwide insecticide market, because of their potent and rapid insect intoxication, low toxicity for mammals, and lim-
insecticide. This latest generation of insecticides includes the pyrazoline-type active against resistant pest strains have been developed. The dent sodium-channel protein (Davies et al., 2007; Dong, 2007; one or several point mutations in the insect voltage-dependent sodium-channel protein (McCann et al., 2001; Silver and Soderlund, 2005a; Silver et al., 2009). In insects, this compound acts as a proinsecticide and is rapidly bioactivated by a hydrolase to produce the N-decarbamethoxylated metabolite DCJW, which is a potent insecticide and is known to block sodium-dependent action potentials in preparations of lepidopteran (Manduca sexta) larval motor nerve (Wing et al., 1998, 2000) and to induce dose-dependent inhibition of voltage-dependent sodium current in cockroach Periplaneta americana neurons (Laped et al., 2001; Zhao et al., 2005). By contrast, although mammalian sodium-channel isoforms display differential sensitivity to pyrazoline-type insecticide (Zhao et al., 2003; Silver and Soderlund, 2005b, 2006), the inability to perform this metabolic bioactivation to DCJW is one of the principal causes of the very favorable selective insect toxicity of indoxacarb (McCann et al., 2001; Silver and Soderlund, 2005a).

Some studies, however, have already demonstrated the emergence of insect strains that are insensitive to indoxacarb. The development of indoxacarb tolerance can be induced by other known resistance mechanisms, such as reduction of cuticular penetration and modification of cytochrome P450 monooxygenase-mediated detoxification (Sugiyama et al., 2001; Shono et al., 2004). More recently, Sayyed and Wright (2006) identified an esterase-associated mechanism of indoxacarb resistance in a field population of Plutella xylostella (Sayyed, 2006) identified an esterase-associated mechanism of indoxacarb resistance in a field population of Plutella xylostella (Choristoneura rosae) larvae and resistant oblique-banded leafrollers (Charistoneura rosae), collected from apple orchards that had never been treated with indoxacarb, exhibited very low sensitivity (>700-fold) to indoxacarb application (Ahmad et al., 2002). In addition, post-transcriptional modifications (RNA editing or alternative splicing) and intracellular regulation of plasma membrane receptor and ion channels targeted by insecticides are processes recently reported to affect sensitivity to insecticides (Courjaret and Laped, 2001; Es-Salah et al., 2008; Laped et al., 2009). Consequently, in the present study, we investigated the influence of sodium-channel intracellular regulation on the efficiency of DCJW using a whole-cell patch-clamp technique adapted on short-term cultured insect pacemaker neurons (Grollleau and Laped, 2000; Wicher et al., 2001). In previous studies, we have demonstrated the coexistence of two inward voltage-dependent sodium currents, named INa1 and INa2, in the same insect pacemaker neuron (Lavialle-Defaix et al., 2006). Here, we report that these two currents differ in their sensitivity to DCJW, with INa2 being 1000-fold less sensitive than INa1. Furthermore, we have demonstrated that activation of an intracellular calcium-dependent phosphorylation process involving CaM-kinase II stimulates the transition from low to very high DCJW sensitivity of the INa2 channel. This fundamental intracellular regulation, which strongly alters DCJW sensitivity, may open an exciting research field that may lead to new strategies of insecticide use.

**Materials and Methods**

**Cells Isolation.** Experiments were performed on DUM neuron cell bodies isolated from the midline part of the terminal abdominal ganglia (TAG) of the nerve cord of adult male cockroaches (P. americana). Insects were obtained from our laboratory stock colony bred at 29°C (photoperiod of 12-h light/12-h dark). Animal care and handling procedures were in accordance with French Institutional and National Health Guidelines. Animals were immobilized ventral side up on a dissection dish. The ventral cuticle and accessory glands were removed to allow access to the ventral nerve cord. The abdominal nerve cord and its TAG, which we carefully dissected under a binocular microscope, were placed in normal cockroach saline containing 200 mM NaCl, 3.1 mM KCl, 5 mM CaCl₂, 4 mM MgCl₂, 50 mM sucrose, and 10 mM HEPES buffer; pH was adjusted to 7.4 with NaOH. We performed isolation of adult DUM neuron cell bodies under sterile conditions using enzymatic digestion (collagenase type I; Worthington Biochemicals, Lakewood, NJ) and mechanical dissection of the median part of the TAG as described previously (Laped et al., 1989). Then, the isolated cell bodies were incubated for 24 h before electrophysiological experiments were carried out.

**Whole-Cell Recordings and Data Analysis.** A whole-cell patch-clamp recording configuration was used to record voltage-dependent sodium currents (voltage-clamp mode) at room temperature (20–22°C). Patch pipettes were pulled from borosilicate glass capillary tubes (GC 150T-10; Clark Electromedical Instruments, Harvard Apparatus, Edenbridge, UK) by use of a PP83 puller (Narishige, Tokyo, Japan). Pipettes had a resistance ranging from 0.8 to 2.0 MΩ when filled with internal solutions (see composition below). The liquid junction potential between bath and internal solution was always corrected before the formation of a gigahm seal (>5 GΩ).

Voltage-dependent sodium currents were recorded with an Axopatch 200A (Molecular Devices, Sunnyvale, CA) amplifier, filtered at 5 kHz using a 4-pole low-pass Bessel filter. Data were stored online on the hard disk of a PC computer (Elonex, Pentium 733 MHz, sampling frequency 33 kHz) connected to a 16-bit analog-to-digital converter (Digidata 1322A; Molecular Devices). The pClamp package (version 8.0.2; Molecular Devices) was used for data acquisition and analysis. DUM neuron cell bodies were voltage-clamped at a steady-state holding potential of −90 mV, and 30-ms test pulses were applied at a frequency of 0.2 Hz. Although leak and capacitive currents were compensated electronically at the beginning of each experiment, subtraction of residual capacitive and leakage currents was performed with an online P/6 protocol provided by pClamp.

**Data Analysis.** We performed data analysis, including fitting procedures, using the software Prism 4 (GraphPad Software, Inc., La Jolla, CA). Data are presented as the mean ± S.E.M. Information regarding which pairs of means were significantly different and which was not was given by using a Student’s t test for multiple comparisons. In this case, statistical analysis was expressed as non-significant (ns) for p > 0.05 and significant for *p < 0.05, **p < 0.01, and ***p < 0.001. The equation used to fit the sigmoid curve was:

\[
Y = \text{Min} + (\text{Max} - \text{Min})/(1 + (IC_{50}/X)^b)
\]

where \( Y \) is the percentage inhibition of the peak inward sodium current; \( \text{Min} \) and \( \text{Max} \) are the minimal and maximal values, respec-
Results

Effects of DCJW on INa1 and INa2 in the DUM Neuron Cell Body. The global sodium current expressed in the DUM neuron cell body was completely blocked by bath application of tetrodotoxin in a dose-dependent manner with a maximum of inhibition at 10 μM (IC50 = 1.96 nM, Fig. 1A). All experiments were carried out with 3 mM ATP added in the patch pipette solution to block the participation of the maintained low voltage-activated current permeable to calcium and sodium identified on DUM neurons (Defaix and Lapied, 2005). It is interesting that an increase of internal ATP concentration from 2 (taken from Lapied et al., 2001) to 3 mM (present study) revealed two distinct components depending on DCJW concentration (Fig. 1B). Whereas the semilogarithmic curve of the global INa reduction induced by bath application of DCJW was best represented by a monophasic curve fitted by the Hill equation (IC50 = 29 nM, see eq. 1 from Lapied et al., 2001) in the presence of 2 mM ATP, the dose-response curve obtained with 3 mM added in the patch pipette solution was best represented by a biphasic curve according to the two-site competition equation (Eq. 1B and eq. 2, IC50,1 = 1.5 nM and IC50,2 = 2.5 μM). These first results seemed to indicate that the two sodium channels, expressed in DUM neuron cell bodies (Lavialle-Defaix et al., 2006), could also be differentiated on the basis of their sensitivity to intracellular ATP concentration, thereby unmasking differing DCJW sensitivities. To substantiate this hypothesis, we designed additional experiments to study the effects of DCJW on INa1 and INa2 in isolation. Figure 1C shows the comparative semilogarithmic curves of INa1 and INa2 inhibition induced by bath application of DCJW. The inhibitory effect of DCJW on INa2 was obtained after bath application of 10 μM l-glutamate, which is known to produce full inhibition of INa1 (Lavialle-Defaix et al., 2006). The effect of DCJW on INa1 was obtained by subtraction of l-glutamate-resistant INa from the corresponding global sodium current (Fig. 1B, closed squares). In both cases, the data were best-fitted through the mean data points according to the Hill equation (eq. 1). The IC50 values estimated for DCJW were 1.7 nM and 1.6 μM for INa1 and INa2, respectively (Fig. 1C). The comparative superimposed semilogarithmic curves we obtained (Fig. 1, D and E) demonstrated that DCJW inhibited two distinct DUM neuron sodium channels mediating INa1 and INa2, reflecting the plateau between the two parts of the curve (Fig. 1, B and E). These results clearly show that INa2 was approximately 1000-fold less sensitive to DCJW than INa1. After 1 μM DCJW treatment, known to produce full inhibition of INa1 (Fig. 1C), bath application of l-glutamate did not produce any additional effect (Fig. 1F). These results indicated that INa1 was completely inhibited and that a higher concentration of DCJW was needed to block the residual l-glutamate-resistant inward sodium current corresponding to INa2 (Fig. 1E).

Calcium-Dependent Intracellular Pathway Modulates INa2 in DUM Neurons. Because previous electrophysiological studies reported that INa1 was regulated by the cAMP/PKA cascade (Lavialle-Defaix et al., 2006) and that global INa was also affected by intracellular calcium concentration (Grolleau et al., 2001), we then investigated the participation of the calcium-dependent intracellular messenger pathways in the regulation of INa2. All experiments were carried out with saline containing 10 μM l-glutamate to inhibit selectively INa1, as reported previously (Lavialle-Defaix et al., 2006). To test whether calcium and calcium-calmodulin complex (CaM) modulated INa2, we first examined the effect of intracellular perfusion of the calcium chelator BAPTA on INa2 amplitude. As illustrated in Fig. 2A, 10 mM

Part1 = (Max - Min)Fraction 1/(1 + X/IC50,1)
Part2 = (Max - Min)(1 - Fraction 1)/(1 + X/IC50,2)
Y = Min + Part1 + Part2

where X corresponds to the DCJW concentration; IC50,1 and IC50,2 are the concentrations that produce 50% INa1 and INa2 inhibition, respectively; Min and Max are the minimal and maximal values, respectively; Fraction 1 is the fraction of all sites that have affinity 1; and Y is the percentage inhibition of the peak inward sodium current.
BAPTA added in the pipette solution produced a slight reduction of INa2 amplitude (residual INa2 amplitude was 87.4 ± 1.7%; n = 6). A similar effect was observed when the CaM inhibitor N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) (0.5 mM) was added in the pipette solution (residual INa2 amplitude was 88.7 ± 2.3%; n = 7).

In contrast, if a relatively high calcium concentration was introduced into the DUM neuron cell body by adding 1 μM calcium into the patch pipette, INa2 amplitude was strongly reduced (residual INa2 amplitude was 64.6 ± 4.9%, n = 9). These results suggested that INa2 was sensitive to change in intracellular calcium concentration. Because it is known that, in DUM neurons, calcium acting through the calcium-receptor protein CaM is an important signal that regulates diverse enzymatic activities, such as calcium/CaM-dependent protein phosphatase (i.e., calcineurin) and calcium/CaM-dependent protein kinase type II, CaM-kinase II (Courjaret and Lapied, 2001), the participation of PP2B and CaM-kinase II in the regulation of INa2 was further examined.

Intracellular application of cyclosporine A (100 nM) reduced (residual INa2 amplitude was 79.8 ± 4.2%, n = 10; Fig. 2B). By contrast, KN-62 (10 μM) produced an increase of INa2 amplitude (111.1 ± 3.9%, n = 6; Fig. 2B). These results indicated that PP2B and CaM-kinase II regulated INa2 amplitude in an opposite way in DUM neurons. In other words, PP2B and CaM-kinase II could up- and down-regulate INa2 amplitude, respectively. We next tested the effect of caffeine, known to stimulate the release of calcium from intracellular stores (Courjaret et al., 2003; Gautier et al., 2008), on INa2 while maintaining PP2B and/or CaM-kinase II inactivated by applying intracellularly 100 nM cyclosporine A and/or 10 μM KN-62, respectively. Under these conditions, bath application of caffeine (10 mM) produced stronger effect on INa2 amplitude in the presence of cyclosporine A added in the pipette solution (residual current was 64.0 ± 2.2%, n = 5, compared with 79.8 ± 4.2% recorded in the absence of caffeine, n = 10; Fig. 2B). From these results, it is reasonable to hypothesize that the caffeine-induced stronger decreased of INa2 was caused by the activation of CaM-kinase II via the release of calcium from the internal store. This theory was substantiated by the absence of any stronger inhibitory effect of caffeine on INa2 amplitude when 10 μM KN-62 was added in the pipette solution (residual current was 96.7 ± 3.3%, n = 3; Fig. 2B). All together, these results indicated that INa2 was modulated by an intracellular calcium-dependent pathway that involved both CaM-kinase II and PP2B.

Does Calcium-Dependent Intracellular Regulation of INa2 Affect the Effect of DCJW? Our previous work performed on insect DUM neurons demonstrated that insecticide efficiency was altered by calcium-dependent phosphorylation/dephosphorylation process (Courjaret and Lapied, 2001; Lapied et al., 2009). To determine whether DCJW-induced INa2 inhibition was affected by changing the internal calcium concentration, we first examined the effect of decreasing the internal calcium concentration.
buffering previously provided a plausible explanation for the intracellular calcium sensitivity of ion channels and/or membrane receptors in numerous preparations. It was demonstrated that the calcium chelator EGTA did not adequately buffer all of the calcium near the inner face of the membrane on a millisecond time scale. By contrast, it was known that BAPTA was a faster and more efficient chelator (Marty and Neher, 1985). Internal application of BAPTA (10 mM) through the patch pipette significantly decreased the inhibitory effect of bath application of DCJW tested at 10 μM on INa2 (21.6 ± 3.1%, n = 5; Fig. 3A) compared with control conditions (53.1 ± 7.3%, n = 6; Fig. 3A). Similar effects were obtained with intracellular application of 0.5 mM W7 (22.5 ± 4.9%, n = 4; Fig. 3A). We also tested the effect of various concentrations of DCJW on INa2 amplitude when intracellular calcium concentration was decreased (using 10 mM BAPTA in the pipette solution) or increased by using high intracellular calcium concentration (1 μM) added in the pipette solution (Fig. 3B). Although BAPTA reduced the effect of DCJW on INa2 (IC50 = 6.69 μM; Fig. 3B), it was interesting to note that an elevation of intracellular calcium concentration (1 μM) increased the DCJW-induced INa2 reduction and shifted the sigmoid curve toward lower DCJW concentrations (IC50 = 0.48 nM) compared with control (IC50 = 1.6 μM) (Fig. 3B). These results indicated that increases in intracellular calcium strongly increase INa2 channel sensitivity to DCJW. We also examined the effect of bath application

**Fig. 2.** Calcium-dependent intracellular signaling pathways involved in the regulation of INa2. A, comparative histogram illustrating the percentage of residual INa2 amplitude recorded at time = 15 min in the presence of BAPTA (10 mM, n = 6), W7 (0.5 mM, n = 7), and 1 μM intracellular calcium added to the pipette solution (n = 9). B, histogram illustrating the percentage of residual INa2 amplitude recorded in the presence of 100 nM cyclosporine A (n = 10), 10 μM KN-62 (n = 6), and 10 mM caffeine applied alone or in combination, as indicated in the graph by + and − symbols below each corresponding bar. INa2 was recorded in the presence of L-glutamate (10 μM) to block INa1. The cells were held at a holding potential of −90 mV and depolarized to 0 mV. Data are means ± S.E.M. * and **, values significantly different, p < 0.05 and p < 0.01, respectively.

**Fig. 3.** Calcium-dependent intracellular pathways affect the DCJW-induced INa2 inhibition. A, histogram summarizing the effect of 10 μM DCJW on INa2 recorded in controls (number 1, n = 6) and after intracellular application of 10 mM BAPTA (number 2, n = 5) and 0.5 mM W7 (number 3, n = 4). Inset shows typical example of superimposed INa2 traces elicited with a 30-ms depolarizing pulse to 0 mV from a holding potential of −90 mV, recorded under each set of experimental conditions. Currents were capacity and leak corrected. B, comparative semilogarithmic dose-response curves of INa2 inhibition induced by DCJW recorded in controls (filled triangles) and in the presence of BAPTA (10 mM, open triangles) and 1 μM intracellular calcium added in the internal solution (open circles). The smooth lines represent the best fit through the mean data points according to the Hill equation (eq. 1). C, comparative histogram illustrating that external application of 9 mM calcium chloride did not change the inhibitory effect of DCJW (10 μM) observed on INa2 compared with control (i.e., 2 mM calcium chloride). In all experiments, INa2 was recorded in the presence of L-glutamate (10 μM) and was evoke by 30-ms depolarizing pulse from a holding potential of −90 mV. * and **, values significantly different, p < 0.05 and p < 0.01, respectively; ns, not significant (p > 0.05). Data are means ± S.E.M.
of DCJW on INa2 amplitude by modification of extracellular calcium concentration. Figure 3C illustrates that an elevation of extracellular calcium concentration (from 2 to 9 mM) does not significantly alter the effect of DCJW (48.0 ± 5.5% (n = 5), compared with control condition [53.1 ± 7.3% (n = 6), p > 0.05]).

Because our results demonstrated that PP2B and CaM-kinase II modulated INa2 current (Fig. 2), we also examined the possible regulatory role of these phosphatases and kinases on DCJW-induced INa2 inhibition. Figure 4, A and B, shows typical examples of normalized Na2 current amplitude recorded as a function of time in the presence of cyclosporine A and KN-62. When cyclosporine A (100 nM) was applied intracellularly through the patch pipette, the inhibitory effect of bath application of DCJW on INa2 amplitude (43.1 ± 8.1%, n = 6) was not significantly different compared with the control (53.1 ± 7.3%, n = 6, p > 0.05; Fig. 4, A and B). By contrast, as indicated in Fig. 4, B and C, the inhibitory effect of DCJW was strongly reduced by KN-62 (8.8 ± 3.2%, n = 7, p < 0.05). These results suggested that direct and/or indirect phosphorylation of the Na2 channel by CaM-kinase II was essential for maintaining the inhibitory effect of DCJW. This finding was also confirmed when the effect of bath application of DCJW (1 µM) on INa2 amplitude was tested in the presence of KN-62 and/or high internal calcium concentration (1 µM), both added in the pipette solution. Under these conditions, the inhibitory effect of DCJW on INa2 amplitude (16.2 ± 0.7%; n = 5) was also strongly reduced compared with the effects observed without KN-62 (Fig. 3, B and D).

Involvement of CaM-Kinase II Increases Na2 Sensitivity to DCJW. Previous studies showed that the blocking action of pyrazoline-type insecticides on sodium channels was voltage-dependent in crayfish giant axons and neurons isolated from thoracic ganglion of the American cockroach, with a selective binding to the slow-inactivated state of the channel (Salgado, 1992; Zhao et al., 2005; Silver et al., 2009). In DUM neurons, however, such a mechanism has never been clearly demonstrated. According to these previous results, we hypothesized that the CaM-kinase II-induced modification of the inhibitory effect of DCJW observed on the Na2 channels could result from change of the sodium channel state regulated by CaM-kinase II. Our results described above indicated that inhibition of CaM-kinase II increased INa2 amplitude (Fig. 2B) and strongly reduced the inhibitory effect of DCJW on Na2 (Fig. 4, A and C). By contrast, activation of CaM-kinase II by intracellular calcium rise decreased INa2 amplitude but increased the DCJW inhibitory effect (Figs. 2A, 3B, and 4, B and C). These results seemed to suggest that CaM-kinase II might affect specific biophysical parameters of the Na2 channel, which thereby increased sensitivity to DCJW. To substantiate this hypothesis, additional experiments were designed to study the influence of steady-state holding-potential change on the inhibitory effect of DCJW. As illustrated in Fig. 5A, when 50% Na2 channels were inactivated by changing the steady-state holding potential from −90 to −40 mV (Lavialle-Defaix et al., 2006), the inhibitory effect of bath application of DCJW on INa2 amplitude was more pronounced (82.3 ± 3.4%; n = 7) compared with control condition [53.1 ± 7.3% (n = 6); holding potential of −90 mV]. From these last results, the emerging question concerned the possible physiological impact of CaM-kinase II on the voltage dependence of the steady-state inactivation parameter of Na2. As shown in Fig. 5B, intracellular application of KN-62 significantly shifted the voltage dependence of steady-state inactivation toward more positive potentials. In this case, V0.5 corresponding to the potential at which half of the Na2 channels were inactivated was −41.3 ± 0.1 mV (n = 11) compared with −31.5 ± 0.2 mV (n = 6) calculated in the presence of KN-62 (p < 0.05). These results indicated that CaM-kinase II activation facilitated an inactivated state of Na2 channels, which thereby potentiated the inhibitory effect of DCJW.

Discussion

Calcium-Dependent Intracellular Signaling Pathways Regulate DUM Neuron INa2. Previous studies have reported that DUM neuron cell bodies expressed two types of voltage-dependent sodium currents. Global INa has been dissociated mainly by means of L-glutamate sensitivity [i.e., a neurotransmitter acting selectively through metabotropic glutamate receptor III activation (Lavialle-Defaix et al.,
Chahine et al., 2005). However, with the exception of data indicating that CaM-kinase II is implicated in various signaling pathways involved in the neuronal excitability in Droso-
phila melanogaster neurons (Park et al., 2002) and the regulation of vertebrate channels, including potassium channels (Sansom et al., 2000; Colinas et al., 2006), calcium channels (Lee et al., 2006; Jiang et al., 2008), and cardiac or neuronal sodium channels (Carlier et al., 2000; Wagner et al., 2006), there are no data indicating the existence of dual regulation attributable to effects of PP2B and CaM-kinase II on insect sodium channels. As summarized in the hypothetical scheme (Fig. 6), elevation in intracellular calcium levels from the intracellular stores, for example, results in activation of CaM-kinase II by intracellular calcium through the calcium/CaM complex. Such phosphorylation is opposed by a dephosphorylation process that involves calcium/CaM-de-
dependent PP2B, also active on Na2 channels. Combining the results of this study with those that we have previously re-
ported (Lavialle-Defaix et al., 2006), we show that the cAMP signaling pathway, PKA, calcium, and PP2B and CaM-kinase II are intimately interconnected in the phosphorylation/dephos-
phorylation process. These signaling pathways are involved in the intracellular regulation of DUM neuron INa1 and INa2, with the modification of DCJW sensitivity attributable to CaM-
kinase II.

**CaM-Kinase II Activation Increases the Sensitivity of the Na2 Channel to DCJW.** Another interesting finding of this study was the physiological implication of the intracellular signaling pathway on the sensitivity of the two voltage-
dependent sodium channels expressed within the same neuron. We have demonstrated that DCJW sensitivity differs in INa1 and INa2 (i.e., INa1 is very sensitive to DCJW compared with INa2). Similar observations have previously been reported in a nonidentified heterogeneous population of cockroach thoracic neurons in which two distinct sodium currents (named type I and type II) were also differently

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**Figure 5.** Effect of DCJW and voltage-dependent inactivation parameter of INa2. A, effect of DCJW (10 μM) on INa2 amplitude. Inward currents were evoked by a 30-ms depolarizing test pulse to 0 mV from a holding potential of −90 mV (1) and after a 10-s depolarizing conditioning pulse of −40 mV (2), according to the protocol illustrated above each current trace. The graph represents the maximum effect of DCJW (10 μM) on INa2 amplitude recorded at a holding potential of −90 mV (filled circles, n = 6) and after a depolarizing conditioning pulse to −40 mV (open circles, n = 7); **,** value significantly different (p < 0.01). B, voltage dependence of inactivation parameters of INa2 studied according to the standard 2-pulse voltage-clamp protocol indicated in the inset in control (Ctr, filled circles, n = 6) and in the presence of KN-62 (10 μM, open circles, n = 6) added in the pipette solution. Smooth curves of inactivation were fitted through data points using Boltzmann equation (eq. 3). The cells were pretreated with l-glutamate (10 μM). Data are mean ± S.E.M.

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**Figure 6.** Hypothetical pattern of intracellular regulation of INa2 in DUM neurons. This scheme summarizes the essential components of PP2B and CaM-kinase II that may regulate INa2. Cyclosp A, cyclosporine A; [Ca2+]i, intracellular calcium concentration; BAPTA, calcium chela-
tor; ER, endoplasmic reticulum.
sensitive to indoxacarb and DCJW (Zhao et al., 2005). In our study, we observed new elements that enhance understanding of how sodium channels differ on the basis on their sensitivity to DCJW. Changes in intracellular ATP concentration, known to be a key factor involved in the phosphorylation process, enable better discrimination between the Na1 and Na2 channels with regard to DCJW sensitivity. As we reported previously (Lapied et al., 2001), the DCJW-induced inhibition of the global inward sodium current was best described by a monophasic dose-response curve. These results were obtained with an internal pipette solution, which contained 2 mM ATP (see Fig. 1B). In the present study, the addition of 3 mM ATP in the pipette solution produced a biphasic DCJW-induced dose-dependent inhibition of the global sodium current. The only difference that explains the modification in the appearance of the dose-response curves is closely related to changes in intracellular ATP concentration (Fig. 1B). The biphasic aspect of the dose-dependent inhibition of the sodium current by DCJW reflects differences in the Na1 and Na2 related to their regulation by the intracellular signaling pathway, which thereby affects the sensitivity to DCJW. In other words, because ATP is a cofactor of many protein and enzyme activities involved in regulatory processes, it can thereby modify sodium current regulation. Increasing intracellular concentration of ATP from 2 to 3 mM will have physiological consequences on intracellular cAMP accumulation. These observations indicate that elevation of the ATP level led to a reduction of INa1 through PKA stimulation after the increase of cAMP concentration (Lavialle-Defaix et al., 2006). This pathway activation unmaps the effect of DCJW on INa2, which appears to be 1000-fold less sensitive than INa1 (see Fig. 1, B, second component of the biphasic dose-response curve, and C).

It is noteworthy that our study further shows that calcium-dependent intracellular pathways may facilitate the transition from a less sensitive Na2 channel to a highly sensitive channel. This observation is confirmed by findings indicating that the inhibitory effect of DCJW on INa2 amplitude is half-reduced in the presence of a calcium chelator and CaM inhibitor, whereas the addition of a high calcium concentration in the patch pipette solution potentiates its inhibitory effect. Moreover, the DCJW-induced inhibition of INa2 amplitude was strongly reduced when CaM-kinase II was inhibited. These results lead us to suggest that CaM-kinase II activated by intracellular calcium through the complex calcium/CaM is involved in phosphorylation process, which will modify channel biophysical properties by inducing conformational change. This effect renders the Na2 channels more sensitive to DCJW, resulting in a shift of the semilogarithmic curve toward lower concentrations. In addition, it has been reported that DCJW is a voltage-dependent inhibitor of insect sodium currents. It appears that the inactivated states of sodium channels are most likely to be sensitive to DCJW (Silver and Soderlund 2005a; Zhao et al., 2005; Silver et al., 2009). Furthermore, results of complementary studies indicate that two German cockroach sodium channel isoforms (BgNav1.1 and BgNav1.4), which exhibit different voltage dependence on fast and slow inactivation, display different DCJW sensitivity and that the amino acid substitution of E1689K in segment 4 of domain IV of the sodium channel is responsible for the difference in the voltage dependence of fast and slow inactivation and DCJW sensitivity between these two isoforms (Song et al., 2006). Based on these findings, an emerging question is whether INa1 and INa2 elicited by DUM neuron sodium channels, differently sensitive to DCJW, result in the combination of different isoforms of α-and/or β-sodium channel subunits. To date, however, such a correlation between native sodium currents and different sodium channel isoforms generated by RNA editing and/or alternative splicing (Dong 2007) remains difficult to establish. This is reinforced by the recent characterization of two distinct sodium channel genes (rather than a unique gene) that encode sodium channels in the P. americana central nervous system (Moignot et al., 2009).

In addition, application of KN-62, an inhibitor of CaM-kinase II, reduced the inhibitory effect of DCJW on Na2 channels. Furthermore, this inhibitor produced a shift of the steady-state voltage dependence of the inactivation curve toward more positive potentials. The most likely explanation for these data is that Na2 channels are continuously activated by CaM-kinase II in the basal state. However, after elevation of the calcium level, the potentiation of CaM-kinase II activation will render Na2 channels more sensitive to DCJW. As observed when the depolarizing conditioning pulse is made more positive, activation of CaM-kinase II via an increase in intracellular calcium stabilizes a more negative voltage dependence of steady-state inactivation of Na2 in a potential range in which sodium channels are more inactivated. In fact, CaM-kinase II activation causes a permanent hyperpolarizing shift of the voltage dependence of inactivation compared with that observed in the presence of CaM-kinase II inhibitor, resulting in a potentiation of the inhibitory effect of DCJW on Na2 channels.

In conclusion, this study is the first investigation to reveal that intracellular regulation mechanisms involving the activation of CaM-kinase II via an elevation of intracellular calcium concentration can potentiate the effect of DCJW on the Na2 channel, a DUM neuron membrane target reported to be relatively insensitive to low concentrations of DCJW. In addition, CaM-kinase II activation seems to be a key factor involved in anthelmintic and insecticide efficiency. It has been reported that the response to levamisole on nicotinic acetylcholine receptors expressed in the nematode Ascaris suum muscle depends on the phosphorylation state of the receptor by CaM-kinase II and tyrosine kinase (Trajilovic et al., 2002). Furthermore, Inagaki et al. (1998) have shown a reduction of CaM-kinase II expression in the kdr-resistant German cockroach Blattella germanica compared with kdr-susceptible cockroaches. These results suggest that manipulating intracellular signaling pathways involved in the regulation of sodium channels can have fundamental consequences for the sensitivity of insects to given insecticides. This finding opens an exciting research area that could lead to improved efficiency of insecticides.

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