Differential Regulation of Two Distinct Voltage-Dependent Sodium Currents by Group III Metabotropic Glutamate Receptor Activation in Insect Pacemaker Neurons

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Lavialle-Defaix, Céline, Hélène Gautier, Antoine Defaix, Bruno Lapied, and Françoise Grolleau. Differential regulation of two distinct voltage-dependent sodium currents by group III metabotropic glutamate receptor activation in insect pacemaker neurons. J Neurophysiol 96: 2437–2450, 2006. First published August 9, 2006; doi:10.1152/jn.00588.2006. Using whole cell patch-clamp technique and immunocytochemistry on adult dorsal unpaired median (DUM) neurons isolated from the cockroach Periplaneta americana CNS, we reported the characterization of a native mGluR, sharing pharmacological properties with vertebrate metabotropic glutamate receptor III (mGluRIII) that regulated voltage-dependent sodium current (INa). The global INa was dissociated by means of l-glutamate sensitivity, deactivation time constant, voltage dependence of activation and inactivation, recovery from inactivation, and intracellular regulation process. These two currents were respectively designated INaL and INaS for l-glutamate-sensitive and -insensitive sodium currents, INaL selectively reduced INaS by an increase of intracellular Ca2+ level. Using different activators and/or inhibitors of G proteins and cAMP/PKA cascade, together with St-Ht31 (an inhibitor of PKA binding to AKAP) and AKAP-79 antibodies, we established that mGluRIII was linked to INaS by a Gi/o and a suspected Gs protein. According to the activated signaling pathway, l-glutamate elevated the cAMP level, which thereby activated cytosolic PKA and released PKA bound to AKAP. As expected from both biophysical and pharmacological studies, we showed that, through an inhibition of INaL, l-glutamate increased DUM neuron spontaneous electrical activity. These results indicated that such mGluRIII-activated dual processes provided a new physiological control of pacemaker neuronal firing.

INTRODUCTION

Metabotropic glutamate receptors (mGluRs) form a family of eight structurally related receptors (mGluR1–8), which can be divided into three groups according to their sequence similarities, pharmacological properties, and signaling cascades (Coutinho and Knopfel 2002; Pin and Acher 2002; Schoepp et al. 1999). Group I is composed of mGluR1 and mGluR5 receptors that activate phospholipase C, whereas group II (mGluR2 and mGluR3) and group III (mGluR4, -6, -7, and -8) can inhibit adenyl cyclase activity by pertussis toxin-sensitive Gi/o proteins, which prevents formation of cyclic adenosine 3’,5’ monophosphate (cAMP) (for review see Moldrich and Beart 2003). Through this molecular diversity, glutamate displays widespread and diverse actions on neuronal excitability by modulating different types of ion channels. Calcium and potassium channels are end targets in a large number of regulatory pathways that are initiated by mGluR activation. Regulation of potassium channels by mGluRs is particularly prominent. mGluR-mediated suppression or enhancement of potassium currents (e.g., calcium-dependent potassium currents or the GIRK family of inwardly rectifying potassium current) has been extensively studied and reviewed (Anwyl 1999; Dutar et al. 2000; Gebremedhin et al. 2003).

Among voltage-dependent ionic channels, which generate inward current, N-type calcium channels have been found to be most commonly inhibited by mGluRs (Anwyl 1999). In addition, recent studies have also described a potent action of mGluR agonists on other voltage-gated calcium channels subtypes. These include low-voltage–activated calcium channels regulated by mGluR2 controlling activation of a protein tyrosine kinase in rat retinal ganglion cells (Robbins et al. 2003) and P/Q-type channels inhibited by mGluR7a through a PKC-dependent phosphorylation cascade in cultured cerebellar granule neurons (Perroy et al. 2000). Although these voltage-dependent ionic channels are known to have specialized functions in cellular excitability, only very few data are available about mGluR-induced regulation of the voltage-dependent sodium channel (Yang and Gereau 2004).

In this study, we have used a well-known insect neuronal model identified as dorsal unpaired median (DUM) neurons, known to express pacemaker activity involving a large number of identified voltage-dependent ionic channels that have specialized function in neuronal excitability (Grolleau and Lapied 2000; Wicher et al. 2001). Among neurotransmitters known to regulate DUM neurons pacemaker activity, it has been reported that glutamate may have important physiological roles. Previous findings have revealed that locust abdominal DUM neurons, for instance, receive significant glutamatergic input from descending intersegmental interneurons (Pflüger and Watson 1995). Furthermore, several arguments lead us to speculate that different groups of mGluRs are expressed in insect and involved in the modulation of electrical activity (Washio 2002).
Finally, the *Drosophila* genome database (Adams et al. 2000) contains at least six members of this G-protein–coupled receptor gene family. To date, a homologue of mammalian group II mGluRs, DmGluRA, has been cloned from the *Drosophila* head cDNA library (Parmentier et al. 1996). It has been shown that DmGluRA is distributed in both larval and adult *Drosophila* CNS (Parmentier et al. 1996; Ramaekers et al. 2001). Using an oocyte expression system, we established that DmGluRA is coupled, by a pertussis toxin–sensitive G protein, to an inwardly rectifying potassium channel (Raymond et al. 1999). In this study, we demonstrate for the first time, that a native neuronal mGluR, sharing pharmacological properties with vertebrate mGluRIII, differentially regulates voltage-dependent sodium currents. We also report that none of the classical mechanisms known to link mGluRIII to the effectors occur in pacemaker DUM neurons.

**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 cockroach (*Periplaneta americana*). Insects were obtained from our laboratory stock colonies maintained under standard conditions (29°C, photoperiod of 12-h light/12-h dark). Animals were pinned 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, carefully dissected under a binocular microscope, were placed in normal cockroach saline containing (in mM) 200 NaCl, 3.1 KCl, 5 CaCl₂, 4 MgCl₂, 50 sucrose, and 10 HEPES buffer; 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 previously described (Lapiéd et al. 1989). According to the cobalt-filling technique together with immunohistochemical mapping and electrophysiological recordings, it was assumed that most of TAG DUM neurons investigated formed a relatively homogeneous population of cells (Lapiéd et al. 1989; Sinakevitch et al. 1994). Ganglia were excised and incubated for 35 min at 29°C in cockroach saline supplemented with 1.5 mg/ml collagenase (type I, Worthington, Lakewood, NJ). After thoroughly washing off the enzyme, ganglia were mechanically dissociated by gentle repeated suctions through fire-polished Pasteur pipettes. Then, the isolated DUM neuron cell bodies were maintained at 29°C for 24 h before experiments were carried out. In this condition, DUM neuron cell bodies never displayed neurite outgrowth.

**Electrophysiological recordings**

Patch-clamp technique in the whole cell recording configuration (Hamill et al. 1981) was used to record voltage-gated sodium currents (voltage-clamp mode; Lapiéd et al. 1990) and spontaneous action potentials (current-clamp mode without current injection). Patch pipettes were pulled from borosilicate 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 (for sodium currents recording) or 1 to 1.5 MΩ (for action potentials recordings) 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 in isolation in the presence of both calcium and potassium channel blockers (see composition of solutions just below). They were recorded with an Axopatch 200A (Axon Instruments, Foster City, CA) amplifier, filtered at 5 kHz using a four-pole low-pass Bessel filter. Data were stored on-line on the hard disk of a PC computer (Elonex, Pentium 733 MHz, sampling frequency 33 kHz) connected to a 16-bit A/D converter (Digidata 1322A, Axon Instruments). The pClamp package (version 8.0.2, Axon Instruments) was used for data acquisition and analysis. 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 on-line P/6 protocol provided by pClamp. Series resistance value was obtained for each experiment from the patch-clamp amplifier settings after compensation and varied between 3 and 5 MΩ.

DUM neuron cell bodies were voltage-clamped at a steady-state holding potential of −90 mV (except when otherwise stated) and 30-ms test pulses were applied from the holding potential. For current-clamp experiments, spontaneous action potentials were displayed on a digital oscilloscope (310, Nicolet Instruments, Madison, WI) and stored on a DAT (DTR-1204, Biologic Science Instruments, Claix, France). Patch-clamp experiments were conducted at room temperature (20–22°C). Data, when quantified, were expressed as means ± SE. Differences between means were tested for statistical significance by Student’s *t*-test. For data analysis including fitting procedures, the software Prism 4 (GraphPad software, San Diego, CA) was used.

**Solutions**

For voltage-clamp experiments, the extracellular solution contained (in mM): 80 NaCl, 100 TEA-Cl, 3.1 KCl, 2 CaCl₂, 7 MgCl₂, 1 CaCl₂, 5 4-aminopyridine (4-AP), and 10 HEPES buffer; pH was adjusted to 7.4 with TEA-OH. Patch pipettes were filled with internal solution containing (in mM): 90 CsCl, 70 CsF, 15 NaCl, 1 MgCl₂, 5 EGTA, 3 ATP-Mg, and 10 HEPES buffer; pH was adjusted to 7.4 with CsOH. Drug solutions were prepared in the external solution and applied in the immediate vicinity of the cell by a gravity perfusion system. In some cases, the tested compounds were added in the internal pipette solution immediately before use. For current-clamp recordings, cells were bathed in external solution containing (in mM): 200 NaCl, 3.1 KCl, 4 MgCl₂, 5 CaCl₂, and 10 HEPES buffer; pH was adjusted to 7.4 with NaOH. The internal solution contained (in mM): 160 K-aspartate, 10 K-fluoride, 10 NaCl, 1 MgCl₂, 0.5 CaCl₂, 10 EGTA, 1 ATP-Mg, and 10 HEPES buffer; pH was adjusted to 7.4 with KOH. The metabolotropic glutamate receptor agonists L-(+)-2-amino-4 phosphonobutyric acid (L-AP₄), trans-azetidine-2,4-dicarboxylic acid (t-ADA), the mGluRs antagonists (R,S)-α-methylserine-O-phosphate (MSOP), (2S,3S,4S)-2-methyl-2-(carboxycyclopentyl)glycine (MCCG), (R,S)-1-amino-1,5-dicarboxylic acid (AIDA), the phosphodiesterase inhibitors, 2-(1-propyloxyphenyl)-8-azapurin-6-one (zapriniast) and 4-(3-(cyclopentylamino)-4-methoxyphenyl)pyrrolidin-2-one (rolipram) were purchased from Tocris Bioscience (Avonmouth, UK). The mGluR agonist (1S,2S,5R,6S)-(+)-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate (LY354740) was a gift from Lilly Research Laboratories (Indianapolis, IN). L-GLutamate, N-[2-[(p-bromocinnamyl-amino)ethyl]-5-isoxazolinesulfonamide (H89), 3-isobutyl-1-methylxanthine (BMBX), cyclic adenosine 3′,5′-monophosphate (cAMP), forskolin, and picrotoxin were purchased from Sigma Chemicals (L’isle d’Abeau Chesnes, France). The pertussis toxin A-protomer (PTX) came from VWR International (Fonteny sous Bois, France). St-H31 (AKAP Stearated-H31 inhibitor peptide) was purchased from Promega (Madison, WI). Zaprinast, rolipram, and IBMX were prepared in dimethyl sulfoxide (DMSO). Final dilution never contained more than 0.1% DMSO. This concentration of solvent was found to be without effect on electrophysiological properties of DUM neurons.
Immunocytochemistry

For light microscope immunocytochemistry, isolated DUM neurons were fixed for 1 h with 4% paraformaldehyde containing 5% (wt/vol) sucrose in phosphate-buffered saline (PBS, pH 7.4). After fixation, cells were washed three times for 5 min each in PBS and 5 min in PBS containing 0.2% Triton X-100 (PBS-T). To block nonspecific binding of the primary antibody, cell bodies were preincubated with 4% bovine serum albumin (BSA) in PBS-T for 1 h. Primary antisera (rabbit anti-cyclic-AMP polyclonal antibodies; Eurodemed, Mundolsheim, France) or goat anti-AKAP-79 polyclonal antibodies (Autogen-Bioclear, Calne, UK), diluted respectively 1/800 and 1/50 in PBS-T, were applied overnight at 4°C or 12 h at 20°C for the anti-AKAP-79 antibodies. The anti-AKAP-79 antibodies were raised against the N-terminal region of human AKAP-79. After repeated washing in PBS-T, the secondary antibody [fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Eurodemed)] or bovine anti-goat IgG (Autogen-Bioclear) diluted 1/300 and 1/30 in PBS-T containing 1% BSA were applied at 20°C for 3 h in the dark. Isolated DUM neuron cell bodies were then rinsed in 4% BSA in PBS and mounted on glass slides in glycerol/PBS. The fluorescence detection was captured using an AxioCam HRC camera mounted on a Zeiss Axioskop microscope. Images were digitized with Axiovision and stored as TIF format files for later analysis. The quantification of the change in immunofluorescence was performed using the software ImageJ 1.34s (Scion, Frederick, MD). The anti-AKAP-79 antibody binding was inhibited by the blocking-peptide (Autogen-Bioclear). The cell bodies were preincubated for 1 h with the blocking peptide (1/20) diluted in PBS containing 4% BSA. Then, the anti-AKAP-79 primary antibodies diluted to 1/100 were incubated with the blocking peptide (1/20) for 12 h at 20°C in PBS-T.

RESULTS

Voltage-dependent sodium current is modulated by activation of a group III metabotropic glutamate receptor

The effect of L-glutamate at a final concentration of 10 μM was examined on isolated adult DUM neuron cell body, in the voltage-clamp mode. Figure 1A (inset) shows a typical example of a transient inward current (I_{Na}) elicited by a voltage step from −90 to 0 mV before and after bath application of 10 μM L-glutamate. In all DUM neuron cell bodies tested, L-glutamate reduced the peak I_{Na} amplitude by 48.0 ± 3.9% (n = 24) (Fig. 1A) without modification of either the potential at which the current was maximum or the extrapolated reversal potential (Fig. 1B). For comparison, the global inward sodium current was completely blocked by 100 nM tetrodotoxin (TTX; Fig. 1C).

The smooth lines of the current–voltage relationship represented the best fit to the mean data (n = 6) according to the equation (Stühmer 1988)

\[ I = G(V - V_{rev}) - \frac{G}{1 + \exp(V - V_{m})} \]  

where I is the amplitude of the inward current; V is the membrane potential; \( V_{rev} \) = −27.2 and −32.6 mV and \( K_a \) = 5.3 and 7.6 are the activation parameters for control and L-glutamate, respectively; \( G = 203 \) and 128.7 nS, representing the conductance; and \( V_{m} \equiv +46.3 \) and +40.9 mV are the extrapolated reversal potentials of the current, for control and L-glutamate, respectively. These last two parameters were obtained from the linear regression (correlation coefficient \( r = 0.9999 \)) through the data points for potentials more positive than 0 mV. Control experiments were also performed to assess the degree of rundown of I_{Na}. When cells were repeatedly pulsed to 0 mV from a holding potential of −90 mV at a frequency of 1 pulse/min, no significant rundown was observed in our experimental condition for ≤25 min (7.1 ± 2.4%, n = 7, Fig. 1A).

FIG. 1. Modulation of dorsal unpaired median (DUM) neuron voltage-dependent inward sodium current by L-glutamate. A: time course of the normalized amplitude of peak sodium current in control (●, n = 7) and under 10 μM L-glutamate treatment (○, n = 24). Inset: representative example of whole cell inward sodium current traces obtained under control condition (●) and in the presence of L-glutamate (10 μM applied during 25 min, the protocol used is indicated above current traces) (○). B: current–voltage relationship constructed from values of maximum current amplitude plotted as a function of test potentials (holding potential −90 mV) under control condition (●, n = 6) and after bath application of L-glutamate (10 μM, ○, n = 6). Smooth curves represent the best fit according to Eq. 1. Data are means ± SE. Inset: family of sodium currents recorded at different test potentials ranging from −80 to +30 mV, from a holding potential of −90 mV. C: semi-logarithmic dose–response curve for the blockade of inward sodium current by tetrodotoxin (TTX). Percentage inhibition of the peak sodium current was plotted as a function of log [TTX]. Smooth line represents the best fit to the mean data according to Hill equation (n = 3 to 6 cells).
Because several groups of mGlurS might be expressed in DUM neuron cell body (Washio et al., 2002), the following experiments were performed to characterize the pharmacological profile of mGlur involved in $I_{Na}$ reduction. Figure 2A shows comparative semi-logarithmic curves of $I_{Na}$ reduction induced by t-glutamate and different mGlur agonists. The solid line fits the data according to the Hill equation (correlation coefficient $r = 0.999$), yielding a half-inhibiting t-glutamate concentration (EC$_{50}$) of 16 nM. The concentration–response curves for three other mGlur agonists (including t-ADA, LY354740, and L-AP4, which are selective ligands for groups I, II, and III, respectively) were also tested on $I_{Na}$ amplitude. As shown in Fig. 2A, only L-AP4 partially mimicked the inhibitory effect of t-glutamate on $I_{Na}$. However, the EC$_{50}$ value estimated for this agonist (760 nM) was about 50-fold higher than EC$_{50}$ calculated for t-glutamate. For comparison, LY354740 and t-ADA used at a final concentration of 10 $\mu$M had no or only a weak effect on $I_{Na}$ amplitude (4.7 ± 1.9%, $n = 5$ and 10.7 ± 3.0%, $n = 5$, respectively). These weak effects were not significantly different compared with control (7.1 ± 2.4%, $n = 7$, $P > 0.05$).

We also assessed the contribution of each mGlur group using specific antagonists. Concentration–response curves for three specific vertebrate mGlur antagonists are shown in Fig. 2B. Only the mGlurIII antagonist MSOP potently blocked, in a dose-dependent manner, the effect of t-glutamate. The IC$_{50}$ value for MSOP, estimated by the Hill equation, was 45 $\mu$M. Neither AIDA nor MCCG, mGlurRI and II antagonists, respectively, totally blocked the effect of t-glutamate when applied in the same concentration range (Fig. 2B). Together, these results demonstrated that $I_{Na}$ was modulated by mGlur sharing pharmacological properties with the vertebrate mGlurRIII.

From this pharmacological study, two important points emerged. First, t-glutamate–induced $I_{Na}$ reduction was always significantly higher than the effect produced by L-AP4 (Fig. 2, C and D). L-AP4 tested at 10 or 200 $\mu$M reduced $I_{Na}$ by 32.7 ± 5.6% ($n = 7$) and 29.8 ± 1.9% ($n = 14$), respectively. After 200 $\mu$M L-AP4 treatment, $I_{Na}$ amplitude was always further reduced by subsequent addition of 10 $\mu$M t-glutamate to 52.3 ± 5.5% ($n = 7$, Fig. 2D, $P < 0.05$). Second, the maximum inhibition obtained with t-glutamate was always <60%. This suggested, among other possibilities, that the total sodium current could be dissociated into two components, one sensitive and another insensitive to t-glutamate. This hypothesis was substantiated by means of tail current analysis reflecting the deactivation kinetics of channel closing on repolarization. Figure 3A shows typical control peak current and deactivation tail current traces evoked by a short depolarizing pulse (1.5 to 2 ms in duration to ensure fully activation). Under control conditions, tail current deactivation was best described by the sum of two exponentials giving the corresponding slow ($r_1$) and fast ($r_2$) time constants ($r_1 = 7.06 ± 1.12$ ms, $r_2 = 0.52 ± 0.04$ ms, respectively, $r = 0.960 ± 0.003, n = 29$; Fig. 3, A and B, Eq. 2).

$$III_{max} = A_1 \exp(-t/r_1) + A_2 \exp(-t/r_2)$$

in which $A_1$ and $A_2$ are the corresponding relative amplitude with time constants $r_1$ and $r_2$. For comparison, the use of one exponential function gave a weak correlation coefficient ($r$) of 0.875 ± 0.015 ($n = 15$, Student’s $t$-test, $P < 0.001$). These results suggested that more than one $I_{Na}$ was present. In 17 of 24 neurons, t-glutamate (10 $\mu$M) strongly reduced $I_{Na}$ to 3.35 ± 0.56 ms ($P < 0.05$) without any significant effect on $r_2$ (0.61 ± 0.07 ms, Fig. 3B). In the same way, the corresponding first component amplitude ($A_1$) was also reduced (62%). Although, this last effect might be related to a poorly clamped sodium current reflecting an inadequate voltage clamp, we present here a number of arguments against a possible voltage-clamp artifact. First, as indicated in the METHODS (also see Lapied et al., 1990), the adequacy of the voltage clamp was assessed by measuring the cell potential directly with an intracellular microelectrode. In none of the experiments did the cell potential differ from the patch pipette potential by >4 mV, even during the largest current flow. Second, as shown in Fig. 2.

**FIG. 2.** Group III metabotropic glutamate receptor (mGlur) modulates DUM neuron voltage-dependent sodium current. A and B: pharmacological characterization of mGlur-induced reduction of the whole cell inward sodium current. A: semi-logarithmic dose–response curves for the reduction of inward sodium current by t-glutamate and 3 vertebrate mGlur agonists L-AP4 (group III), t-ADA (group I), and LY354740 (group II). B: semi-logarithmic dose–response curves for the blockage of t-glutamate–induced sodium current reduction by 3 mGlur antagonists MSOP (group III), AIDA (group I), and MCCG (group II). Curves in A and B represent the best fit to the mean data points according to the Hill equation yielding the corresponding EC$_{50}$ (A) and IC$_{50}$ (B) indicated in the text. Values are means ± SE ($n = 5$ to 24 cells). C: graph illustrating the additional effect induced by t-glutamate (10 $\mu$M) on the inward sodium current recorded in the presence of L-AP4 (200 $\mu$M). Straight line represents the theoretical correlation ($r = 1$). D: histogram summarizing the significant effect of t-glutamate on the sodium current amplitude in the presence of L-AP4 (7, significantly different, $P < 0.05$). Each bar of histogram represents the means ± SE ($n = 7$ to 24 cells). In all experiments, the sodium currents were elicited with a 30-ms depolarizing pulse to 0 mV from a holding potential of −90 mV.
functions after application of L-glutamate (10 μM) yielded half-maximum tail current, and \( k \) is the slope factor. As illustrated in Fig. 4A, \( I_{Na2} \) was activated in a more negative potential range than \( I_{Na1} \). The current observed at \(-100 \) mV was 1.5 mV shifted to the left as \( V_{0.5} \) for \( I_{Na1} \) (estimated from \( V_{0.5} \)) and showed a slower voltage dependency because the slope factor \( k \) was about twice as low as the \( k \) value calculated for \( I_{Na1} \) (Fig. 4A, Table 1). The voltage dependence of steady-state inactivation (\( h^{\infty} \)) was also examined using a double-pulse protocol (inset, Fig.

1B, the inward current was seen to be progressively activated at all tested potentials, contrary to what was expected under very poor voltage-clamp conditions. In this case, a loss of voltage control was seen as an all-or-none inward current and an instantaneous increase of current to maximum values. Finally, parallel experiments were performed using 20 mM external sodium concentration to reduce the driving force. In these conditions we always obtained similar results as described above (data not illustrated). All together, these results indicated that L-glutamate preferentially blocked the slower component of the total \( I_{Na} \) and led us to conclude that the global \( I_{Na} \) can be further dissociated by means of glutamate sensitivity and deactivation time constants as \( I_{Na1} \) and \( I_{Na2} \) for L-glutamate–sensitive and –insensitive currents, respectively.

Interestingly, in seven to 24 neurons treated with L-glutamate, the deactivation tail current recorded at \(-100 \) mV was found to be best fitted with a one-exponential function (0.58 ± 0.07 ms, \( n = 7 \), Fig. 3B), which was very close to the value of \( \tau_2 \) determined in control. This indicated that in about 29% of neurons tested, \( I_{Na1} \) was completely blocked by L-glutamate, at this potential (Fig. 3C). In fact, the percentage of cells displaying single-exponential function of deactivation tail current versus neurons exhibiting two exponential deactivation tail current was dependent on the membrane potential at which the cell was stepped back after short depolarizing pulse. At \(-50 \) mV, which is the physiological DUM neuron membrane potential, 60% of neurons tested exhibited a deactivation tail current described by a single exponential (Fig. 3C). These results might indicate that L-glutamate was more potent in affecting \( I_{Na1} \) in a voltage-dependent manner.

\[ \frac{I(t)}{I_{Na\max}} = \frac{1}{1 + \exp[(V_{0.5} - V)/k]} \]

where \( I(t) \) is the tail current, \( I_{Na\max} \) is the maximum tail current amplitude (measured at 0 mV), \( V_{0.5} \) is the step potential yielding half-maximum tail current, and \( k \) is the slope factor. As illustrated in Fig. 4A, \( I_{Na2} \) was activated in a more negative potential range than \( I_{Na1} \). The current observed at \(-40 \) mV was almost purely \( I_{Na2} \). This was more obvious at \(-30 \) mV. Furthermore, the curve for \( I_{Na2} \) was 1.5 mV shifted to the left of \( I_{Na1} \) (estimated from \( V_{0.5} \)) and showed a slower voltage dependency because the slope factor \( k \) was about twice as low as the \( k \) value calculated for \( I_{Na1} \) (Fig. 4A, Table 1).

\( I_{Na1} \) and \( I_{Na2} \) are further separated by means of activation, inactivation, and recovery from inactivation studies.

To reinforce the idea that two separate voltage-dependent sodium channels were present in DUM neurons, we next focused on the study of activation and inactivation properties of \( I_{Na1} \) and \( I_{Na2} \). \( I_{Na1} \) can be studied in isolation by subtracting the residual current after L-glutamate (10 μM) from the control current. As a result of the findings presented above, only neurons displaying a single exponential tail current after L-glutamate treatment were considered in this biophysical analysis. The voltage range for activation of the two distinct \( I_{Na1} \) and \( I_{Na2} \) can be assayed from the amplitude of the tail currents after pulses of various amplitudes (−60 to 0 mV, in 10-mV increments), reflecting current flow through all open channels before any have been able to close. The resulting tail current amplitude curves should be proportional to the fraction of channels that are activated at a given potential. Figure 4A shows normalized plots of the two tail current amplitudes (determined by fitting exponentials) as a function of the voltage activation. To compare more quantitatively the voltage dependency of the two tail currents, the voltage relationships are fitted by a Boltzmann equation

\[ \frac{I(t)}{I_{Na\max}} = \frac{1}{1 + \exp[(V_{0.5} - V)/k]} \]

where \( I(t) \) is the tail current, \( I_{Na\max} \) is the maximum tail current amplitude (measured at 0 mV), \( V_{0.5} \) is the step potential yielding half-maximum tail current, and \( k \) is the slope factor. As illustrated in Fig. 4A, \( I_{Na2} \) was activated in a more negative potential range than \( I_{Na1} \). The current observed at \(-40 \) mV was almost purely \( I_{Na2} \). This was more obvious at \(-30 \) mV. Furthermore, the curve for \( I_{Na2} \) was 1.5 mV shifted to the left of \( I_{Na1} \) (estimated from \( V_{0.5} \)) and showed a slower voltage dependency because the slope factor \( k \) was about twice as low as the \( k \) value calculated for \( I_{Na1} \) (Fig. 4A, Table 1).
Smooth curves were fitted to the mean data points using Boltzmann values were obtained from tail currents recorded after 2-ms depolarizing membrane potential was stepped back to the holding potential (inset) whole cell inward sodium currents were plotted as a function of the L-glutamate-sensitive sodium currents were obtained by subtracting currents recorded after 10 ms then brought back to the recovery potential (Fig. 4B) with a slope factor k 7.65 and 7.08 per e-fold change for I_{Na2} and I_{Na1}, respectively (Fig. 4B). Finally, a two-pulse protocol (inset, Fig. 4C), used to determine the rate of recovery from inactivation of both I_{Na1} and I_{Na2}, consisted of two identical depolarizing pulses applied from a holding potential of −90 mV (or from the recovery potentials) to 0 mV and separated by a variable time (1–15 ms in 1-ms steps). The first depolarizing pulse (conditioning pulse) was long enough (10 ms in duration) to give >95% inactivation of the current. Between the two pulses, the membrane was repolarized to −55 mV (i.e., DUM neuron membrane potential). To determine the time constant for recovery from inactivation, the curves were fitted using a single- or double-exponential function (see Eq. 2). At −55 mV, the mean values (n = 9) were best fitted either by one exponential function for I_{Na2} or two exponential functions for I_{Na1} (Fig. 4C, Table 1).

### Does L-glutamate–induced inhibition of I_{Na1} involve the cyclic AMP pathway?

It is well known in the literature that among mGluRs, some of them (e.g., mGluRIII) suppress the activity of voltage-dependent ion channels by a mechanism involving a heterotrimeric pertussis toxin-sensitive guanine nucleotide-binding protein (G protein) of the Gα subtype negatively coupled to adenylyl cyclase, which thereby modulates the intracellular cAMP level. To examine the possibility that the reduction in I_{Na} amplitude (resulting in the suppression of I_{Na1}) involved an intracellular cAMP level, antibodies against cAMP (De Vente et al. 1993) were used. The control experiments in the presence of AIDA (500 μM) and MCCG (500 μM), two antagonists of mGluRI and mGluRII, respectively, did not occlude the elevation of cAMP level observed in the presence of L-glutamate (Fig. 5, A and B, n = 6). By contrast, pretreatment with MSOP (500 μM), a mGluRII antagonist, in the presence of AIDA and MCCG completely abolished the intensity of fluorescent cAMP immunostaining produced by L-glutamate (Fig. 5, C and D, n = 6). These results provided evidence that the effect of the mGluRIII activation on I_{Na1} involved an unexpected rise in internal cAMP level. To more deeply explore this hypothesis, DUM neurons were dialyzed using an internal solution containing different concentrations of cAMP, ranging from 0.1 to 2 mM (Fig. 6A). It should be mentioned that the cAMP concentrations in the pipette solution did not necessarily reflect the actual concentrations of the cyclic nucleotide inside the cell arising from access resistance to the cell and the presence of endogenous cyclic nucleotide phosphodiesterases. Moreover, the above experiments were performed in adenosine triphosphate (ATP)–free intracellular solution, to limit the extent of endogenous cAMP synthesis by the neuron, which might interfere with the effects of exogenous...
cAMP introduced through the patch pipette. As shown in Fig. 6A, a concentration-dependent decrease of the global $I_{Na}$ amplitude was observed. Maximal inhibition was obtained with 2 mM cAMP (40.6 ± 3.1%, $n = 7$), a percentage close to that obtained with L-glutamate (Fig. 2D). An alternative way of increasing internal cAMP level was to use inhibitors of phosphodiesterase (PDE). We therefore tested the effect of IBMX, a broad-spectrum PDE inhibitor applied alone. As expected, a reduction of the global $I_{Na}$ amplitude was observed during bath application of 10 μM IBMX. The average decrease was 30.5 ± 1.8% ($n = 4$, Fig. 6B). In an attempt to narrow the identity of PDE subtype, more specific pharmacological inhibitors were used. PDE that preferentially hydrolyzes cAMP was first considered by using rolipram (for review see Beavo 1995). Bath application of 100 μM rolipram caused a reduction of $I_{Na}$ of 27.1 ± 3.8% ($n = 7$, Fig. 6B). For comparison, we tested zaprinast, known to block PDE, that converts cGMP to 5’ GMP. At 20 μM, zaprinast did not cause a significant effect on $I_{Na}$ amplitude compared with control experiments (7.2 ± 1.2%, $n = 6$, $P > 0.05$, Fig. 6B). These results confirmed the key role played by intracellular cAMP in the modulation of $I_{Na}$.

It is worth mentioning that the effect of L-glutamate on $I_{Na}$ amplitude was completely inhibited when DUM neurons were dialyzed using high internal cAMP concentration (2 mM) compared with 0.3 mM cAMP (Fig. 6C). In addition, the effects of L-glutamate were partially or almost completely blocked by intracellular application of 0.1 or 0.3 mM H89, respectively, known to inhibit PKA (Engel et al. 1996) (Fig. 6D). These results indicated that $I_{Na}$ reduction observed with L-glutamate occurred by activation of PKA, after elevation of cAMP level.

**Pretreatment with the active A-protomer of pertussis toxin and forskolin affects the L-glutamate–induced $I_{Na}$ inhibition**

As indicated just above, mGluRIII might suppress the activity of voltage-dependent ion channels by a mechanism involving a Gi/o protein subtype. To examine this possibility, we used the active A-protomer of PTX in the pipette solution, which inactivates the Gi/o protein (Kaslow and Burns 1992). After establishing the whole cell configuration, a time-dependent decrease in total $I_{Na}$ amplitude was observed and usually stabilized within 15 min. The average percentage of $I_{Na}$ inhibition reached 32.0 ± 2.0% ($n = 12$, Fig. 7A) in the presence of PTX (10 nM). Interestingly, L-glutamate applied in the presence of PTX further decreased current amplitude (17.7 ± 1.0%, $n = 8$, Fig. 7A and B), indicating that besides this PTX-dependent mechanism, an additional pathway could be involved in the $I_{Na}$ reduction. Moreover, as shown in Fig. 7C, PTX did not produce any effect on $I_{Na}$ amplitude after DUM neuron pretreatment with 10 μM L-glutamate. These unexpected results seemed to indicate that the PTX-sensitive Gi/o proteins were already blocked by L-glutamate. Based on these findings, it was tempting to suggest that elevation in cAMP underlies the reduction of $I_{Na}$ amplitude. This was confirmed by the experiments shown in Fig. 7B. The percentage of $I_{Na}$ reduction was more important in DUM neurons dialyzed using internal solution containing 0.1 mM cAMP and PTX (30.8 ± 1.9%, $n = 6$) instead of 0.1 mM cAMP alone (12.05 ± 3.1%,

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**TABLE 1. Activation, inactivation, and recovery from inactivation parameters of $I_{Na1}$ and $I_{Na2}$ in DUM neurons**

<table>
<thead>
<tr>
<th></th>
<th>$I_{Na1}$</th>
<th>$I_{Na2}$</th>
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<tbody>
<tr>
<td></td>
<td>$V_{1/2}$</td>
<td>$k$</td>
</tr>
<tr>
<td>$I_{Na1}$</td>
<td>$-23.1 ± 0.1$</td>
<td>$2.4 ± 0.1$</td>
</tr>
<tr>
<td></td>
<td>($n = 9$)</td>
<td>($n = 9$)</td>
</tr>
<tr>
<td>$I_{Na2}$</td>
<td>$-24.6 ± 0.2$</td>
<td>$5.3 ± 0.2$</td>
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<tr>
<td></td>
<td>($n = 9$)</td>
<td>($n = 9$)</td>
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**FIG. 5.** Cyclic adenosine 3’5’ monophosphate (cAMP) immunoreactivity of isolated DUM neuron cell bodies. A: cells were pretreated with 500 μM AIDA and 500 μM MCCG, the mGluRI and mGluRII antagonists, respectively, for 20 min before (a) and after application of 10 μM of L-glutamate for 25 min (b). B: comparative quantification of the change in immunofluorescence measured in control (a, $n = 6$) and after application of 10 μM of L-glutamate (b, $n = 6$; * significantly different, $P < 0.05$). C: after pretreatment of DUM neuron cell bodies with 500 μM MSOP (c), the mGluRIII antagonist, cAMP immunostaining induced by L-glutamate was completely abolished (d). D: histogram illustrating the quantification of the fluorescence intensity measured in both experimental conditions (ns, not significant, $P > 0.05$). Green fluorescence was replaced by gray level. Scale bar: 50 μm.
n = 12, Fig. 7B). This indicated that the PTX-induced removal of adenylyl cyclase inhibition was sufficient to produce an increase in cAMP concentration.

Finally, it has also been demonstrated that some metabotropic receptors (i.e., adrenergic receptors) are coupled to both inhibitory G protein (Gi/o) and stimulatory G protein (Gs) to modulate adenylyl cyclase and thereby the internal cAMP level (Brink et al. 2000; Eason et al. 1992; Hermans 2003). The origin of the additional effect observed with l-glutamate in the presence of PTX was then examined by studying the effect of an activator of adenylyl cyclase, forskolin, known to mimic the activation of a Gs protein (Fig. 7D). Experiments were carried out in the presence of PTX to ensure that the Gi/o protein pathways did not interfere with each other. In this case and as shown in Fig. 7D, I_{Na} amplitude decreased during bath application of 10 μM forskolin (19.0 ± 5.6%, n = 4) but I_{Na} was not further reduced when l-glutamate (10 μM) was coapplied with forskolin. This was also confirmed more directly by studying the effect of forskolin alone and in combination with l-glutamate on cAMP immunostaining under the same experimental conditions as previously described (see Fig. 5). As we observed with l-glutamate (Figs. 5A and 7E), forskolin (10 μM) increased cAMP immunoreactivity in DUM neuron cell bodies in the same order of magnitude (Fig. 7, E and F). Furthermore, in the presence of 10 μM forskolin, l-glutamate did not produce any significant increase of the intensity of cAMP immunoreactivity (Fig. 7, E and F; P > 0.05).

**l-glutamate affects I_{Na} by an additional PKA-AKAP interaction-dependent pathway**

Although l-glutamate increased the intracellular cAMP level, our data demonstrated that the inhibitory effect of l-glutamate on I_{Na} was mediated through the activation of PKA by high cAMP levels. There is now an increasing body of evidence that indicates that cAMP signaling is compartmentalized in many if not all cells (for review see Taske and Aandahl 2004). Various enzymes such as PKA have been shown to be anchored to specific intracellular sites such as A-kinase anchoring proteins (AKAPs). Furthermore, in the presence of high internal cAMP level, AKAP is able to release PKA, which induces physiological functions. This indicates the fundamental role of intracellular cAMP gradients in this highly organized signaling pathway. These findings also explained why, in our case, a relatively high concentration of H89 was needed to ensure full inhibition of PKA (i.e., classical cytosolic PKA and PKA released from AKAP; see Fig. 6D).

Two different sets of experiments were therefore designed to explore the hypothesis that the effect of l-glutamate also involved a PKA anchored to an AKAP. The importance of PKA-mediated phosphorylation in the modulation of I_{Na} was determined from experimental inhibition of the anchoring of PKA by St-Ht31, a cell-permeable stearated Ht31 counterpart (Vijayaraghavan et al. 1997), known to release PKA from the AKAP (Carr et al. 1991). When the St-HT31 was applied (Vijayaraghavan et al. 1997), known to release PKA from the AKAP, the sodium currents were elicited with a 30-ms depolarizing pulse to 0 mV applied from a holding potential of −90 mV.

Figure 6: Involvement of cAMP/protein kinase A (PKA) pathway in the mGluR3-induced I_{Na} modulation. A: histogram illustrating the dose-dependent decrease of the whole cell inward sodium current recorded in the presence of different cAMP concentrations imposed in the pipette solution. Concentrations are indicated above each bar. Insert: examples of sodium current traces recording in control (τ = 0 min after whole cell established) and after 15 min of cAMP perfusion (0.1 or 0.3 mM). Each bar histogram represents the mean values ± SE (n = 5 to 19 cells) (*, significantly different, P < 0.05 and ns, not significant). B: effects of bath application of phosphodiesterase inhibitors, 3-isobutyl-1-methylxanthine (IBMX, 10 μM), rolipram (100 μM), and zaprinast (20 μM) on the whole cell global I_{Na}. C: typical examples of sodium current traces recorded in the presence of 2 concentrations of cAMP (0.3 or 2 mM, n = 4) before and after application of 10 μM l-glutamate (L-glu). D: comparative histogram summarizing the effects of bath application of l-glutamate (10 μM) on sodium current amplitude in control and after intracellular application of the PKA inhibitor N-[2-(p-bromocinnamyl- amino)ethyl-5-isooquinolinesulfonamide (H89). At 0.1 and 0.3 mM, H89 partially blocked or abolished l-glutamate-induced I_{Na} reduction, respectively. Experiments were performed with an adenosine triphosphate (ATP)-free intracellular solution containing 0.1 mM cAMP. Each bar histogram represents the mean values ± SE (n = 5) (*, significantly different, P < 0.05). For experiments illustrated in A–D, the sodium currents were elicited with a 30-ms depolarizing pulse to 0 mV applied from a holding potential of −90 mV.
antibodies showed fluorescence represented by small points preferentially localized at the apical pole of the cell near the initial segment (Fig. 8Ca). Negative control experiments were performed in parallel using the specific blocking peptide. In this case, we never observed AKAP79 immunoreactivity (Fig. 8Cb).

**Regulation of $I_{Na1}$ and spiking**

The physiological significance of the inhibition of $I_{Na1}$ on pacemaker activity was examined by measuring both action potential amplitude and spontaneous firing frequency before and after L-glutamate or L-AP4 application (Fig. 9). L-glutamate was applied on DUM neurons pretreated with picrotoxin (1 μM), AIDA (500 μM), and MCCG (500 μM) to avoid possible interference with another glutamatergic receptor (i.e., ionotropic receptors, group I and group II metabotropic receptors, respectively) expressed in DUM neurons. As shown in Fig. 9, L-glutamate (10 μM) significantly reduced spike amplitude from 139.5 ± 2.1 mV (control, Fig. 9A) to 123.8 ± 4.4 mV (n = 6, p < 0.05, Fig. 9B), whereas L-AP4 (100 μM) produced only a very slight effect on action potential amplitude compared with the control (from 138.3 ± 3.9 to 132.1 ± 9.4 mV, n = 5, Fig. 9, D and E) that was not significant. This spike amplitude reduction was always associated with an increase in action potential discharge frequency (74.2 ± 20.8%, n = 6 and 71.8 ± 12.1% for L-glutamate and L-AP4, respectively; Fig. 9, B and E). Finally, pretreatment of DUM neuron cell bodies by 500 μM MSOP completely suppressed the L-glutamate effects observed on pacemaker activity (Fig. 9, C and F).

**DISCUSSION**

In the present study we reported that group III metabotropic glutamate receptor activation allowed us to identify two distinct voltage-dependent sodium currents in pacemaker DUM neurons. They differed from each other on the basis of their sensitivity to L-glutamate, electrophysiological properties, and intracellular signaling pathways involving cAMP, both cytosolic and anchored PKA, by an unexpected inhibition of a Gi/o protein and activation of a suspected Gs protein.

**Identification of $I_{Na1}$ and $I_{Na2}$ in pacemaker DUM neurons**

Our findings demonstrated, in the same pacemaker neurosecretory neuron, the coexistence of two distinct voltage-dependent inward sodium currents defined as $I_{Na1}$ and $I_{Na2}$, which had a specialized function in the pacemaker activity. Very
recently, previous findings obtained from neurons acutely disso-
ciated from thoracic ganglia of Periplaneta americana re-
ported the characterization of two types of TTX-sensitive
sodium currents, designated type I and type II, with different
pharmacological and biophysical properties (Zhao et al. 2005).
However, a direct comparison of our results with those de-
scribed in this study is not easy because, in this last case, they
were obtained from a nonidentified heterogeneous population
of neurons. Interestingly, although saxitoxin and tetrodotoxin
were known to block the global inward sodium current in
DUM neurons (Lapied et al. 1990, 2001), L-glutamate sup-
pressed only a fraction of the global sodium current. Using
specific mGluR agonists and antagonists, it was possible to
establish that the pharmacological profile of the mGluR in-
volved in the reduction of the voltage-dependent inward so-
dium current was related to group III mGluR. Apart from only
one investigation reporting that a group II mGluR activation
negatively regulated a TTX-resistant sodium current in mouse
dorsal root ganglion neurons (Yang and Gereau 2004), our
study was the first example that demonstrated the involve-
ment of a group III mGluR in the regulation of the neuronal sodium
current. In our case, L-glutamate, by mGluRIII activation,
discriminated these two currents as $I_{Na1}$ for L-glutamate-sensitive
current and $I_{Na2}$ for L-glutamate-insensitive current. To ex-
amine their specific biophysical properties, a subtraction
procedure was used. L-glutamate-sensitive current was studied
by subtracting inward current recorded in the presence of
L-glutamate from inward currents recorded in the absence of
L-glutamate. $I_{Na1}$ and $I_{Na2}$ differed on the basis of their voltage
dependence of activation and inactivation, time constant of
deactivation, and recovery from inactivation. $I_{Na1}$ activated in
the more positive potential range than $I_{Na2}$ and exhibited a
steep,er voltage dependency. In addition, its voltage-dependent
inactivation was about 20 mV shifted toward more positive
potential. This gave rise to a larger “mh” overlap than for $I_{Na1}$.
Finally, study of the recovery from inactivation showed that the recovery process of $I_{Na1}$ was lower than that of $I_{Na2}$ because the recovery process for $I_{Na1}$ occurred in two phases, a fast one and a slow one. Taken together these specific biophysical properties of $I_{Na1}$ and $I_{Na2}$ should influence the DUM neuron pacemaker activity as discussed in the following text in the *Physiological significance* section below. In accordance with these results, recent studies demonstrated that alternative splicing and RNA editing of German cockroach sodium channel gene exhibited a broad range of functional and pharmacological properties (Liu et al. 2004; Song et al. 2004; Tan et al. 2002). Moreover, functional expression of two splicing variants paraCSMA (BgNav) sodium channels in Xenopus oocytes revealed distinct electrophysiological properties regarding their voltage dependency of activation and inactivation as well as their recovery from inactivation (Tan et al. 2002). Consequently, it is tempting to suggest that the two sodium channel populations identified in DUM neurons could result from molecular diversity and generate two splicing and/or RNA-editing variants of a sodium channel gene in cockroach *Periplaneta americana*.

**Involvement of PTX-sensitive Gi/o protein and cAMP/PKA pathway in the mGluRIII-induced $I_{Na1}$ inhibition**

The mechanism by which mGluRIII inhibited $I_{Na1}$ in DUM neurons was complex. It is well assumed in the literature that mGluRIIIs are negatively coupled to adenylyl cyclase by the activation of a PTX-sensitive Gi/o protein. This normally mediates an inhibitory effect on cAMP accumulation. We showed here, using electrophysiological and immunocytochemistry studies, that L-glutamate inhibited $I_{Na1}$ by an increase in cAMP level. This blockade did not really match with the classical mGluRIII model because, in DUM neurons, mGluRIII activation involved the inhibition of Gi/o proteins. This inhibition of Gi/o proteins by PTX applied alone mimicked the reduction of $I_{Na1}$ observed in experiments in which DUM neuron cell body was dialyzed with high cAMP concentration added in the patch pipette solution. This indicated that 1) the PTX-sensitive Gi/o protein was tonically active in DUM neurons in the absence of agonist and 2) basal activation of this Gi/o protein was necessary to maintain a low internal cAMP level for optimizing pacemaker activity by full activation of voltage-dependent sodium currents. These results were mimicked with DUM neurons pretreated with l-glutamate. In these conditions, PTX failed to produce any effect on $I_{Na1}$, suggesting that Gi/o proteins were already inhibited by l-glutamate. As a finding of these results, the molecular events involved in the regulation of $I_{Na1}$ might be summarized in Fig. 10. Inhibition of PTX-sensitive Gi/o proteins by mGluRIII activation led to inhibition of $I_{Na1}$ through cytosolic PKA stimulation after the increase of cAMP level (Fig. 10). In vertebrates the phosphorylation of sodium channels by PKA leading to a reduction of inward sodium current was previously described (Catterall 2000; Li et al. 1992; Smith and Goldin 1997). Moreover, neurotransmitters such as dopamine also modulated voltage-gated sodium channels by cAMP/PKA pathway in neostriatal and hippocampal neurons (Cantrell et al. 1997, 1999a; Schifflmann et al. 1995).

In insects, different PKA phosphorylation sites were identified in para sodium channel α subunit from *Drosophila melanogaster* (Loughney et al. 1989), Vssc1, and BSC1 from *Musca domestica* and *Blattella germanica* respectively (Ingle et al. 1996; Liu et al. 2001). Although direct evidence for the implication of G protein was not shown, Wicher (2001) demonstrated, in *Periplaneta americana* DUM neurons, a dose-dependent reduction of sodium current amplitude through the cAMP/PKA pathway, by neurohormone D. In our case we reported that PKA stimulation-induced reduction of $I_{Na1}$ might occur through elevation of cAMP level by both inhibition of Gi/o and activation of suspected Gs proteins. This last assumption was based on findings reporting that L-glutamate further reduced $I_{Na1}$ amplitude in DUM neurons pretreated with PTX. Using forskolin, which activates adenylyl cyclase and mimics Gs protein activation, we also established that, in cells pretreated with PTX, forskolin-stimulated cAMP formation decreased $I_{Na1}$ amplitude in an order of magnitude similar to that observed with L-glutamate under the same experimental conditions. These results seemed to be indicative of a mGluRIII also coupled to another G protein such as Gs protein involved in the stimulation of the cAMP/PKA pathway. In the literature, the coupling between a G-protein–coupled receptor (i.e., adrenergic, muscarinic, glutamatergic, and serotoninergic receptors) and two G proteins are known (Cussac et al. 2002; Dittman et al. 1994; Eason et al. 1992; Selkirk et al. 2001). However, in these studies, the effect produced by L-glutamate, by elevation of cAMP level after both inhibition of Gi/o and activation of Gs proteins, has not been previously reported.

According to these results mGluRIII activated by L-glutamate regulated $I_{Na1}$ through a parallel pathway that could involve different types of PKA. Besides classical cytosolic PKA, subcellular localization of PKA mainly arises from anchoring of the PKA regulatory subunits to AKAP. This complex is known to produce optimal regulation of ion channels (Tasken and Aandahl 2004). For instance, AKAP-15 is involved in targeting PKA to rat brain sodium channels (Tibbs et al. 1998) and it is also required for dopaminergic modulation of sodium currents in hippocampal neurons (Cantrell et al. 1999, 2002). In addition, the effectors regulated by PKA through AKAP interactions are very sensitive to changes in

![FIG. 10. Proposed model for the inhibition of Na1 channels by mGluRIII activation. This scheme summarizes the essential components of the intracellular signaling pathways that may regulate Na1 channels by the effects of L-glutamate on mGluRIII (see text for details). Na2, glutamate-insensitive sodium channels; PKAa, PKA controlled through association with AKAP; PKAc, cytosolic PKA; mGluRIII, metabotropic glutamate receptor III; AC, adenylyl cyclase; Gs and Gi/o, stimulatory and inhibitory G proteins.](image-url)
cAMP concentration. In our case, a high elevation in cAMP level seemed to play a key role in the release of PKA from the AKAP, which thereby potentiated the l-glutamate–induced inhibition of $I_{\text{Na}1}$. We established here that St-H31, known to release PKA from its site of localization, decreased $I_{\text{Na}}$ amplitude when applied alone. In addition, St-H31 completely occluded the effect of l-glutamate in the presence of 0.1 mM H89, which inhibited cytosolic PKA at this concentration (Courjaret and Lapied 2001). According to the hypothetical scheme shown in Fig. 10, we thus proposed an additional parallel model in which l-glutamate–induced stimulation of adenyl cyclase, by suspected Gs proteins, led to a further increase in cAMP level. This in turn converted inactive forms of PKA (i.e., PKA–AKAP complex) into active forms (PKA released from an AKAP). Then PKA directly and/or indirectly inhibited $I_{\text{Na}1}$. Together, these results indicated that such a dual process involving cytosolic and anchored PKA would provide an original physiological control of neuronal firing by a single neurotransmitter (l-glutamate) acting on mGluRIII coupled to two different G proteins (Fig. 10).

Physiological significance

Although previous findings reported that the DUM neuron action potential depolarizing phase was mediated by the activation of voltage-dependent sodium channels (Grolleau and Lapied 2000; Lapied et al. 1990; Wicher et al. 2001), this refined electrophysiological study demonstrated that two distinct inward sodium currents, rather than one, were involved in the pacemaker activity. The specific biophysical and pharmacological properties of $I_{\text{Na}1}$ and $I_{\text{Na}2}$ were expected to have important consequences for cellular excitability. For instance, the DUM neurons expressing only the Na2 channel would be expected to respond to slow depolarizing inputs that Na1 channel DUM neurons could not respond to. In addition, a study of the voltage dependency of both activation and inactivation showed an enlarged steady-state window current for $I_{\text{Na}1}$. In this case, $I_{\text{Na}1}$ can give rise to a larger “mh” overlap over a potential range more positive compared with $I_{\text{Na}2}$ “mh” overlap. Then, we expected that an inhibition of $I_{\text{Na}1}$ would result in hyperexcitability. This was reinforced by the study of the recovery from inactivation measured at a recovery voltage of −55 mV, corresponding to the DUM neuron physiological membrane potential. The recovery from inactivation played a major role in the repriming of sodium currents because the firing rate was limited by the repriming rate. Thus because the Na1 channels reprimed more slowly than did Na2 channels, l-glutamate–treated DUM neurons expressing only a pure population of Na2 channels should be able to sustain higher firing rates than neurons expressing Na1 channels. According to these findings, activation of mGluRIII was expected to influence pacemaker activity.

L-Glutamate reduced action potential amplitude but also increased spontaneous firing. Under these conditions, the variation of the global sodium current produced changes in the pacemaker activity. It is interesting to note that previous observations reported that modification of the sodium current inactivation property was always accompanied by a reduction of sodium current (Wicher 2001). In addition, it was also demonstrated, using a model reproducing the modulation of the sodium current, that changing inactivation properties of the sodium current associated with such reduction of sodium conductance caused a steep increase in firing frequency (Wicher et al. 2006). In light of these results, the physiological consequences of 1) the reduction of the peak inward current observed with l-glutamate and 2) the modification of the inactivation property (i.e., inactivation time constants for $I_{\text{Na}1}$ and $I_{\text{Na}2}$ were $\tau = 8.5$ ms, $\tau'_f = 0.72$ ms and $\tau = 6.8$ ms, $\tau'_f = 0.81$ ms, respectively) on the firing frequency, matched previous findings (Wicher et al. 2006) and confirmed the simulated situation describing an increase of spike frequency under such conditions. These results, together with previous findings on the specific physiological targets innervated by TAG DUM neurons in Periplaneta americana (e.g., accessory glands, heart wall muscles) (Sinaevitch et al. 1994, 1996), indicate that the regulation of voltage-dependent sodium channels involved in the pacemaker activity may play crucial roles in the adaptation of DUM neurons to different behavioral situations, which thereby will influence vital physiological functions.

Finally, we also suggested, among other possibilities, that l-glutamate, by mGluRIII activation, could inhibit $I_{\text{Na}1}$ in a voltage-dependent manner. Although further experiments are needed to confirm these results, it was previously demonstrated that M2 muscarinic receptor could also be voltage sensitive (Ben Chaim et al. 2003). In this case, the apparent affinity of M2 muscarinic receptors toward acetylcholine was reduced on depolarization. This voltage sensitivity could reside in the receptor region that coupled to the G protein rather than in the acetylcholine binding site. Moreover, Cantrell et al. (1999a) demonstrated that dopaminergic receptors (D1) regulated sodium current by PKA in a voltage-dependent manner, in which membrane depolarization facilitated inhibition of sodium current by dopamine agonist. We report here that l-glutamate was more potent in inhibiting $I_{\text{Na}1}$ in a positive potential range (i.e., −50 mV), suggesting that the apparent affinity of DUM neuron mGluRIII toward l-glutamate was enhanced in the physiological membrane potential range (i.e., −55 to −45 mV). This exciting feature, never reported until now, may provide a new mechanism for modulation of the signal transduction process in excitable cells.

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