Complex Intracellular Messenger Pathways Regulate One Type of Neuronal α-Bungarotoxin-Resistant Nicotinic Acetylcholine Receptors Expressed in Insect Neurosecretory Cells (Dorsal Unpaired Median Neurons)

Raphael Courjaret and Bruno LapiéD
Laboratoire de Neurophysiologie Unité Propre de Recherche et de l’Enseignement Supérieur, Equipe d’Accueil 2647 (Récepteurs et Canaux IoniQues Membranaires), Université d’Angers, Unité de Formation et de Recherche Sciences, Angers, France

Received January 22, 2001; accepted March 14, 2001 This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT
Although molecular biology provides new insights into the subunit compositions and the stoichiometries of insect neuronal nicotinic acetylcholine receptors (nAChRs), our knowledge about the phosphorylation/dephosphorylation mechanisms of native neuronal nAChRs is limited. The regulation of α-bungarotoxin-resistant nAChRs was studied on dissociated adult dorsal unpaired median neurons isolated from the terminal abdominal ganglion of the cockroach Periplaneta americana, using whole-cell, patch-clamp technique. Under 0.5 μM α-bungarotoxin treatment, pressure ejection application of nicotine or acetylcholine onto the cell body induced an inward current exhibiting a biphasic current-voltage relationship. We found that two distinct components underlying the biphasic curve differed in their intrinsic permeability and pharmacology (one being sensitive to d-tubocurarine, and the other affected only by mecamylamine and α-conotoxin ImI). This indicated that two types of α-bungarotoxin-resistant nAChRs (named nAChR1 and nAChR2) mediated the nicotinic response. These two components were also differentially sensitive to rundown and intracellular messengers. Intracellular application of 0.1 mM cAMP only increased the current amplitude mediated by nAChR1. Using forskolin (1 μM), W7 and H89, we demonstrated that adenyl cyclase, sensitive to calcium/calmodulin complex, regulated nAChR1 via a cAMP/cAMP-dependent protein kinase cascade. By contrast, intracellular concentration higher than 0.1 mM reduced the current amplitude. This effect, mimicked by high external concentration of forskolin (100 μM) and IBMX, was reversed by okadaic acid, suggesting the implication of a protein phosphatase. Using KN-62, we demonstrated that calmodulin-Kinase II also modulated directly and indirectly nAChR1, via an inhibition of the phosphatase activity. Finally, we reported that phosphorylation/dephosphorylation of nAChR1 strongly affected the action of the widely used neonicotinoid insecticide imidacloprid.

The nicotinic acetylcholine receptor (nAChR) has served as a model system for the study of the structure, function, and regulation of ligand-gated ion channels in vertebrates as well as in invertebrates (Osborne, 1996; Mongan et al., 1998; Swope et al., 1999). In vertebrates, nAChRs can be subdivided into two subgroups, end-plate nAChRs and neuronal nAChRs. To date, 10 known members of the gene family encode the subunits of neuronal nAChR [i.e., eight α subunits (α2–α9) and three β subunits] and different neuronal nAChRs can be formed from the members of this gene family (Cordero-Erausquin et al., 2000). These nAChRs can be further classified according to their sensitivity to α-bungarotoxin into α-bungarotoxin-sensitive and -insensitive nAChRs. The nAChRs formed of α7, α8, and α9 subunits are blocked by α-bungarotoxin, whereas those composed of α2 to α6 and β2 to β4 subunits are α-bungarotoxin-resistant (Cordero-Erausquin et al., 2000). The regulation of nAChR function by intracellular messengers plays a key role in the modulation of neuronal activity (e.g., Paterson and Nordberg, 2000). Abundant evidence indicates that the nAChR is a phosphoprotein that has been shown to be phosphorylated and regulated by protein kinases such as cAMP-dependent protein kinase (PKA), protein kinase C, calcium-calmodulin-dependent protein kinase (CaM kinase), and endogenous protein tyrosine kinase (Margiotta et al., 1987; Eilers et al., 1997; Liu and Berg, 1999; Paterson and Nordberg, 2000). In addition, protein phosphatases, such as phosphatases PPI/
In contrast to vertebrates neuronal nAChRs, phosphorylation/dephosphorylation of nAChRs in insect neuronal preparations has not been examined in detail. It is known that acetylcholine (ACh) is the predominant excitatory neurotransmitter in insect central nervous system (Satellite, 1985). Among insect AChRs, both α-bungarotoxin-sensitive and -insensitive nAChRs have been widely studied in their pharmacological and physiological aspects (Lapied et al., 1990; Benson, 1992; David and Pitman, 1993; Grolleau et al., 1996; Osborne, 1996). Although the molecular structure of insect nAChRs is not as well characterized as that of their vertebrates counterparts, many recent studies have focused on characterizing the subunit composition of insect nAChRs. Although several different nAChR α-type and β-type subunit genes and cDNAs have been isolated from various insect species, such as the fruit fly Drosophila melanogaster (Gun- dellfinger and Schulz, 2000; Lansdell and Millar, 2000a), the tobacco hornworm Manduca sexta (Eastham et al., 1998), the locusts Schistocerca gregaria and Locusta migratoria (Mar- shall et al., 1990; Hermsen et al., 1998), and the aphid Myzus persicae (Sgard et al., 1998; Huang et al., 2000), the manner in which these native insect nAChRs can be modulated by phosphorylation and/or dephosphorylation induced by protein kinases and protein phosphatases remains unknown. Because of the importance of insect nAChRs as target sites for the major highly effective and widely used neonicotinoid insecticides, such as imidacloprid (Yamamoto and Casida, 1999), it seems necessary to better understand the intracellular messenger pathways involved in the regulation of native nAChRs. Such unknown intracellular mechanisms underlying the nAChR functional properties should undoubtedly alter the mode of action of this new class of insecticides.

Consequently, in this study, we have begun to study, for the first time in insect neuronal preparations, the phosphor- ylation/dephosphorylation mechanism involved in the regulation of the α-bungarotoxin–insensitive neuronal nAChRs using whole-cell, patch-clamp technique. We have found that two pharmacologically distinct types of native somatic α-bun- garotoxin–insensitive nAChRs are differentially modulated by complex intracellular mechanisms involving PKA, an oka- daic acid-sensitive protein phosphatase, and CaMKII. The possible cross talk between these intracellular messenger cascades is discussed. Moreover, we also report, for the first time, that phosphorylation/dephosphorylation process could strongly affect the mode of action of imidacloprid, known to act as agonist at the cockroach DUM neuron nAChRs (Buckingham et al., 1997).

**Materials and Methods**

**Preparation.** Experiments were performed on DUM neuron cell bodies isolated from the midline of the terminal abdominal ganglia (TAG) of the nerve chord of adult male cockroaches (Periplaneta americana) obtained from our laboratory stock colony maintained at 29°C on a 12-h light/dark cycle. Animals were immobilized ventral side up on a dissection dish. The ventral cuticle and the accessory gland were removed to allow access to the TAG. The abdominal nerve cord and its TAG, carefully dissected under a binocular microscope, were placed in normal cockroach saline containing 200 mM NaCl, 3.1 mM KCl, 5 mM CaCl\(_2\), 4 mM MgCl\(_2\), 50 mM sucrose, 10 mM HEPES; pH was adjusted to 7.4 with NaOH.

**Cell Isolation.** Isolation of adult DUM neuron cell bodies was performed under sterile conditions using enzymatic digestion and mechanical dissociation of the median part of the TAG as described previously (Lapied et al., 1989). DUM neuron cell bodies were maintained at 29°C for 24 h before electrophysiological experiments were carried out. The DUM neuron cell bodies used in the present study were chosen as indicated previously (Lapied et al., 1989).

**Whole-Cell Recording and Data Analysis.** Nicotine-, acetyl- choline- and imidacloprid-induced ionic currents were recorded using the patch-clamp technique in the whole-cell recording configuration (Hamill et al., 1981) under voltage-clamp mode. Signals were recorded with an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA). Patch pipettes were pulled from boro- silicate glass capillary tubes (GC 150T-10; Clark Electromedical Instruments, Harvard Apparatus, Edenbridge, UK) using a PP83 puller (Narishige, Tokyo, Japan). Pipettes had resistances ranging from 0.8 to 1 MΩ when filled with internal solutions (see composition below). The liquid junction potential between bath and internal solutions was always corrected before the formation of a giantOm seal (>5 GΩ). Ionic currents induced by the cholinergic agonists were recorded on an NEC Celeron 333 computer with software control pClamp (version 6.03; Axon Instruments) connected to a 125-kHz Labmaster DMA data acquisition system (TL1–125 interface; Axon Instruments). DUM neuron cell bodies were voltage-clamped at a steady-state holding potential of −50 mV (except when otherwise stated).

**Pneumatic Pressure Ejection Application of Agonists and Insecticide.** Nicotine hydrogen tartrate (10 mM), acetylcholine (100 mM), and imidacloprid (0.1 mM) were applied by pneumatic pressure ejection (McCaman et al., 1977; Lapied et al., 1990) (15 pounds per square inch gauge, 100-ms pulses for nicotine and imidacloprid and 300-ms pulses for acetylcholine), with a pneumatic pressure system (Miniframe; Medical Systems Corporation, Greenvale, NY) to mini- mize receptor desensitization resulting from bath application of agonists. The pressure ejection was made through a glass micropipette (resistance of 2 MΩ when filled with agonists) positioned in solution within 50 μm from the isolated neuron cell body. Droplets were ejected under oil and the diameter was measured with an ocular micrometer. There was a linear relationship between the volume delivered and both of the pulse duration parameters (McCaman et al., 1977). In this situation, the logarithmic concentration of cholinergic agonists at any point of the cell body will be approximately proportional to the pulse duration of the cholinergic agonist applications (at constant pressure), as previously reported on the same preparation (Lapied et al., 1990). In no experiment did the pressure ejection of normal saline realized with the same protocol affect the current baseline. The steady-state recordings were made 5 min after the setting of the whole cell recording configuration and repeated applications of the cholinergic agonists were made with an interval of 2 min between the end of one application and the beginning of the next. Isolated neuron cell bodies were continuously bathed with saline (see composition below) using a gravity perfusion system positioned within 100 μm from the cell body. Experiments were performed at room temperature (20°C) and results, when quantified, were expressed as means ± S.E.M. Differences between means were tested for statistical significance by Student’s t test.

**Solutions and Drugs.** The extracellular solution contained 200 mM NaCl, 3.1 mM KCl, 5 mM CaCl\(_2\), 4 mM MgCl\(_2\), and 10 mM HEPES buffer, and pH was adjusted to 7.4 with NaOH. To inhibit the ionic currents induced by the activation of the α-bungarotoxin–sensitive mixed nicotinic-muscarinic acetylcholine receptors (Lapied et al., 1990), 0.5 μM α-bungarotoxin was added in the saline superfusing the cell. Patch pipettes were filled with internal solution containing 160 mM K+/β-glucuronate, 10 mM NaCl, 1 mM MgCl\(_2\), 0.5 mM CaCl\(_2\), 10 mM KF, 3 mM ATP Mg, 10 mM EGTA, 20 mM HEPES, pH was adjusted to 7.4 with KOH. In some cases, the tested
compounds were added in the internal pipette solution immediately before use. Imidacloprid stock solution (100 mM) was prepared in dimethyl sulfoxide. Final dilution contained at most 0.1% dimethyl sulfoxide. These concentration of solvent were found to be without effect on electrophysiological properties of DUM neurons. All compounds were purchased from Sigma Chemicals (L’isle d’Abeau Chesnes, France), except N-2-1(β-bromocinnamyl-amino)ethyl]-5-isoquinolinesulfonamide (H89) and 5'-adenylyl-β-γ-imidophosphate (AMP-PNP) were obtained from ICN Biochemicals (Orsay, France); 3-isobutyl-1-methylxanthine (IBMX), N-6(aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7), and water soluble forskolin from RBI (L’isle d’Abeau Chesnes, France); and α-conotoxin Iml was from American Peptide Company (Sunnyvale, CA).

Results

Previous electrophysiological studies indicated that adult DUM neuron cell bodies isolated from the cockroach TAG expressed both nAChR resistant to α-bungarotoxin and AChR with “mixed” nicotinic-muscarinic pharmacology (Lapied et al., 1990; Grolleau et al., 1996). Consequently, the electrophysiological properties of the ionic current mediated by α-bungarotoxin-resistant nAChR can be studied in isolation in the presence of extracellular 0.5 μM α-bungarotoxin. As illustrated in Fig. 1A, pressure application of nicotine (10 mM) onto the soma of isolated DUM neuron cell body induced a transient inward current. When the mean amplitudes of the nicotine-induced inward current were plotted against the different holding potentials, an unexpected biphasic aspect was observed (Fig. 1B) that differed from those usually reported for other insect nAChRs (Sgard et al., 1998; Goldberg et al., 1999). A similar biphasic aspect was observed (Fig. 1C) when the current-voltage relationship was plotted with charge entry calculated from the integral of the current according to the equation CE = It, where CE is the charge entry, z is the ion charge, F is the Faraday constant, I is the inward current, and t is the time.

Furthermore, it should be indicated that no significant change of the biphasic aspect of the curve was observed after external application of the voltage-dependent sodium and calcium channel blockers such as tetrodotoxin (500 nM) and ω-conotoxin GVIA (1 μM), respectively (data not shown). The current-voltage relationship indicated that the peak amplitude of the inward current decreased linearly between −90 mV and −30 mV before increasing again between −30 mV and +20 mV. Similar results were obtained after application of ACh (100 mM, 300-ms duration), which appeared, however, less effective as an agonist of nAChR compared with nicotine (Fig. 1D). For comparison, when ACh and nicotine were applied on the same DUM neuron cell body (holding potential −50 mV) for 300 ms, the charge entry calculated from the integral of an average of five current traces in each condition (see equation above) was 0.001 pC and 0.081 pC, respectively. This indicated that nicotine was a more potent agonist than ACh in DUM neurons. This apparent low potency of ACh compared with nicotine might be a result of its predisposition to be rapidly hydrolyzed by acetylcholinesterase present in insect neurons, as indicated previously (Bai et al., 1992; David and Pitman, 1993).

It was interesting to note that the two components of the biphasic current-voltage curve were differentially affected by prolonged recording performed in whole cell configuration. Twenty-five minutes after disruption of the membrane patch, the second component observed in a positive potential range (between −30 mV and +20 mV) progressively disappeared, whereas the first component (between −90 mV and −30 mV) seemed to be unaffected (Fig. 1B). This effect might be attributable to the loss of important cellular components essential in maintaining the global nicotinic response, as already described for invertebrate nAChRs (Goldberg et al., 1999). To minimize rundown of nicotine-evoked inward current, an additional 10 mM phosphocreatine di-tris (Alkondon et al., 1994) was added in the pipette solution immediately before use. The effect of phosphocreatine on the rundown of the second component was monitored by measuring the nicotinic-induced current amplitude during a 25-min recording period. As illustrated in Fig. 1B, the rundown of the nicotinic response was significantly attenuated by the addition of phosphocreatine in the internal solution. It should be indicated that phosphocreatine also increased (72.8 ± 15.3%, measured at −50 mV; n = 6) the amplitude of the nicotinic response between −90 mV and −30 mV, which was previously shown to be insensitive to rundown. Therefore, it was essential to determine whether or not this unusual biphasic
aspect of the current-voltage relationship reflected the existence of two distinct types of α-bungarotoxin-resistant nAChRs that differed in their phosphocreatine sensitivity.

The Two Components of the Biphasic Current-Voltage Relationship Differ by Their Ionic Permeability and Pharmacology. To examine the possibility that the nicotine-evoked inward current was mediated by two distinct types of α-bungarotoxin–resistant nAChRs, we studied, at first, the ionic permeability of the two components of this biphasic curve. Previous electrophysiological studies performed on the same preparation (Grolleau et al., 1996) reported that nicotine was unable to produce an increase in the intracellular level of free calcium in the presence of α-bungarotoxin. This indicated that the global nicotine-induced inward current was not dependent on the calcium ions. Steady-state recordings indicated that the second component (between −30 mV and +20 mV) of the current-voltage curve seemed to be dependent on the potassium ions. As illustrated in Fig. 2A, a and b, the inward current was strongly reduced in the presence of intracellularly applied cesium chloride (10 mM) or bath application of 4-aminopyridine (5 mM). Interestingly, these potassium channel blockers did not affect the first component of the biphasic curve. These results, together with recordings indicating that an increase in input resistance (by 16 ± 5%; n = 3) was observed in this membrane potential range (not shown), led us to suggest that the second component could be caused by a diminution in potassium conductance. By contrast, the inward current underlying the first component appeared to be carried largely by sodium. As shown in Fig. 2Ac, when 100 mM NaCl in the saline (corresponding to half external sodium concentration) were replaced by 100 mM Tris-hydrochloride, the nicotine-induced current, recorded in the presence of 10 mM cesium chloride, was strongly decreased. This result gave direct evidence for a major contribution of sodium to the inward current activated by nicotine application between −90 mV and −30 mV.

To substantiate that the inward currents measured over the two different ranges of membrane potential reflected the activation of two distinct nAChRs, we next focused on the selective inhibitory effects of different nAChR antagonists,

![Fig. 2. Ionic permeability and pharmacological separation of the two components of the biphasic current-voltage curve of the nicotine-induced inward current. A, a and b, selective effects of the potassium channel blockers cesium chloride (10 mM; ●, Aa, n = 4) and 4-aminopyridine (4-AP; 5 mM; ●, Ab, n = 4) on the second component of the current-voltage relationship. Ac, lowering external sodium concentration from 200 mM (control conditions) to 100 mM in the presence of intracellular application of cesium chloride (10 mM, n = 5), strongly affected the first component of the biphasic current-voltage curve. B, a–c, effects of specific nAChR antagonists (●) on the nicotine-induced inward currents. Current-voltage relationships of the nicotine-induced inward current amplitudes are plotted against different holding potentials before (○) and after application of 10 μM d-tubocurarine (Ba; n = 6), 50 μM mecamylamine (Bb; n = 6), and 10 μM α-conotoxin ImI (Bc; n = 6). Note that d-tubocurarine affected only the first component, whereas mecamylamine and α-conotoxin ImI reduced the second one. Controls illustrated in B are the same as those shown in A. Data are means ± S.E.M..](https://molpharm.aspetjournals.org/doi/10.1124/mol.106.083271)
such as d-tubocurarine, mecamylamine, and α-conotoxin ImI. All experiments were performed in the presence of extracellular 0.5 μM α-bungarotoxin and 10 mM phosphocreatine added in the pipette solution to prevent rundown of the second component. Figure 2Ba shows that bath application of 10 μM d-tubocurarine for 35 min strongly reduced the amplitude of both inward current and first component of the biphasic curve (79.8 ± 6.7% measured at −70 mV; n = 6). By contrast, d-tubocurarine did not affect the second phase. This effect was compared with those obtained by the noncompetitive antagonist mecamylamine and α-conotoxin ImI, isolated from the venom of Conus imperialis (McIntosh et al., 1999) and known to block both α-bungarotoxin-sensitive and -insensitive nAChRs in both vertebrate (Broxton et al., 1999; McIntosh et al., 1999) and invertebrate preparations (Kehoe and McIntosh, 1998; van den Beukel et al., 1998). As can be seen in Fig. 2B, b and c, mecamylamine at 50 μM and α-conotoxin ImI at 10 μM induced opposite effects compared with d-tubocurarine. Both compounds, bath-applied for 10 min, selectively reduced the second phase of the current-voltage curve (50.3 ± 6.4% and 71.7 ± 9.1% measured at +20 mV; n = 6, for mecamylamine and α-conotoxin ImI, respectively). These antagonists did not reduce the first component (between −90 mV and −30 mV). It should be noted that higher concentrations of mecamylamine and α-conotoxin ImI (e.g., 100 μM) affected both components (not illustrated). Taken together, these results strongly suggest the existence of two distinct types of α-bungarotoxin-resistant nAChRs in DUM neuron cell body defined, for clarity, as nAChR1 and nAChR2. They differ from each other on the basis of their ionic permeability and sensitivity to different nAChR antagonists but also phosphocreatine.

Effects of AMP-PNP, cAMP and H89 on the Inward Current Mediated by nAChR1. As indicated above, phosphocreatine added in the internal pipette solution increased the amplitude of the first component mediated by nAChR1 (Fig. 1B). Consequently, this study was mainly focused on the intracellular regulation of this first component. The detailed study of the direct or indirect control of potassium channels together with the mechanisms underlying the regulation of nAChR2 will be the topic of a forthcoming article.

Because phosphocreatine is well known in the literature to represent a major source of ATP, this observation could be consistent, among other possibilities, with the fact that supply of ATP could be one of the key factors in modulating this nicotinic response. We examined the effect of the nonhydrolyzable ATP analog AMP-PNP on this first component, particularly on the nicotine-evoked inward current recorded at a holding potential of −50 mV [a value that corresponds to the resting membrane potential of TAG DUM neuron (Lapied et al., 1989)] using a phosphocreatine-free solution in the pipette. As illustrated in Fig. 3A, substitution of all internal ATP with equimolar concentration of AMP-PNP reduced the current amplitude from −0.46 ± 0.03 nA (n = 30) to −0.14 ± 0.03 nA (n = 10). This result suggested that a metabolite produced by ATP hydrolysis was essential in the regulation of the nicotinic response. Among metabolites of ATP, it is reasonable to hypothesize that cAMP which is known to modulate the nicotinic response through the activation of PKA (Margiotta et al., 1987; Swope et al., 1999) could be involved in the modulation of this nicotinic response. When intracellular cAMP (0.1 mM) was introduced into cell body by diffusion through the patch pipette, the peak inward current recorded at −50 mV was increased above standard level from −0.46 ± 0.03 nA (n = 30) to −0.92 ± 0.05 nA (0.1 mM cAMP added in the pipette solution; n = 15) (Fig. 3A). The time elapsed between establishing the whole-cell configuration and the recording was 5 min, suggesting a short latency for
the cAMP-dependent change from normal to high nicotinic response sensitivity. It is interesting to note that 1) similar enhancement of the nicotinic response was observed with 10 mM phosphocreatine when present in the pipette solution and 2) as with phosphocreatine, only the first component of the current-voltage curve was affected by cAMP (Figs. 1B and 2B). To test whether the increased nicotinic-induced current amplitude observed in the presence of cAMP (0.1 mM) was specific for a cAMP-dependent process, similar experiments were performed with cGMP (0.1 mM). No significant change ($p > 0.05$) was seen when the intracellular solution contained cGMP instead of cAMP (Fig. 3A).

In general, it is thought that the signaling pathway of cAMP is mediated through the activation of PKA. To study whether PKA was involved in the regulation of this nicotinic response, we applied H89, a well-known inhibitor of PKA, intracellularly. As illustrated in Fig. 3A, the stimulatory effect of cAMP was abolished by H89 (0.1 mM). Again, the first component of the current-voltage curve was reduced whereas H89 did not diminish the second phase (Fig. 3C). Thus, PKA seems to only exert its modulatory action on the first component.

**Effect of an Okadaic Acid-Sensitive Protein Phosphatase on the Inward Current Mediated by nAChR1.**

We further examined the effect of cAMP on the nicotine-induced inward current recorded at a holding potential of $-50$ mV. Interestingly, intracellular application of higher cAMP concentrations (i.e., 0.3 and 1 mM) did not enhance the current amplitude, as expected, but it reduced the nicotinic current in a dose-dependent manner (Fig. 4A). The amplitudes of the inward currents after a 5-min perfusion period in 0.1, 0.3, and 1 mM cAMP were $-0.92 \pm 0.05$ nA ($n = 15$), $-0.40 \pm 0.03$ nA ($n = 7$) and $-0.18 \pm 0.01$ nA ($n = 5$), respectively. Because we reported that cAMP increased the nicotinic response through PKA pathway, the reduction of the inward current observed with high cAMP levels could be due either to activation of phosphodiesterase (PDE), known to convert cAMP to 5'-nucleotide phosphate or a dephosphorylation process involving a protein phosphatase. IBMX, an inhibitor of PDE, was first tested on the nicotine-induced current using 0.1 mM cAMP in the pipette. Pressure application of nicotine was performed at two different times (10 and 20 min) during bath application of 10 $\mu$M IBMX. As shown in Fig. 4B, the inward current amplitude decreased in a time-dependent manner. These results indicated that the decrease in current amplitude observed with high concentration of cAMP was not related to the activation of PDE. IBMX (which blocked PDE) increased cAMP levels, which thereby decreased the inward current amplitude as observed when high cAMP concentrations were directly added in the pipette solution (Fig. 4A).

To check whether a protein phosphatase was involved in the reversal of the phosphorylated nAChR1, intracellular application of the potent protein phosphatase inhibitor okadaic acid, known to inhibit phosphatases PP1/2A (Herzig and Neumann, 2000) was tested on the inward current (holding potential $-50$ mV) using 0.1, 0.3, and 1 mM cAMP in the patch pipette. As can be seen in Fig. 4C, the dose-dependent reduction of the inward current observed with 0.3 and 1 mM cAMP was completely reversed by 1 $\mu$M okadaic acid. It should be noted that the slight, okadaic acid-induced increase in current amplitude observed with 0.1 mM cAMP was

**Fig. 4.** Effects of high internal cAMP concentration, IBMX and okadaic acid on the nicotinic response mediated by nAChR1. A, Histogram illustrating the dose-dependent decrease of the nicotine-induced inward current recorded at a holding potential of $-50$ mV, following pressure application of nicotine (10 mM). The different internal cAMP concentrations tested are indicated above each bar. B, Effect of bath application of 10 $\mu$M phosphodiesterase inhibitor IBMX versus time of application (indicated above each bar) on the inward current recorded at a holding potential of $-50$ mV ($n = 3$). Note that IBMX mimicked the effect of high cAMP concentration. C, Superimposed histogram illustrating the reversal effect of 1 $\mu$M phosphatase PP1/2A inhibitor okadaic acid (OA) on the cAMP-induced reduction of the inward current amplitude recorded at a holding potential of $-50$ mV. ** indicates that it is not significantly different ($p > 0.05$). Data are means $\pm$ S.E.M. (bars); values in parentheses indicate number of experiments in each condition.
not statistically significant \((p > 0.05)\), suggesting very low or no phosphatase activity under these experimental conditions. These results indicate that the dephosphorylation mechanism via an okadaic acid-sensitive phosphatase could have important functional consequences on such DUM neuron nAChR1, particularly when cAMP levels are increased.

**Effects of Forskolin and W7 on the Nicotinic Response Mediated by nAChR1.** The effects of regulating cAMP-dependent PKA phosphorylation were monitored by comparing the amplitude of nicotinic-inward current before (standard condition; i.e., 3 mM ATP in the pipette solution) and after external application of forskolin tested at 1, 10, and 100 \(\mu\)M. Figure 5A, a and b, shows typical inward current evoked by pressure application of nicotine at a holding potential of \(-50\) mV. Application of forskolin (1 \(\mu\)M), which directly activates adenylyl cyclase, produced an increase in current amplitude from \(-0.46 \pm 0.03\) nA \((n = 30)\) to \(-0.94 \pm 0.26\) nA \((n = 6); p < 0.05;\) Fig. 5, Aa and Ab. By contrast, as indicated in Fig. 5B, 10 \(\mu\)M forskolin did not influence the nicotinic response \((p > 0.05)\), whereas 100 \(\mu\)M forskolin strongly reduced the inward current amplitude from \(-0.46 \pm 0.03\) nA \((n = 30)\) to \(-0.20 \pm 0.04\) nA \((n = 6); p < 0.05;\) Fig. 5, Ab and Bb. These results confirmed that the amplitude of the nicotinic response was regulated through a dose-dependent forskolin-stimulated level of cAMP accumulation.

We have previously demonstrated that the level of cAMP in DUM neuron cell body was mainly governed by two different pathways, one through the activation of adenylyl cyclase (Fig. 5, A and B) and the other regulated by the degradative enzyme cAMP-phosphodiesterase (Fig. 4B). The following experiments revealed that a third mechanism could also strongly influence the nicotinic response via the level of cAMP. In insect, it is known that calcium acting through the calcium-receptor protein CaM could be an important signal that regulates diverse enzymatic activities such as adenylyl cyclase (Roeder, 1999). To test the possible regulatory role of the calcium/CaM complex in the adenylyl cyclase activity, we examined the effect of the calmodulin inhibitor W7 on the forskolin-induced increase in the current amplitude. As illustrated in Fig. 5B, 0.5 mM W7 applied intracellularly completely suppressed the effect of 1 \(\mu\)M forskolin on the inward current. This suggests that the calcium/CaM complex is a major factor accounting for the regulation of the cAMP level induced by stimulation of adenylyl cyclase.

**Effect of KN-62 on the Nicotinic Response Mediated by nAChR1.** It is well established that the protein phosphatase activities can be directly or indirectly influenced by intracellular calcium concentration (Herzig and Neumann, 2000). We next focused on the effect of caffeine, known to stimulate the release of calcium from internal stores, on the nicotine-evoked inward current. All the following experiments were carried out at a holding potential of \(-50\) mV with 0.3 mM cAMP added in the pipette solution to ensure that the okadaic acid-sensitive phosphatase was activated (Fig. 4, A and C). In these conditions, bath application of 5 mM caffeine increased the current amplitude from \(-0.40 \pm 0.03\) nA \((n = 7)\) to \(-0.92 \pm 0.14\) nA \((n = 4);\) Fig. 6A). The participation of cAMP-dependent PKA phosphorylation were monitored by comparing the amplitude of nicotine-evoked response. Aa-b, Typical example of nicotine-induced inward current recorded at a holding potential of \(-50\) mV in control condition and after bath application of 1 \(\mu\)M and 100 \(\mu\)M forskolin (a and b, respectively). Nicotine (Nic, 10 mM) was applied by pressure ejection. B, histogram summarizing the dose-dependent effect of forskolin on the nicotinic response measured at a holding potential of \(-50\) mV. The different concentrations of forskolin tested are indicated above each bar. Intracellular application of W7 (0.5 mM) inhibited the forskolin-induced increase in the current amplitude. For these experiments, pipette solution contained 3 mM ATP. *, significantly different; ** not significantly different. Data are means \pm S.E.M. (bars); values in parentheses indicate number of experiments in each condition.
of the CaM kinase II via the release of calcium from internal store. Moreover, it is interesting to point out that the percentage of the current amplitude reduction produced by KN-62 was more important when 0.3 mM cAMP was added in the pipette solution instead of 0.1 mM cAMP (69.6 ± 5.6%, n = 7 and 36.1 ± 5.7% n = 15 with 0.3 and 0.1 mM cAMP, respectively) (Fig. 6A and B). Although a direct effect of CaM kinase II on the nicotinic response underlying the first component of the current-voltage curve (Fig. 6C) was predicted (Fig. 6B), these results suggest that CaM kinase II could also exert its modulatory action of the nicotinic response via the modulation of the phosphatase activity.

To address this issue, an additional set of experiments was performed either in the presence of okadaic acid, which blocked phosphatase (Fig. 7Aa) or KN-62, which inhibited CaM kinase II (Fig. 7Ba). We first investigated the effect of KN-62 on the nicotinic-induced inward current while maintaining phosphatase inactivated by applying intracellularly 1 μM okadaic acid (Fig. 7Aa). In this condition, the percentage
of KN-62-induced reduction of the current amplitude in the presence of okadaic acid was smaller (41.4 ± 9.1%, n = 7; Fig. 7Aa) than that calculated without okadaic acid (69.6 ± 5.6%, n = 7; Fig. 7Ab). This result indicated that CaMK kinase II could affect directly and indirectly the nicotinic response. The second set of experiments was performed to examine the effect of okadaic acid while maintaining, this time, CaMK kinase II inactivated (Fig. 7Ba). Comparison of the average percentage changes in the current amplitude during drugs application (Fig. 7B, a and b) indicated that the phosphatase-induced modulatory effect of the nicotinic response was more important when CaM kinase II was inhibited. In other words, CaM kinase II exerts a modulatory effect on the nicotinic response via an inhibition of phosphatase.

**Phosphorylation/Dephosphorylation Process Influences the Effect of the Insecticide Imidacloprid.** Insect nAChRs are well known to be the target sites for insecticides such as the neonicotinoid imidacloprid. This insecticide compound is one of the most important neonicotinoid insecticide known to act as agonist at the α-bungarotoxin–resistant cockroach DUM neuron nAChRs (Buckingham et al., 1997). In this study, we tested whether the imidacloprid-induced current could be affected by intracellular phosphorylation/dephosphorylation process as was the case for the inward current evoked by nicotine. Under the same experimental conditions described above, pneumatic pressure ejection application (Fig. 7B, a and b) indicated that the phosphatase-induced modulatory effect of the nicotinic response was more important when CaM kinase II was inhibited. In other words, CaM kinase II exerts a modulatory effect on the nicotinic response via an inhibition of phosphatase.

**Discussion**

Although different native neuronal nAChR subtypes have previously been characterized in both vertebrates and invertebrates (Benson, 1992; Kehoe and McIntosh, 1998; van den Beukel et al., 1998; Paterson and Nordberg, 2000), the existence of two native neuronal α-bungarotoxin–resistant nAChRs (i.e., nAChR1 and nAChR2) expressed in the same neuron has never been electrophysiologically and pharmacologically reported. The inward currents mediated by these nAChRs differ from each other on the basis on their voltage dependence, ionic permeability, and selective sensitivity to different nAChR antagonists, such as d-tubocurarine (nAChR1), mecamylamine (nAChR2), and α-conotoxin ImI (nAChR2). This C. imperialis peptide, known to preferentially inhibit α7 homomeric complexes (McIntosh et al.,
In this case, a neuronal α-bungarotoxin-insensitive nAChR, as previously reported in both vertebrate and invertebrate preparations (Kehoe and McIntosh, 1998; Broston et al., 1999). It has been established that a strong relationship between pentameric functional motif of nAChRs and nAChR subtype selectivity to pharmacological ligands exists. Although our knowledge about the subunit composition of insect native neuronal nAChRs is still limited, previous molecular biological studies allowed to identify different cDNA clones encoding nAChR α and β subunits expressed in the insect central nervous system. In the insect aphid M. persicae, five nAChR α subunits (Mpa1–5) and one β subunit (Mpb1) were identified (Sgard et al., 1998; Huang et al., 2000). Expression of Mpa1 and Mpb2 cDNAs in Xenopus laevis oocytes indicated that they were distinguished by different pharmacological profiles, because AC and nicotine were found to be more potent agonists on Mpa1 than Mpb2. Furthermore, α-bungarotoxin, which antagonized the nicotinic induced current of oocytes injected with Mpb2. Coexpression of Mpa1–4 subunit cDNAs with the rat β2 or coexpression of Mpa1–2 subunit cDNAs with the Mpb1 subunit in D. melanogaster S2 cells indicated an important pharmacological diversity (Huang et al., 2000). In the locust (L. migratoria) central nervous system, at least four ligand-binding α subunits (Loca1–4) and one β subunit were identified (Hermesen et al., 1998). Three genes (loca1, loca2, and locb1) were expressed in similar areas of the head ganglia and retina of the locust. Finally, two different types of D. melanogaster nAChR α subunits (ALS and Do2) can also be coassembled within a single receptor complex (Gundelfinger and Schulz, 2000). Such assembly yielded an nAChR that displayed distinct pharmacological properties from other “classical” nAChRs. Taken together, these studies demonstrated that insect nAChRs exhibit complex subunit diversity like their vertebrate counterparts. This might explain the diversity of pharmacological properties related to the presence of several nAChR subtypes in the same neuron. According to these results, it is tempting to suggest that different nAChR subunits can be coassembled in DUM neuron cell body to form distinct α-bungarotoxin-resistant nAChRs that differ in their pharmacological profile. This hypothesis is further reinforced by the finding that nAChR1 is the only one nAChR modulated by intracellular messengers such as cAMP, PKA, okadaic acid-sensitive phosphatase, and CaM kinase II. To the best of our knowledge, the present investigation is the first attempt to investigate electrophysiologically the phosphorylation/dephosphorylation process involved in the regulation of insect α-bungarotoxin-resistant neuronal nAChR.

A Calcium/CaM-Sensitive Adenylyl Cyclase Modulates Action of Nicotine on Nachr1 via a cAMP/PKA Cascade. At relatively low internal concentration (0.1 mM), cAMP increases the nicotinic response. Because this effect is mimicked by 1 μM forskolin and blocked by H89, we assume that it occurs through cAMP/PKA cascade via the activation of adenylyl cyclase. Only few studies have shown that ionic currents flowing through vertebrate neuronal α-bungarotoxin–resistant nAChRs could be affected by an increase in internal cAMP concentration (Margiotta et al., 1987; Paterson and Nordberg, 2000). In insects, very recent works have suggested a modulation of nicotinic responses by the cAMP-dependent pathway. In D. melanogaster, the elevation of the cAMP levels modulates the frequency of miniature excitatory postsynaptic potentials at a cholinergic central synapse (Lee and O’Dowd, 2000). In cockroach P. americana motoneuron Df, the modulation of the ACh response observed with the biogenic amine octopamine, known to be coupled to adenylyl cyclase (Roeder, 1999), probably involved a cAMP-dependent signaling pathway (Pitman et al., 1999); in this case, however, the nAChRs involved are sensitive to α-bungarotoxin. The implication of cAMP has also been proposed to explain the octopamine-induced increases of the postsynaptic response amplitude in the cholinergic synapses of the honeybee mushroom bodies (Oleskevich, 1999). Using specific pharmacological tools, we have given experimental evidences for a modulation of insect neuronal nAChR by the cAMP/PKA cascade. Although the phosphorylation sites on the cockroach neuronal nAChRs have not been mapped, the implication of PKA in the regulation of insect neuronal nAChRs is consistent with previous findings that report the existence of a putative phosphorylation site for PKA in the Locb1 subunit of the locust nAChRs (Hermesen et al., 1998). We also report, that calcium/CaM exerts a stimulatory regulation of adenylyl cyclase. These results are consistent with a complex interplay between cAMP/PKA and calcium signaling pathways. It is known in both vertebrates and invertebrates that adenylyl cyclase can be either activated or inhibited by calcium and/or calcium/CaM (Roeder, 1999; Chern, 2000). In DUM neurons, the ability of adenylyl cyclase to be activated by two pathways, including 1) extracellular signals (e.g., locustatachytokinins, octopamine) via heterotrimeric G proteins (Roeder, 1999) and/or 2) calcium through calcium/CaM complex via, for instance, the activation of the calcium-permeable AChR exhibiting mixed nicotinic-muscarinic pharmacology (Grolleau et al., 1996), could provide a coordinated system to maintain the cAMP level necessary to activate PKA, which thereby enhances sensitivity of nAChR1.

Inhibitory Effect of an Okadaic Acid-Sensitive Protein Phosphatase. A decrease of the current amplitude is observed when intracellular concentration of cAMP is increased either directly (using high internal cAMP concentration) or indirectly by using high concentration of forskolin or IBMX, both of which mimic the effect of high cAMP concentration. This transient effect is probably caused by a strong activity of protein phosphatase, which can reverse the cAMP-mediated phosphorylation. This is supported by the reversed effect of okadaic acid observed only for high internal cAMP concentration. In this case, the reduction of the effect of PKA by such phosphatases provides a very important mechanism for switching off PKA-mediated phosphorylation of nAChR1 particularly when the cAMP level is too elevated. In other words, an important increase in intracellular cAMP concentration could be concomitant with an activation of okadaic acid-sensitive protein phosphatase, thus limiting the phosphorylation of nAChR1. This synchronous regulatory mechanism could make nAChR1 very sensitive to changes in cAMP level. Although the relationship between cAMP/PKA and activation of phosphatase is not directly assessed in our experiments, it is interesting to mention that previous findings have reported the existence of a strong relationship between cAMP-mediated phosphorylation and phosphatases PP1/2A activity (Smith et al., 1993). In most cases, the PKA regulatory subunit RII inhibits phosphatase activity, which thereby prolongs the effect of PKA. But recent experiments
have reported that PKA could activate the protein phosphatase 2A via the phosphorylation of its 74-kDa B’ subunit (Usui et al., 1998). Although further experiments are needed, our results suggest a possible concomitant activation of PKA and phosphatase when high cAMP level is reached. However, we cannot exclude a regulation of DUM neuron nAChR1 by an associated phosphatase-kinase signaling pathway, as previously reported for rat brain N-methyl-d-aspartate receptors (Westphal et al., 1999). In this case, a scaffold protein that physically attaches phosphatase (PP1) and PKA to N-methyl-d-aspartate receptors regulates channel activity.

**Direct and Indirect Modulation of DUM Neuron**

**nAChR1 by CaM Kinase II.** We also demonstrate that the calcium/CaM complex could activate CaM kinase II, which modulates the nicotinic response. nAChR1 seems to be directly and indirectly regulated by CaM kinase II. This notion is supported by at least three different findings. First, the effect of KN-62 observed in the presence of okadaic acid is consistent with that observed when experiments are performed with 0.1 mM cAMP in the patch pipette (i.e., when phosphatase is not activated; see Figs. 4C and 6B). This strongly suggests that CaM kinase II directly regulates nAChR1 function. Second, under experimental conditions in which phosphatase is active, the current response to nicotine observed in the presence of KN-62 is strongly decreased compared with that illustrated previously (Fig. 4A) where CaM kinase II is not inhibited. In other words, phosphatase activity is increased when CaM kinase is inhibited by KN-62. This notion is reinforced by the third set of experiments, in which the effect of okadaic acid is compared before and after treatment with KN-62 (see Fig. 7Ba, b). These results indicate that CaM kinase II can inhibit phosphatase, because the effect of okadaic acid on the nicotinic response (reflecting the phosphatase activity) is more important when CaM kinase II is inhibited by KN-62. Taken together, these results confirm 1) a direct interaction of CaM kinase II on nAChR1 and 2) the existence of inhibitory cross talk by CaM kinase on phosphatase activity. Such similar interplay has recently been characterized in hippocampal long-term potentiation (Fukunaga et al., 2000).

In conclusion, this study provides new insights into the complex regulation of phosphorylation/dephosphorylation mechanisms of insect neuronal α-bungarotoxin–resistant nAChRs. Both intracellular calcium and cAMP seem to play a key role in this regulation. According to the hypothetical scheme shown in Fig. 9, we propose that the nAChR1 or a closely associated regulatory molecule can exist either in a phosphorylated or a dephosphorylated form. Increased cAMP concentration via a calcium/CaM-sensitive adenylyl cyclase modulates the nicotinic response by activating PKA, which phosphorylates the molecule and maintains nAChR1 in a functional form. Phosphorylation is opposed by a dephosphorylation process, which renders the nAChR1 nonfunctional. For elevated internal cAMP concentration, the dephosphorylation is catalyzed by an okadaic acid-sensitive phosphatase regulated by a CaM kinase II also active on nAChR1. CaM kinase II is activated by intracellular calcium acting through the complex calcium/CaM. The resulting complex is also implicated in the regulation of adenylyl cyclase, which catalyze cAMP production.

Such complex regulation could have fundamental consequences, particularly in the mode of action of insecticides. Insect nAChRs are the targets of imidacloprid, a neonicotinoid insecticide with high insecticidal potency (Buckingham et al., 1997; Yamamoto and Casida, 1999). It was demonstrated that imidacloprid sensitivity of nAChRs was dependent on nAChR subunit composition and nAChR molecular structure (Landsdell and Millar, 2000b; Matsuda et al., 2000). However, the phosphorylation state of the receptor could also affect the mode of action of toxic compounds. Such a mechanism could explain the anthelmintics pyrantel resistance known to alter the nematode nAChR properties (Robertson et al., 2000). In our context, we have clearly demonstrated that phosphorylation, which plays an important role in modulating channel opening, could alter the insecticide efficiency. These preliminary results indicate that the binding of insecticide may be dependent on the phosphorylation mechanism, which thereby strongly modifies its expected toxicity. This opens an exciting research field to provide a better understanding of the action of this new class of highly effective insecticides.

**Acknowledgments**

The authors thank Dr. D. B. Sattelle (Oxford University, Oxford, UK) for the gift of imidacloprid.

**References**


Address correspondence to: Professor B. Lapied Laboratoire de Neurophysiologie UPRES EA 2647 (RCIM), Université d'Angers, UFR Sciences, 2 boulevard Lavoisier, F-49045 Angers cedex, France. E-mail: bruno.lapied@univ-angers.fr