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Inhibition of protein kinase C decreases sensitivity of GABA receptor subtype to fipronil insecticide in insect neurosecretory cells

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ABSTRACT

Phosphorylation by serine/threonine kinases has been described as a new mechanism for regulating the effects of insecticides on insect neuronal receptors and channels. Although insect GABA receptors are commercially important targets for insecticides (e.g. fipronil), their modulation by kinases is poorly understood and the influence of phosphorylation on insecticide sensitivity is unknown. Using the whole-cell patch-clamp technique, we investigated the modulatory effect of PKC and CaMKinase II on GABA receptor subtypes (GABAR1 and GABAR2) in DUM neurons isolated from the terminal abdominal ganglion (TAG) of *Periplaneta americana*. Chloride currents through GABAR2 were selectively abolished by PMA and PDBu (the PKC activators) and potentiated by Gö6983, an inhibitor of PKC. Furthermore, using KN-62, a specific CaMKinase II inhibitor, we demonstrated that CaMKinase II activation was also involved in the regulation of GABAR2 function. In addition, using $CdCl_2$ (the calcium channel blocker) and LOE-908, a blocker of TRPγ, we revealed that calcium influx through TRPγ played an important role in kinase activations. Comparative studies performed with CACA, a selective agonist of GABAR1 in DUM neurons confirmed the involvement of these kinases in the specific regulation of GABAR2. Furthermore, our study reported that GABAR1 was less sensitive than GABAR2 to fipronil. This was demonstrated by the biphasic concentration–response curve and the current–voltage relationship established with both GABA and CACA. Finally, we demonstrated that GABAR2 was 10-fold less sensitive to fipronil following inhibition of PKC, whereas inhibition of CaMKinase II did not alter the effect of fipronil.

1. Introduction

To understand how insecticides exert neurotoxic actions, it is necessary to understand how the pest targets normally function and to characterize the cellular factors that can lead to affect insecticide efficacy. Among membrane receptors and/or ion channels targeted by insecticides, ionotropic γ-aminobutyric acid receptors (GABARs) are considered as vital complex protein subunits, mediating fast inhibitory transmission in the central nervous system. They are targeted by several important classes of insecticides, including cyclodienes (e.g. dieldrin) and phenylpyrazoles (e.g. fipronil). By blocking the inhibitory action of GABA and in some cases glutamate (Raymond et al., 2000; Janssen et al., 2010; Narahashi et al., 2010), these compounds induce neuronal hyperexcitation and convulsions by reducing the flow of chloride ions through the receptor channel complex (Raymond-Delpech et al., 2005; Law and Lightstone, 2008).

There is substantial evidence indicating that many cellular factors can modulate insecticide sensitization. Among such factors, the development of resistance mechanisms in many pest species is one of the most well-known molecular factors involved in the reduction of insecticide efficacy. For insect GABARs, cyclodiene/fipronil resistance is associated with a point substitution (A301S/G) in a GABAR subunit, named RDL (for resistance to dieldrin), that is widespread in the central nervous system of insects (ffrench-Constant et al., 1993, 2000; Hosie et al., 1995; Le Goff et al., 2005). In addition and in contrast to resistance, another factor involved in the reduction of insecticide effect is defined as tolerance of insecticides. This is a natural tendency and is not a result of forced change in a genetic makeup of a given insect population. In this

Abbreviations: CaMKinase II, calcium/calmodulin-dependent protein kinase II; DAG, diacylglycerol; DUM, Dorsal Unpaired Median; GABARs, gamma-aminobytiric acid receptors; Gö6983, 3-[1-[3-(dimethylamino) propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione; KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; PDBu, phorbol 12,13-dibutyrate; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; RDL, resistant to dieldrin; TAG, terminal abdominal ganglion; TRPgamma, transient receptor potential.

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context, a recent study has revealed that sensitivity of insect GABARs to the insecticide fipronil can be altered by posttranscriptional modifications, such as RNA editing (Es-Salah et al., 2008). In addition, phosphorylation and/or dephosphorylation processes, which are thought to play significant roles in the maintenance of receptors and/or ion channels, can also modulate insecticide sensitivity of insect membrane targets, such as nicotinic acetylcholine receptors and voltage-dependent sodium channels (Courjaret and Lapied, 2001; Lapied et al., 2009; Lavialle-Defaix et al., 2010).

It has previously been reported that (i) PKA participates in the maintenance of GABAR function in cockroach unidentified neurons (Watson and Salgado, 2001) and (ii) calcium modulates GABA-induced currents via an unidentified kinase pathway in the mushroom body Kenyon cells and antennal lobe cells of the honeybee (Grünewald and Wersing, 2008; Dupuis et al., 2010). Furthermore, previous findings have indicated that GABAR subtypes, expressed in cockroach DUM neuron cell bodies (here referred as GABAR2), are positively regulated by calcium entry through a channel incorporating a TRPγ protein (Wicher et al., 2006), which thereby activates a CaMKinase II pathway (Alix et al., 2002). Based on these findings and because it is not known if insect GABARs are affected by other members of the serine/threonine kinase family, such as PKC, we have investigated the effects of PKC activation and/or inhibition on GABA-induced chloride currents in cockroach DUM neurons isolated from the terminal abdominal ganglion (TAG). Furthermore, we have studied the possible roles of both PKC and CaMKinase II in modulating the sensitivity of GABAR subtypes to phenylpyrazole insecticide, fipronil.

2. Materials and methods

2.1. Cell isolation

DUM neuron cell bodies from the TAG of adult male cockroaches, *Periplaneta americana*, were prepared under sterile conditions, using enzymatic digestion and mechanical dissociation, as previously described (Lapied et al., 1989). Isolated cell bodies were maintained at 29 °C for 24 h before experiments were carried out.

2.2. Electrophysiology

GABA- and CACA-induced chloride currents were recorded using the whole-cell patch-clamp technique, under voltage-clamp mode. Ionic currents were evoked by applying GABA (100 μM) and CACA (1 mM) through a glass micropipette connected to a pneumatic pressure ejection system (15 psig, 100 ms in duration, Miniframe Medical Systems Corporation, Greenvale, NY, USA). The tip of this pipette (resistance 2 MΩ, when filled with agonist solution) was positioned within 50 μm from the examined cell body. GABA- and CACA-induced currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) and filtered at 5 kHz. Data were stored on-line on the hard disk of a PC computer (sampling frequency: 10 kHz) connected to a 16-bit A/D converter (Digidata 1322A, Axon Instruments). The pClamp package (pClamp 8.0.2., Axon Instruments) was used for data acquisition and off-line analysis. Patch pipettes were pulled from borosilicate glass capillary tubes (GC 150T-10; Clark Electromedical Instruments, Harvard Apparatus, Edenbridge, UK) using a P-97 puller (Sutter Instruments, Novato, CA, USA) and displayed resistances ranging from 1 to 1.2 MΩ when filled with internal solution (see composition below). The liquid junction potential between bath and internal pipette solution was always corrected before the formation of a gigaohm seal (>2 GΩ). DUM neuron cell bodies were voltage-clamped at a holding potential of −50 mV (except when otherwise stated). Patch-clamp experiments were conducted at room temperature (20 °C).

2.3. Solutions and chemicals

The cells were continuously superfused by a gravity perfusion system with a chloride-isotonic solution containing (in mM): 167 NaCl, 33 D-gluconic acid, 3.1 KCl, 4 $MgCl_2$, 5 $CaCl_2$ and 10 HEPES; pH was adjusted to 7.4 with NaOH. The internal pipette solution contained (in mM): 170 KCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 15 pyruvic acid sodium salt, 10 EGTA, 20 HEPES and 10 phosphocreatine-di-Tris; pH was adjusted to 7.4 with KOH. All chemicals were purchased from Sigma–Aldrich Chemicals (l'Isles d'Abeau Chenes, France). Pyruvic acid (sodium salt, Research Organics Inc., Cleveland, USA) was used to prevent rundown of GABA currents. Fipronil (CIL Cluzeau, France) was first dissolved in dimethylsulfoxide (DMSO) at 1×10^{-2} M and then diluted in bath solution to the final required concentration. Stock solutions containing the PKC activators (PMA, PDBu) and inhibitors (Gö6983) were dissolved in the bath solution, just before use. The CaMKinase II inhibitor KN-62 and the TRPγ inhibitor LOE 908, first dissolved in DMSO, were finally diluted in internal pipette solution.

2.4. Statistical analysis

When quantified, data were expressed as mean ± S.E.M., where *n* is the number of experiments. Curves were fitted using the software Prism 2 (GraphPad Software Inc., San Diego, USA). The equation used to fit the fipronil inhibition curve through the mean data points is shown below.

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom})\left[\frac{\text{Fraction\_1}}{1+10^{\text{X}-\log \text{EC50\_1}}} + \frac{1-\text{Fraction\_1}}{1+10^{\text{X}-\log \text{EC50\_2}}}\right] \quad (1)$$

Statistical analysis was performed using unpaired ANOVA-test and differences were expressed as non significant (NS: $p > 0.05$) or significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Results

Pressure application of 100 μM GABA onto the voltage-clamped DUM neuron somata induced a transient inward current (IGABA) that reversed at +1.6 mV, a value close to the calculated Nernstian equilibrium potential for chloride ions (−2.1 mV; Fig. 1a). The current–voltage (*I–V*) relationship was linear between −70 and −30 mV but deviated from linearity for potentials above −30 mV (Fig. 1b). The best linear fit through the mean data points gave a slope conductance of 111.4 ± 14.6 nS ($n = 9$) and 66.3 ± 4.6 nS ($n = 9$) between −70 and −30 mV and −30 and 0 mV, respectively. As previously reported (Alix et al., 2002), the biphasic shape of the *I–V* curve reflected the presence of two distinct GABAR subtypes (here referred as GABAR1 and GABAR2) in DUM neuron somata.

3.1. Effect of PKC activation on the inward chloride current mediated by GABARs

DUM cells were pretreated with 300 nM PMA (a PKC activator) for 4 h, according to the protocol previously used in the same preparation to activate PKC (Courjaret et al., 2003). IGABA amplitude was not significantly modified by PMA at a holding potential of −30 mV, but was reduced by 37.9 ± 4.7% at −70 mV (control: $n = 9$; PMA: $n = 5$; $p < 0.05$) (Fig. 1c and d). Under these conditions, the current–voltage relationship became linear between −70 mV and 0 mV, with a slope conductance of 55.1 ± 2.4 nS ($n = 5$), a