Calcium-dependent inward current induced by nicotinic acetylcholine receptors in mesencephalic dopamine neurons

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CONTENTS

1. Summary

Mesencephalic dopamine (DA) neurons receive a keen interest of many researchers because of their physiological and patho-physiological importance. Especially, the mechanism to modulate their firing pattern has been investigated for many years but still remains elusive. DA neurons are supposed to be modulated by excitatory and inhibitory inputs from other parts of the brain. It has been shown that N-metyl-D-aspartate receptors (NMDARs) play an important role in switching the firing pattern from tonic to burst firing mode, but it has been also claimed that the activation of NMDARs dose not explain the whole mechanism of the induction of bursting activity. On the other hand, cholinergic afferent projection from the pedunculopontine nucleus has recently been highlighted because both nicotinic and muscarinic acetylcholine receptors were reported to function in DA neurons. In this article, we will show that Ca^{2+} influx through nicotinic acetylcholine receptors in DA neurons subsequently activate inward current which is sensitive to fulfenamic acid (FFA) and phenytoin and this current is presumed to be a subtype of calcium-activated nonselective cation current, I_{CAN}. This channel exhibits a negative slope conductance and induces a dramatic

change in firing pattern of DA neurons from tonic single spikes to a multiple bursting spikes by markedly enhancing their depolarizing responses. Furthermore, the activation of this channel depends not only on intracellular Ca^{2+} but also on the calmodulin-Ca^{2+}/calmodulin dependent protein kinase II pathway. In addition, we found that the activation of the FFA- and phenytoin-sensitive current plays a major role in enhancement of the synaptic response to glutamatergic inputs when they are coincident with the cholinergic inputs. Taken together, our results strongly suggest that cholinergic inputs to DA neurons modulate the firing pattern of DA neurons by the activation of I_{CAN} induced by Ca^{2+} influx through the nicotinic receptors.

2. General introduction

We have general interest in cholinergic input to mesencephalic dopamine (DA) neurons because of the main two reasons. DA neurons have been extensively studied in relation to control of muscle tone and pathogenesis of Parkinson's disease (Olanow and Tatton, 1999) and more recently highlighted by its role in reward-based reinforcement learning process (Schultz, 1998) and addiction (Dani and Heinemann, 1996; Nicola et al., 2000). Such physiological and patho-physiological importance has attracted a keen interest of many researchers. Especially, the neuronal mechanism of dramatic change of their firing pattern from tonic single spikes to multiple bursting spikes has been intensively investigated but still remains elusive (White, 1996; Overton and Clark, 1997; Kitai et al., 1999).

On the other hand, it is generally accepted that acetylcholine (ACh) in central nervous system (CNS) are involved in cognitive functions, such as memory (Huerta and Lisman, 1993; Winkler et al., 1995), attention (Risbrough et al., 2002). Recently, the role of nicotinic acetylcholine receptors (nAChRs) has been highlighted partly because they exhibit high Ca^{2+} permeability (Rathouz et al., 1996; Albuquerque et al., 1997).

Needless to say, Ca^{2+} is a very important second messenger in neurons (Ghosh and Greenberg, 1995; Berridge, 1998). Accordingly it is highly likely that Ca^{2+} influx through nAChRs would be related to many types of Ca^{2+} signaling and induce various effects in the cell. Thus we paid attention to Ca^{2+} influx through the nAChRs.

Considering the fact that mesencephalic DA neurons receive strong cholinergic afferent projections from the pedunculopontine nucleus (Inglis and Winn, 1995; Takakusaki et al., 1996), we thought nAChRs on DA neurons might have a very important role in modulating the firing pattern of DA neurons. However, in DA neurons the Ca^{2+} influx through postsynaptic nAChRs and its downward cascade have not fully been studied. Thus we decided to address this problem at the beginning of this study.

We have disclosed generally three important points in this study. First, we have reported that Ca^{2+} influx from nAChRs subsequently activate fulfenamic acid (FFA)- and phenytoin-sensitive Ca^{2+} dependent current, presumably calcium activated nonselective cation current, I_{CAN} (Colquhoun et al., 1981; Maruyama and Peterson, 1982; Yellen, 1982; Siemen, 1993; Kuriyama et al., 1998). This current has negative

slope conductance from -80 to -40 mV (Fraser and MacVicar, 1996; Haj-Dahmane and Andrade, 1996, 1998, 1999), which is quite different from that of nAChRs (Yawo, 1989; Mathie et al., 1990) (part 1). Next, this FFA- and phenytoin-sensitive current is dependent not only on Ca^{2+} but also Ca^{2+}/calmodulin (Ca^{2+}/CaM)-calmodulin dependent protein kinase (CaMKII pathway) (part 2). Third, coactivation of cholinergic and glutamatergic receptors in DA neurons induce the dramatic change from tonic single spikes to multiple bursting spikes by the activation of FFA- and phenytoin-sensitive current (part 1 and part 2). In addition, to confirm these results, we examined whether this FFA- and phenytoin-sensitive current caused by cholinergic inputs actually enhance the glutamatergic excitatory postsynaptic potentials (EPSPs). As expected, the depolarizing phase of EPSPs was enhanced by FFA- and phenytoin-sensitive current (part 3).

Taken together, our results have suggested that cholinergic input to DA neurons is one of the main factors in modulating the firing patterns. And in DA neurons Ca^{2+} increase through nAChRa would subsequently activate FFA- and phenytoin-sensitive current, which might contribute to the burst firing of DA neurons by its negative slope

conductance.

3. Material and Methods

Slice preparations

Parasagittal midbrain slices were prepared from young (12 - 17 days old) Wistar rats. The experiments were approved by the Animal Research Committee in the Okazaki National Research Institutes. The rats were deeply anesthetized with diethylether and rapidly decapitated. The brain was removed and submerged immediately in ice-cold sucrose Ringer's solution and bubbled with 95% O_2 and 5% CO_2 for 5 to 10 minutes. Parasagittal slices 250-300 μm thick were cut using a microslicer (DTK-2000, Dosaka EM, Kyoto, Japan). They were then incubated in the standard Ringer's solution. After incubation for more than 1 hour, slices to be used for recording were placed individually in a recording chamber on an upright microscope (Axioskop FS, Zeiss, Germany) and continuously superfused with standard Ringer's solution at a rate of 3 to 5 mL/min using a peristaltic pump (Minipuls 3, Gilson, Villiers, France).

Electrophysiological recordings

For whole-cell patch clamp recording, individual DA neurons in the SNc and VTA were visualized using Nomarski optics and a 63x water-immersion objective. Whole-cell

patch clamp recording was performed in large neurons in the SNc and VTA using an EPC-7 amplifier (List, Darmstadt, Germany) under visual control of the patch pipettes. Patch pipettes were prepared from borosilicate glass capillary tubes (GC150TF-15, Clark Electromedical Instruments, Pangbourne, England) using a micropipette puller (P-97, Sutter Instrument Co., Novato, CA). The resistance of the electrodes was 2.5 to 7 MΩ in the bath solution and the series resistance during recording was 10 to 25 MΩ. Data were discarded if the series resistance changed by more than 30 %. The liquid junction potential was not corrected. In some experiments we applied perforated patch clamp recording using amphotericin B in the patch pipettes. In these experiments, 300 μg/ml amphotericin B was dissolved in the intracellular solution just before recordings. The experiments were performed as described in an earlier paper (Rae et al., 1991). Acetylcholine (ACh; 1 mM) was applied to the recorded cell by air pressure (5-10 psi, 5-30 ms) through puff pipettes placed in the close proximity of the soma (Alkondon and Albuquerque, 1993) using PV-820 (World Precision Instruments, Sarasota, FL). In some experiments, glutamate (Glu, 0.5-1 μM) was applied in the same manner. The whole cell patch clamp recordings of part 1 were performed at room temperature (22 -

25℃). It was because at higher bath temperature, the recording tended to become unstable after application of FFA in the voltage clamp recording. On the other cases, experiments were performed at 32-33 ℃.

In part 3, to induce EPSPs in DA neurons, electrical stimulation was applied through a bipolar electrode (Clark Electromedical Instruments, Pangbourne, UK) placed 200-300 μm dorso-caudal to the SNc, which were presumed to stimulate the afferent fibers from the PPTN. Gamma-aminobutyric acid A ($GABA_A$) receptor antagonist, picrotoxin (50 μM, PTX) was contained in all the external solution to suppress the inhibitory postsynaptic potentials (IPSPs). The duration of the stimulation pulse was 200 μs and the current intensity was 0.1-1 mA. The stimulation was performed every 20 - 30 seconds. In most of the cases a single stimulation pulse was applied, but in some cases two or three pulses were applied at a frequency of 50 Hz. In most cases, electrical stimulation was applied just after the ACh-induced depolarization returned to the baseline level. In this case, the interval between the ACh application and electrical stimulation ranged from 0.8 - 2.0 s (referred to as “short interval”). For comparison, the electrical stimulation was performed at 3s interval (referred to as “long interval”;

Part 3, Fig. 2). Data were acquired and analysed using a pClamp hardware/software system (Axon Instruments, Inc., Foster City, CA). All quantitative data were expressed as mean ± S.D. Student's t-test was used for statistical analysis.

Solutions

The standard Ringer's solution contained (mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4 and 25 glucose, and continuously bubbled with 95% O_2 and 5% CO_2 (pH 7.4). The concentration of $MgCl_2$ was increased to 3 mM in case of Ca^{2+}-free solution. The sucrose-Ringer's solution contained (mM): 234 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 10 $MgSO_4$, 0.5 $CaCl_2$, 26 $NaHCO_3$, and 11 glucose. The intracellular solution of the patch pipette contained (mM): 140 K-gluconate, 20 KCl, 0.02 EGTA, 2 $MgCl_2$, 2 Na_2ATP, 10 HEPES, and 0.1 spermine (pH 7.3) or 130 Cs-gluconate, 20 CsCl, 0.02 EGTA, 2 $MgCl_2$, 2 Na_2ATP, 10 HEPES, and 0.1 spermine (pH 7.3). To stain the recorded neurons, biocytin (5 mg/mL, Sigma, St. Louis, MO) was dissolved in the internal solution just before recording.

ACh, apamin, atropine, carbachol, methyllycaconitine (MLA), α-bungarotoxin (αBgt), mecamylamine (Mec), thapsigargin, phenytoin, N-(6-aminohexyl)-5-chloro-1-

naphthalene-sulfonamide hydrochloride (W-7), trifluoperazine (TFP), calmidazolium, KN93, KN92, fulfenamic acid (FFA), picrotoxin (PTX), 6-nitro-7-cyanoquinoxaline-2,3-dione (CNQX), D-aminophosphonovaleric acid (APV), bis (o-aminophenoxy)ethane-N,N,N',N'-tetracetic acid (BAPTA) and NiCl were purchased from Sigma (St. Louis, MO). Dihydro-β-erythroidine (DHβE) was from RBI (Natick, MA) and tetrodotoxin (TTX) was from Sankyo (Tokyo, Japan).

In our experimental condition of part 2, application of W-7 and TFP became effective in 2-3 min, while it took more than 10 min for calmidazolium to be effective. In addition, it was difficult to wash out the effect of calmidazolium. Thus, we tested the effect of calmidazolium only in the experiments in which we tested the FFA-sensitive current (Part 2, Fig. 1). In this case, we incubated the slices in the solution containing calmidazolium for 10 min and then started whole cell recordings. We did not test calmidazolium in the other experiments because we were unable to obtain stable control responses during the long period of time before calmidazolium became effective.

Histology

After recording was terminated, the slices were fixed with 4% paraformaldehyde in 0.1

M phosphate buffer solution. Then the recorded cells were visualized with ABC methods (Horikawa and Armstrong, 1988).

4. Part 1

Fulfenamic acid sensitive, Ca^{2+}-dependent inward current induced by nicotinic acetylcholine receptors in dopamine neurons

Summary

Nicotinic acetylcholine receptors (nAChRs) exhibit high Ca^{2+} permeabilities and the Ca^{2+}-influx through nAChRs may be involved in regulation of a variety of signal processing in the postsynaptic neurons. The mesencephalic dopamine (DA) neurons receive cholinergic inputs from the brainstem and express abundant nAChRs. Here we report that the Ca^{2+} influx induced by a transient pressure application of ACh activates an inward current mediated by nAChRs and subsequently an inward current component that is sensitive to fulfenamic acid (FFA) and phenytoin, presumably a Ca^{2+}-activated nonselective cation current in the DA neurons in the midbrain slices of the rat. The FFA- and phenytoin-sensitive current exhibits a negative slope conductance below -40 mV, suggesting its role in significant enhancement of depolarizing responses. In the current clamp recordings with perforated patch clamp configuration, bath application of carbachol markedly enhanced the glutamate-induced depolarization, which led to a long lasting depolarizing hump. Activation of nAChRs is involved in this process, in cooperation with muscarinic receptors that suppress afterhyperpolarization caused by Ca^{2+}-activated K^+ channels. The long-lasting depolarizing hump was suppressed by

FFA. All these results suggested a potential role of the FFA-sensitive current triggered by nAChR activation in marked enhancement of the excitatory synaptic response in DA neurons.

Introduction

The dopamine (DA) neurons in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) are involved in initiation and termination of movements and regulation of the muscle tone (Olanow and Tatton, 1999). In addition, they are thought to have a role in reward-based reinforcement learning and addiction (Schultz, 1998; Nicola et al., 2000). The DA neurons exhibit either tonic single spike activities or multiple spike bursts *in vivo* (Wilson et al., 1977; Shepard and Bunney, 1988), and it has been shown that burst activities increases the DA release in the forebrain (Chergui et al., 1994; Garris and Wightman, 1994; Gonon and Buda, 1985). Thus, changes in firing pattern of DA neurons may represent a physiological mechanism through which DA neurons alter their influence on target neurons. Previously tonic activation of NMDA receptors through glutamatergic inputs from the prefrontal cortex (Gariano and Groves,

1988; Sesack and Pickel, 1992; Naito and Kita, 1994) and the subthalamic nucleus (Hammond et al., 1978; Smith and Grace, 1992) has been considered to regulate the firing pattern of DA neurons. However, later studies showed that the activation of NMDA receptors is not the sole factor that induces burst firing (Chergui et al., 1993; Christoffersen and Meltzer 1995), but instead the cholinergic projection from the pedunculopontine tegmental nucleus has become more and more highlighted as another major excitatory input (Futami et al., 1995; Inglis and Winn, 1995; Takakusaki et al., 1996; Lockwan et al., 1999; Kitai et al., 1999). Activation of muscarinic acetylcholine receptors (mAChRs) has been shown to suppress the afterhyperpolarization following a spike generation and induce bursts in DA neurons by suppression of SK type Ca^{2+}-activated K^+ channels (Scroggs et al., 2001). Besides mAChRs, a number of reports have shown that activation of nicotinic acetylcholine receptors (nAChRs) significantly modulates the DA neuron function. The chronic nicotine treatment produces a variety of effects on the DA system. For instance, nicotine sensitizes the rewarding effect of drugs (Shippenberg et al., 1996) and increases in the number of nAChRs that mediate nicotinic addiction by smoking (Dani and Heinemann, 1996). A recent study by

Picciotto et al. (1998) showed that an application of nicotine caused DA release in the striatum in wild type mice, while not in nAChRs-β2-subunit knockout mice. Self-administration of nicotine was attenuated in these mutant mice. All these results have suggested a significant role of nicotinic receptors in regulation of DA neuron activities, however the physiological action of nicotinic receptor activation on the membrane properties of DA neurons has not been fully understood. It is well known that activation of nicotinic acetylcholine receptors (nAChRs) on the presynaptic terminals of DA neurons increases the DA release in the striatum (Mogg et al., 2002; Zhang et al., 2002). Further, activation of nAChRs on the presynaptic terminals of the afferents to the DA neurons modulates synaptic inputs to DA neurons (Marsvelder and McGehee, 2000; Marsvelder et al., 2002). In addition to these mechanisms, the role of nAChRs on the somatic and dendritic membrane of DA neurons has attracted much attention (Calabresi et al. 1989; Sorenson et al., 1998; Pidoplichko et al., 1997; Klink et al., 2001).

Among various physiological properties of nAChRs, much interest has particularly been focused on their relatively high permeabilities to Ca^{2+} (Rathouz et al., 1996;

Albuquerque et al., 1997). It has been shown that the activation of nAChRs can substantially increase intracellular free Ca^{2+} levels (Mulle et al., 1992; Vijayaraghavan et al., 1992). Such high Ca^{2+} permeability of nAChRs may have special implications for their receptor functions in DA neurons. Activation of non-selective cation currents in DA neurons has been suggested before (Calabresi et al., 1989; Lacey et al., 1990), however their activation mechanism has not been well characterized. Here we report that the Ca^{2+}-influx through nAChRs in the DA neurons subsequently activates an inward current component that is sensitive to fulfenamic acid (FFA) and phenytoin, presumably I_{CAN}. To further study the physiological properties of this FFA-sensitive current, we investigated its role in modulation of voltage response of DA neurons in current clamp recording. Preliminary results have been published in an abstract form (Yamashita and Isa, 2001).

Results

The present study consists of two major sections. First, we analysed characteristics of an inward current component, which is subsequently activated by Ca^{2+}-influx through

nAChRs in DA neurons. Because of its sensitivity to fulfenamic acid (FFA), this current component is referred to as FFA-sensitive current. In these experiments, whole cell patch clamp recording techniques were applied and transient pressure application of ACh was used to induce the current component. The physiological and pharmacological properties of the FFA-sensitive current were analysed using voltage clamp configuration. In the second section of this study, we investigated the role of the FFA-sensitive current in modulating the firing pattern of DA neurons. In these experiments, we applied perforated patch clamp recording technique and investigated the firing response of DA neurons to a brief pressure application of glutamate (Glu), which mimics the response to excitatory synaptic inputs. In this case, bath application of carbachol (CCh) was used to activate both nicotinic and muscarinic acetylcholine receptors.

Identification of DA neurons

Whole-cell patch clamp recordings were made from the DA neurons in slices from young Wistar rats (12-17 days old). The DA neurons were identified by their location in the SNc or VTA and their morphological and electrophysiological properties. Since

we did not see any marked difference in responses to application of ACh and its agonists between the two groups of DA neurons, we did not differentiate them in this article. A total of 127 DA neurons were recorded in the present study. Eleven of these neurons were successfully stained with biocytin and they had relatively large soma and widely spread dendrites (Fig. 1A) (Jones and Kauer, 1999). In response to depolarizing current pulses, they exhibited spike firing followed by a large afterhyperpolarization and in response to hyperpolarizing current pulses, they exhibited voltage sag due to a hyperpolarization-activated current (I_h) (Fig. 1B) (Mercuri et al., 1995). The neurons examined in the present study exhibited these characteristics of typical DA neurons.

Section 1. Characterization of an inward current component accompanying the nAChR activation

Effect of FFA on ACh-induced current in DA neurons

In voltage clamp recording (holding potential: Vh = -60 mV), a brief pressure application of 1 mM ACh induced a fast inward current in these neurons that was completely suppressed by 10 μM mecamylamine (Mec), a broad antagonist of nAChRs

(Alkondon et al., 1993). On the other hand, the ACh-induced inward current was also partially suppressed by 200 μM FFA (Fig. 1C), which is an antagonist of a subgroup of Ca^{2+}-activated nonselective cation currents (I_{CAN}) (Lee et al., 1996; Yellen et al., 1982; Partridge and Swandulla, 1988; Partridge et al., 1994; Perrier and Hounsgaard, 1999). On average, the amplitude of the ACh-induced current was reduced to about 70% by 200 μM FFA (n = 9). The efficacy of FFA did not differ in the presence of 10 μM atropine (Fig. 1E and Fig.4), indicating that ACh can activate the FFA-sensitive current component without involvement of mAChRs in the present experimental condition (see Discussion). The effect of FFA was dose dependent. The mean (± S.D.) amplitude of the ACh-induced current in the solution containing 20 μM, 100 μM and 200 μM FFA was 101.7 ± 4.2 % (n = 6), 91.3 ± 11.8 % (n = 12) and 70.3 ± 7.4 % (n = 9) of the control response, respectively (Fig. 1E). Significant increase in effect ($p < 0.01$, Student's t-test) was observed at 200 μM. When 500 μM FFA was used, the effect of FFA was not reversible, which suggested that 500 μM FFA affected unknown additional process in the cascade between the Ca^{2+} influx and the channel activation in the cell (Voilley et al., 2001; Spehr et al., 2002). Thus, we used 200 μM as a standard dose of

FFA in all the other experiments.

These results indicate that ACh activates an inward current in the DA neurons that wholly depends on nAChRs but includes a current component that is sensitive to FFA (presumably I_{CAN}). This suggests the possibility that Ca^{2+}-influx through nAChRs can subsequently activate I_{CAN} in the DA neurons.

FFA also significantly reduced the firing responses of the DA neurons caused by a transient application of ACh in the current-clamp recordings (Fig. 2A). In the solution containing 0.25 μM tetrodotoxin (TTX) and/or in the case of using the intracellular solution containing 5 mM Qx-314 to block the generation of action potentials, the depolarizing response was found to be markedly suppressed by the FFA application (Fig. 2B). Thus, the FFA-sensitive current component has a significant role in enhancing the effects of ACh-induced depolarization in the DA neurons.

Ca^{2+}-dependence of the FFA-sensitive current

A possible mechanism underlying the FFA effect on the ACh-induced currents is that FFA partially suppressed nAChRs themselves, as suggested in a previous study using recombinant receptors expressed in *Xenopus* oocytes (Zwart et al., 1995). To test this

possibility, we performed two experiments described below. First, we chelated intracellular free Ca^{2+} by adding 10 mM bis-(*o*-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA) to the intracellular solution (Fig. 3A). FFA had no significant effect on the ACh-induced currents under this condition (n = 8, Fig. 3A, Fig. 4). We then tested the effect of FFA in the Ca^{2+}-free extracellular solution (Fig. 3B). Under this condition, the ACh-induced current was markedly suppressed. It is well known that the reduction in nAChR currents after lowering extracellular Ca^{2+} concentration is due to an allosteric-like mechanism (Mulle et al., 1992; Vernino et al., 1994). Here the remaining current response was likely primarily Na^{+} influx via nAChRs. In this case FFA did not affect the remaining currents (n = 8, Fig. 3B, Fig. 4). These results indicated that the FFA-sensitive current was dependent on both intra- and extracellular Ca^{2+}, suggesting that FFA did not block nAChRs themselves, but suppressed a current component that was subsequently activated by Ca^{2+} influx through the nAChRs.

I-V relationship of the FFA-sensitive current

To characterize the I-V relationship of the FFA-sensitive current, we first applied ACh to the cells at a holding potential of -60 mV, allowing the same amount of Ca^{2+} influx,

and then after the peak of the current, applied voltage steps to -80 mV, -60 mV (unchanged), and -40 mV (Fig. 5A). Subtraction of the current responses before and after FFA application yielded the I-V relationship of the FFA-sensitive current between -80 and -40 mV. The FFA-sensitive current exhibited a negative slope conductance in this voltage range (n = 11, Fig. 5B and 5C). This I-V relationship was markedly different from that of nAChRs which was investigated under application of 200 μM FFA, the I-V relationship of nAChRs tested in this condition exhibited inward rectification as previously shown (Yawo, 1989; Mathie et al., 1990) (n = 4, Fig. 5D and E). These results also suggested the possibility that FFA did not inhibit nAChRs directly.

Effect of phenytoin

We also tested the effect of phenytoin, another antagonist of I_{CAN} (Haj-Dahmane and Andrade, 1999) (Fig. 6). We found that 2, 10 and 50 μM phenytoin significantly suppressed a portion of the ACh-induced current to 98.3 ± 2.6 % (n = 4), 84.1 ± 4.9 % (n = 6), and 74.9 ± 13.6 % (n = 5) (Fig. 6D). Significant increase in effect was observed ($p < 0.05$) as the dose increased. Thus, the effect of phenytoin was also dose-

dependent. The phenytoin-sensitive current also exhibited a negative slope conductance between -80 and -40 mV as well as the FFA-sensitive current (Fig. 6B and C, n = 5).

Possible contribution of Ca^{2+}-release from the internal store

It is also possible that Ca^{2+} release from the endoplasmic reticulum is essential for activation of the FFA-sensitive current, in addition to Ca^{2+} influx through nAChRs. To test this possibility, we preincubated the slices with a solution containing 1 μM thapsigargin, a SERCA (smooth ER Ca^{2+} ATPase) pump blocker (Berridge, 1998), for 10 minutes and then tested the effect of FFA. Under these conditions, the effect of FFA was unchanged (Fig. 4, n = 6) suggesting that the FFA-sensitive current can be activated without Ca^{2+}release from the internal Ca^{2+} store.

Section 2. The role of FFA-sensitive current in modulating the firing response of DA neurons

Based on the above results, we expected that the negative slope conductance of the FFA-sensitive current would markedly enhance depolarizing responses of DA neurons. It has been reported that the I_{CAN} with negative slope conductance causes plateau-like

long-lasting depolarization in some neurons (Di Prisco et al., 2000; Fraser and MacVicar, 1996). However, a transient pressure application of ACh could induce the depolarizing response in DA neurons but by itself could not induce large potentiation of the depolarization as expected (Fig. 2). We supposed that the huge afterhyperpolarization caused by SK type Ca^{2+} activated K^{+} channels, which is prominent in DA neurons, might have suppressed maintenance of depolarization. In DA neurons, it has been reported that mAChRs can reduce the slow afterhyperpolarization and cause the increase in the number of action potentials during depolarization (Scroggs et al., 2001). Thus, we hypothesized that coactivation of nAChRs and mAChRs might be necessary to induce the long-lasting depolarization in DA neurons. To test this possibility, we adopted a perforated patch clamp technique, which prevents dialysis of the internal solution and protects the mAChRs-mediated response. However, even if we switched to the perforated patch clamp configuration, a transient pressure application of ACh did not induce a long-lasting depolarization (data not shown). We also tried a transient application of 1 mM carbachol (CCh), which is an agonist of both nAChRs and mAChRs, but it was also unsuccessful (n = 3).

Therefore we abandoned a transient pressure application of ACh and/or CCh, and hypothesized that continuous activation of mAChRs may be necessary to suppress the SK channels (Scroggs et al. 2001). Then we switched to bath application of CCh, which is hard to be dissolved by acetylcholine esterase, to mimic the diffuse transmission of ACh. The diffuse transmission has been reported in many cholinergic synapses in the brain (Descarries et al., 1997). We investigated the effect of bath application of CCh (30 - 50 μM) on the depolarizing response caused by a pressure application of glutamate (Glu; 0.5-1 mM, frequency; 1/30s), which was performed to mimic the excitatory synaptic inputs on the postsynaptic cells. Application of CCh induced depolarization of the base-line membrane potential by 5 – 10 mV. To compare the effect at the same baseline level, we usually applied constant hyperpolarizing current to set the baseline membrane potential at the control level. As shown in Fig 7A1, a brief pressure application of Glu induced depolarizing response and repetitive spike firing. When CCh was bath applied, the Glu application led to induction of a long-lasting depolarizing hump and larger number of spike responses (n = 5, arrow in Fig. 7A2), and these recovered to the control level after washing out CCh (in Fig. 7A3).

mAChRs were supposed to contribute to the long-lasting depolarizing hump, because such response was not observed with the whole cell configuration and moreover, the bath application of atropine (1 μM) suppressed this response (n = 3, Fig. 7 B). Then we tested the involvement of FFA-sensitive current in the induction of the long-lasting depolarizing hump. As shown in Fig. 7C, 200 μM FFA suppressed the long-lasting depolarizing hump (n = 5, arrow in Fig. 7C2). Further, 5 μM mecamylamine (Mec) suppressed the long-lasting depolarizing hump (n = 6, arrow in Fig. 7D). All these results have suggested that activation of the FFA-sensitive current subsequently activated by the Ca^{2+}-influx through the nAChRs (described in section 1) contributes to the marked enhancement of the depolarizing response of DA neurons under the condition that co-activation of mAChRs suppressed the afterhyperpolarization.

Discussion

FFA- and phenytoin-sensitive current subsequently activated by nAChRs

The present study has shown that the ACh-induced current in the DA neurons includes a current component that is sensitive to FFA and phenytoin. The effect of FFA may not

be the direct effect on the nAChRs because first, FFA had no effect when the extracellular solution did not contain Ca^{2+}, which indicated that FFA did not suppress Na^{+} influx through the nAChRs. Second, FFA did not suppress the ACh-induced current when the intracellular Ca^{2+} was chelated by BAPTA. Third, the I-V relationship of the FFA- and phenytoin-sensitive current exhibited negative slope conductance between -40 and -80 mV, which was different from that of nAChRs that should exhibit positive slope conductance and inward rectification in this voltage range (Fig. 5D and E) (Yawo, 1989; Mathie et al., 1990).

It can be claimed that the negative slope conductance of the FFA-sensitive current can be attributed to the possibility that FFA enhanced the Ca^{2+}-activated K^{+}-channels, which are known to be abundantly expressed in the DA neurons (Ping and Shepard, 1996). To exclude this possibility, we used Cs-gluconate (n = 7) based intracellular solution (the above experiments were done with K-gluconate based intracellular solution). In this case the negative slope conductance of the FFA-sensitive currents was again observed. We also performed the experiments under the presence of 200 nM apamin with Cs-gluconate based intracellular solution, which suppressed the Ca^{2+}-activated K^{+}-

channels. In this case, we could still observe that the FFA-sensitive current exhibited the negative slope conductance (n = 5, data not shown). All these observations suggested that contribution of K^+-conductance such as Ca^{2+}-activated K^+-channels to the current component with the negative slope conductance like the FFA-sensitive current was unlikely. The biophysical mechanism of the negative slope conductance of the FFA-sensitive current is currently unknown. Voltage dependent blockade of the channels by divalent cation such as Mg^{2+} as in the case of NMDA receptor channels (Mayer ad Westbrook, 1984) should be tested in future.

The process for the Ca^{2+} influx through nAChRs to activate the FFA-sensitive current can be very fast; the FFA-sensitive current component appeared approximately 10 ms (measured in 6 neurons, data not shown) following the onset of the ACh-induced current.

The ion permeability of the FFA- and phenytoin-current was not investigated because of difficulty in measurement of the equilibrium potentials. However, it is likely the FFA-sensitive current is caused by calcium-activated nonselective cation (CAN) channels, which have been found in many neuronal and non-neuronal cells (Colquhoun et al.,

1981; Maruyama and Peterson, 1982; Yellen, 1982; Siemen, 1993; Kuriyama et al., 1998). According to the literature, they exhibit three unique characteristics. First, they are activated by intracellular Ca^{2+}, which is regulated by membrane flux and pumping, Ca^{2+} release from the intracellular organelle, uptake and cytoplasmic buffering. Second, CAN channels do not undergo voltage- or Ca^{2+}-dependent inactivation and thus are able to maintain depolarization. Third, they can also provide a route for Ca^{2+} entry from extracellular space. In addition, most of CAN channels studied by a single channel recording technique exhibited no voltage dependency (Maruyama and Peterson, 1982; Yellen, 1982). However, the FFA-sensitive CAN channels studied in prefrontal cortical and hippocampal neurons were shown to exhibit voltage dependency (Fraser and MacVicar, 1996; Haj-Dahmane and Andrade, 1996, 1998, 1999), as in the case of the FFA- and phenytoin-sensitive current in our study. The discrepancy between the channels investigated in the single channel recording studies and neurons in situ remains to be studied.

Contribution of mAChRs to the induction of FFA-sensitive current

It has been shown that activation of mAChRs subsequently activates TRP channels that

may be related to I_{CAN} (Inoue et al. 2001). Thus, involvement of mAChRs in activation of the FFA-sensitive current should be considered. In this study, however, we did not observe the inward current caused by activation of mAChRs (Fig. 1C) and could not obtain the experimental results that suggest the possible involvement of mAChRs in inducing the FFA-sensitive current (Fig. 4). This might be due to dialysis of the internal solution during the whole cell patch recordings and the results do not always indicate the DA neurons lack the muscarinic receptor-mediated responses.

Contribution of Ca^{2+} release from internal store to the induction of FFA-sensitive current

Our present results showed that the FFA-sensitive current remained after depletion of internal Ca^{2+} store by thapsigargin, which indicated that the Ca^{2+}-influx through nAChRs alone could activate the FFA-sensitive current without release of Ca^{2+} from the internal store in our experimental condition. However, Tsuneki et al. (2000) studied the Ca^{2+} mobilization in the mice DA neurons by Ca^{2+}-imaging technique and showed that increase in intracellular Ca^{2+} by activation of β2-containing non-α7 and α7 type nAChRs. The former elicited rise in $[Ca^{2+}]_i$ via activation of Na^+- and T-type Ca^{2+}-

channels, while the latter directly increased the $[Ca^{2+}]_i$. The authors further showed thus induced rise in $[Ca^{2+}]_i$ was not caused without Ca^{2+} release from the intracellular Ca^{2+} store in both cases, which suggested that the Ca^{2+} signals mainly reflect the indirect effect of Ca^{2+} influx through the nAChRs. In these experiments, they used continuous superfusion of nicotine and its agonist. These results suggest that the cascades of $[Ca^{2+}]_i$ can be different if the way to apply the ACh agonists is changed.

Characteristics of the FFA-sensitive current

One functionally important aspect of the FFA- and phenytoin-sensitive current in the DA neurons was the negative slope conductance. The negative slope conductance may endow the DA neurons with a coincidence detector-like function as well as the NMDA-type glutamate receptors that are known to mediate excitatory synaptic transmission in DA neurons (Christoffersen and Meltzer, 1995).

The molecular identity of the FFA- and phenytoin-sensitive current is not yet known. Recent studies suggest that some channel molecules belonging to the TRP family are responsible for nonselective cation channels including I_{CAN} (Hofmann et al., 2000). It has been reported that among the TRP family proteins, TRP7 is inhibited by 100 μM

FFA in smooth muscle cells (Inoue et al., 2001). However the negative slope conductance as observed in the present study has not been shown for TRP7 (Okada et al., 1999). It remains to be solved whether other molecules than TRP7 are responsible for the FFA-sensitive current in DA cells, or some unknown modulatory mechanisms can change the voltage-dependence of the TRP channels.

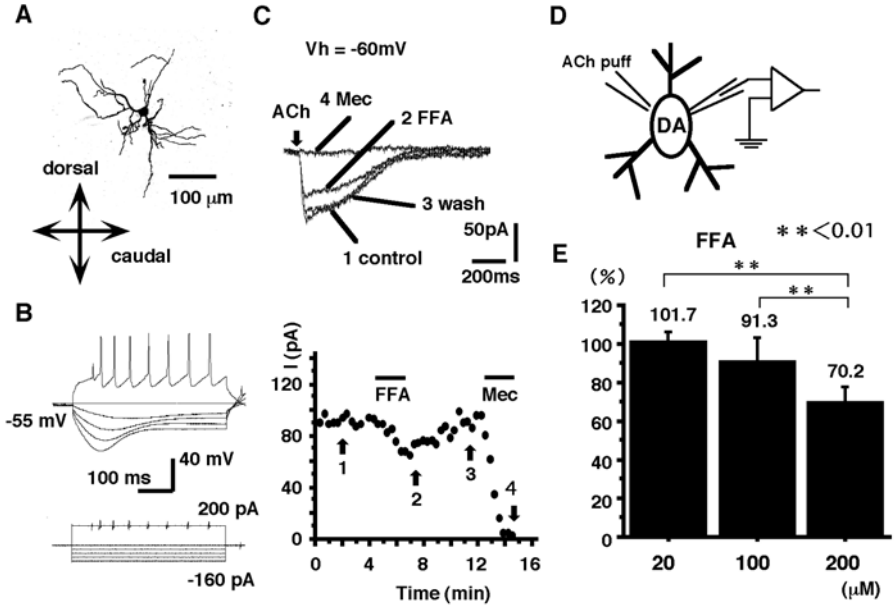
Functional implication

The FFA-sensitive I_{CAN} has been reported to induce plateau-potentials in the lamprey reticulospinal neurons (Di Prisco et al., 2000). The negative slope conductance of the FFA-sensitive current could be a crucial factor for initiating the plateau potential. It has also been proposed that the Ca^{2+}-dependent plateau potential elicits the burst spiking in the DA neurons (Overton and Clark, 1997; Amini et al., 1999). As shown in Fig. 7, the continuous activation of nAChRs and mAChRs markedly enhanced glutamate-induced depolarization and caused a long-lasting depolarizing hump in DA neurons. Suppression of slow afterhyperpolarization of DA neurons by activation of mAChRs was essential to induce the long-lasting depolarizing hump. Transient pressure application of ACh was not sufficient, but bath application of CCh was necessary. A

likely explanation for this would be that continuous activation of mAChRs was necessary to trigger the downstream cascade to cause suppression of SK channels (Scroggs et al. 2001). The present study has clarified that in addition to such effect of mAChRs, the activation of FFA-sensitive current, presumably I_{CAN}, contributes to the long-lasting depolarizing hump. All these results suggest that the activation of the FFA-sensitive current by the cholinergic inputs might play a crucial role in enhancing the excitatory response of DA neurons and that such effect would potentiate the reward-related signalling of the DA neurons (Schultz, 1998; Picciotto et al., 1998, Kobayashi et al. 2002).

It is well known that ACh enhances DA release by its action on the presynaptic terminals in the striatum (Mogg et al., 2002; Zhang et al., 2002). In this study we propose that in addition to these effects on the presynaptic terminals of the DA neurons in the striatum, the cooperative action of the postsynaptic nicotinic and muscarinic receptor activation could significantly modulate the depolarizing response of DA neurons, to which the activation of the FFA- and phenytoin-sensitive current by nAChRs would contribute by its voltage-dependent activation property.

Fig.1



T. Yamashita & T. Isa
Fulfenamic acid sensitive, Ca^{2+}-dependent inward current induced by nicotinic acetylcholine receptors in dopamine neurons
Fig. 1

Fig. 1 **A**, A representative DA neuron stained with biocytin. **B,** Voltage responses to depolarizing and hyperpolarizing current pulses in a DA neuron. The intensities of the current pulses are indicated below. **C**, The effect of fulfenamic acid (FFA; 200 μM) and mecamylamine (Mec; 10 μM) on the ACh-induced current. The ACh-induced current was partially blocked by FFA and completely blocked by Mec. Cells were recorded in voltage-clamp mode at a holding potential (Vh) of –60 mV. Superimposed sample waveforms (top panel; average of 3 records) were recorded under respective conditions at individual timings indicated by arrows with the corresponding numerals in the bottom panel. **D,** Schematic diagram of the experimental arrangement. **E,** Dose-response relationship of the FFA effect. The relative amplitude of the ACh-induced currents under each condition is indicated (mean ± S.D.). Statistical significance was examined by Student's t-test (**; $p < 0.01$, *; $p<0.05$).

Fig. 2

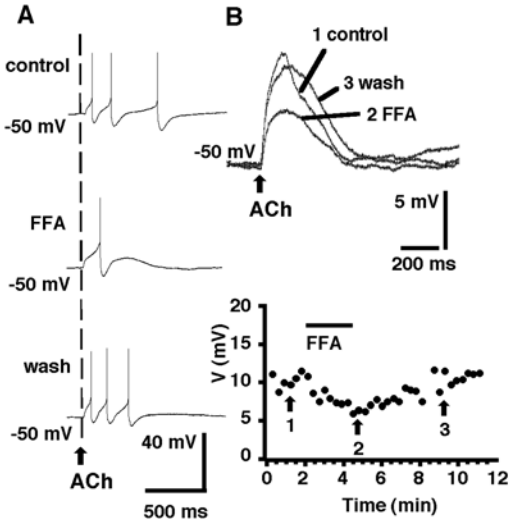


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Fig. 2

Fig. 2 The FFA-sensitive current enhanced the depolarizing effects of ACh. **A**, FFA (middle panel) suppressed the ACh-induced increase in firing rate. **B,** Under the solution containing TTX (0.25 μM), FFA significantly suppressed the ACh-induced depolarizing response. Other details as in Fig. 1**C**.

Fig. 3

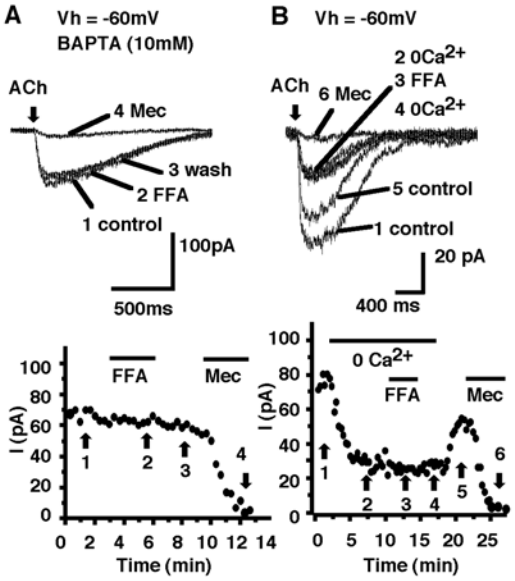

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Fig. 3

Fig. 3 The effect of 200 μM FFA and 10 μM Mec on the ACh-induced current with (**A**) BAPTA-containing intracellular solution, and (**B**) Ca^{2+}-free extracellular solution. Cells were recorded in the voltage-clamp mode at a holding potential (Vh) of –60 mV. FFA was ineffective in both cases.

Fig. 4

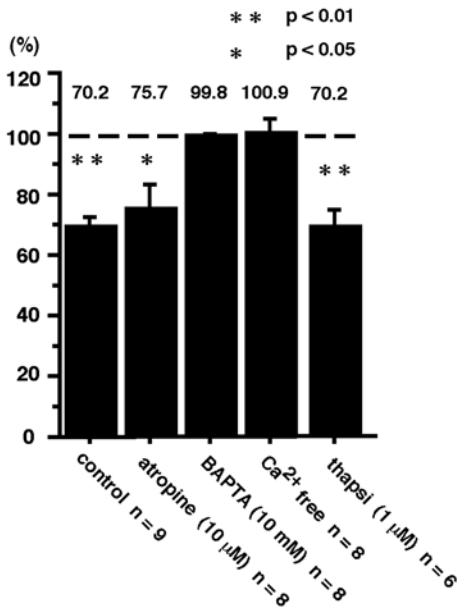

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Fig. 4

Fig. 4 Pharmacology of the FFA -sensitive current. Average effects of FFA in control solution, with 10 μM atropine in the extracellular solution, with 10 mM BAPTA in the intracellular solution, in Ca^{2+}-free extracellular solution, and in extracellular solution containing 1 μM thapsigargin. In case of thapsigargin, the slices were preincubated for 10 min with the solution containing it.

Fig. 5

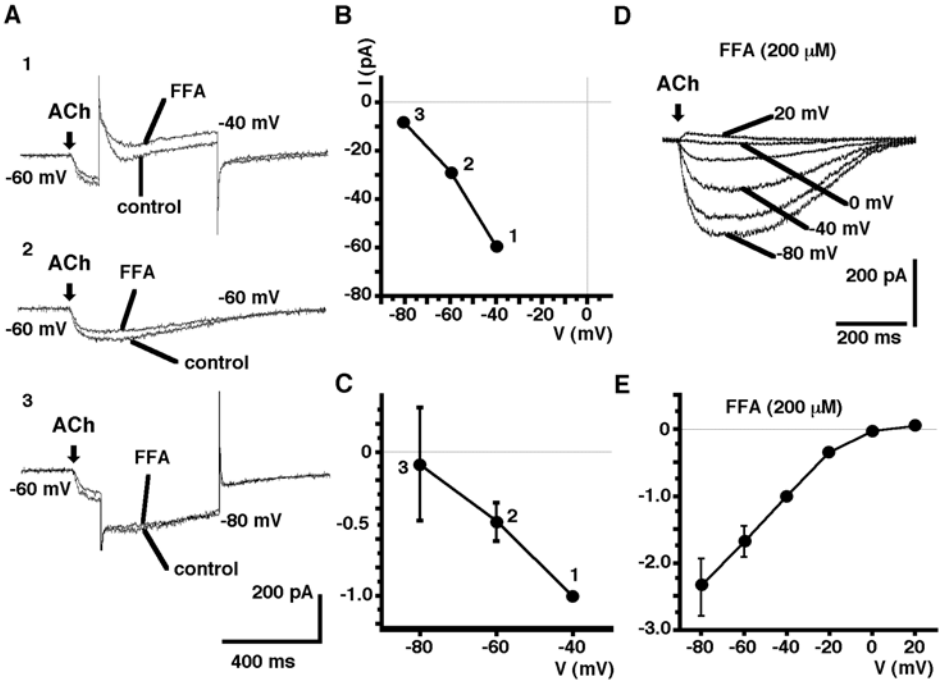

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Fig. 5

Fig. 5 Current-voltage (I-V) relation of the FFA-sensitive current. **A**, The membrane voltage was stepped from –60 mV to –80 mV (**3**) or –40 mV (**1**) or left unchanged at –60 mV (**2**) 100 ms after application of ACh. The responses recorded in the control solution and in the presence of FFA are superimposed. **B**, I-V plots of the FFA-sensitive currents obtained by subtracting the current responses in the FFA-containing solution from those obtained in the control solution in **A**. **C**, The average (±S.D.) of the normalized I-V relationship of 11 DA neurons (The current records at –40 mV were normalized to be –1.0). Note that the FFA-sensitive current exhibited a negative slope conductance between –80 and –40 mV. **D** and **E,** I-V relationship of the nAChR-mediated current response, which was measured under the presence of 200 μM FFA. D, an example of the ACh-induced current response. E, I-V curve of the nAChR-induced current (mean ± S.D., n = 4).

Fig. 6

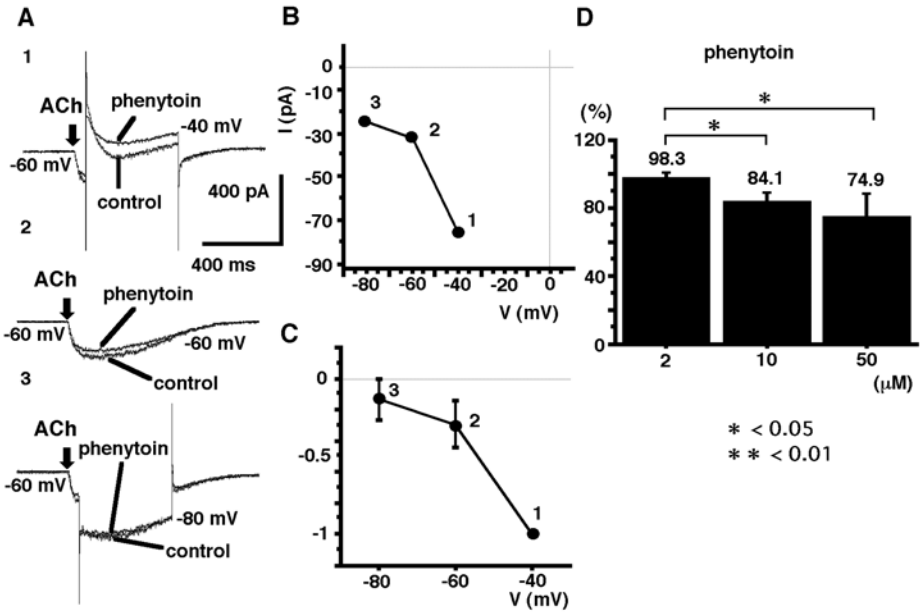
A
1
ACh
phenytoin
-40 mV
-60 mV
control
400 pA
400 ms
2
ACh
phenytoin
-60 mV
-60 mV
3
control
ACh
phenytoin
-60 mV
-80 mV
control
B
I (pA)
0
-10
-30
-50
-70
-90
3
2
1
-80 -60 -40 -20 0
V (mV)
C
0
-0.5
-1
3
2
1
-80 -60 -40
V (mV)
D
phenytoin
(%)
*
*
120
80
40
0
98.3
84.1
74.9
2 10 50
(μM)
* < 0.05
** < 0.01

Figure 6, I-V relationship of the phenytoin-sensitive current. **A-C**; the same arrangement as in Fig. 6. Dose-response relationship of the phenytoin effect. The relative amplitude of the ACh-induced currents under each condition is indicated (mean ± S.D.). **D,** the dose-response relationship of the phenytoin effect. Statistical significance was examined by Student's t-test (**; $p < 0.01$, *; $p<0.05$).

Fig. 7

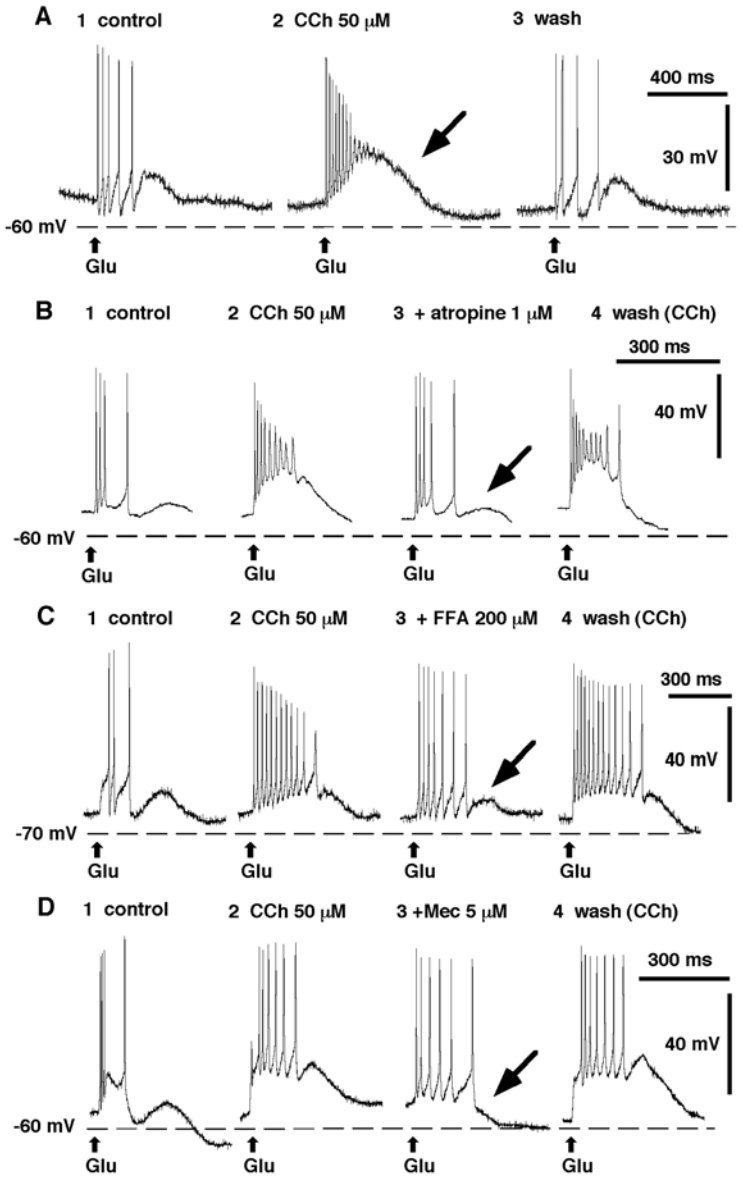

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Fig. 7

Figure 7. The effect of carbachol-induced current in depolarizing response of DA neurons investigated using the current clamp recording mode with perforated patch clamp technique. **A1,** Control response to a brief application of glutamate (Glu 1-0.5 mM, 10 ms). **A2,** During application of 30-50 μM carbachol (CCh), the Glu application induced high frequency burst firing overriding the plateau-like depolarizing hump. **A3.** After washing of CCh. **B,** Effect of 1 μM atropine (B3) on the CCh-induced enhancement of Glu-induced depolarization. **C,** Effect of 200 μM FFA (C3) on the CCh-induced enhancement of Glu-induced depolarization. **D,** Effect of 5 μM Mec (D3) on the CCh-induced enhancement of Glu-induced depolarization. Note these antagonists significantly suppressed the CCh-induced plateau-like depolarizing hump of DA neurons.

5. Part 2

Ca^{2+}-dependent inward current induced by nicotinic receptor activation depends on Ca^{2+}/calmodulin-CaMKII pathway in dopamine neurons

Summary

It is well known that mesencephalic dopamine neurons receive massive projection from cholinergic neurons in the brainstem. In Part 1, we showed that Ca^{2+}-influx through nicotinic acetylcholine (ACh) receptors in the dopamine neurons subsequently activated an inward current that was sensitive to fulfenamic acid (FFA) and phenytoin, presumably a Ca^{2+}-activated non-selective cation current. The FFA-sensitive current exhibited a negative slope conductance and predominantly enhanced the depolarizing responses of dopamine neurons. In this study, we showed that the inward FFA-sensitive current was eliminated by antagonists of Ca^{2+}/calmodulin (Ca^{2+}/CaM), N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride (W-7; 1 μM), trifluoperazine (TFP; 1.5 μM) and calmidazolium (100 nM). Application of W-7 and TFP reduced the ACh-induced inward current and the current component suppressed by these drugs exhibited negative slope conductance, as well as the FFA-sensitive current. Further, intracellular application of KN-93, an antagonist of Ca^{2+}/CaM-dependent protein kinase II (CaMKII), but not KN-92 eliminated the FFA-sensitive current. All these results suggest that Ca^{2+}/CaM-CaMKII pathway is involved in an activation of the

FFA-sensitive current.

Introduction

Acetylcholine (ACh) plays an important role in cognitive functions, such as memory (Huerta and Lisman, 1993; Winkler et al., 1995), attention (Risbrough et al., 2002), and sleep and arousal cycles (Perry et al., 1999). Among the cholinergic neurons in the central nervous system, those in the pedunculopontine and laterodorsal tegmental nuclei (PPTN and LDTN) have been highlighted as major input sources to dopamine (DA) neurons in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) (Inglis and Winn, 1995; Takakusaki et al., 1996; Kitai et al., 1999; Lokwan et al., 1999). They are supposed to be related to DA neuron functions such as reinforcement learning and addiction (Schultz, 1998; Nicola et al., 2000) and control of muscle tone (Olanow and Tatton, 1999). Recently Scroggs et al. (2001) reported that muscarinic ACh receptors (mAChRs) in DA neurons had a significant role in regulating the firing pattern of DA neurons by modulating the small type of Ca^{2+}-activated K channels (SK channels). Moreover, it has been shown that ACh regulates neurotransmitter release

from glutamatergic and GABAergic synaptic terminals on DA neurons via an activation of presynaptic nicotinic ACh receptors (nAChRs) (Mansvelder and McGehee, 2000; Mansvelder et al., 2002). Furthermore, following studies focused on the existence of the nAChRs on the somatodendritic membrane of DA neurons (Calabresi et al., 1989; Pidoplichko et al., 1997; Sorenson et al., 1998; Klink et al., 2001).

Previous studies showed that nAChRs exhibited high Ca^{2+}-permeabilities (Rathouz et al., 1996; Albuquerque et al., 1997). In our preceding study, we reported that Ca^{2+}-influx through nAChRs subsequently activated an inward current which was sensitive to fulfenamic acid (FFA) and phenytoin in DA neurons by using whole cell patch clamp recording obtained from the rat brain slice preparation (part 1; Yamashita and Isa, 2003a). These results have suggested that the inward current is a subtype of Ca^{2+}-activated non-selective cation currents (I_{CAN}) (Colquhoun et al., 1981; Maruyama and Peterson, 1982; Yellen, 1982; Siemen, 1993; Kuriyama et al., 1998). We showed that the FFA-sensitive current exhibited negative slope conductance at the membrane potentials between -40 and -80 mV. Previous studies showed that a class of I_{CAN} sensitive to FFA exhibited negative slope conductance in prefrontal cortical and

hippocampal neurons (Fraser and MacVicar, 1996; Haj-Dahmane and Andrade, 1996, 1998, 1999). The negative slope conductance may endow the FFA-sensitive current with the potential to significantly enhance the depolarizing response of the DA neurons. Actually, depolarizing responses of DA neurons induced by a brief pressure application of 1 mM glutamate was predominantly enhanced and switched to long lasting plateau-like depolarizing hump after bath application of carbachol (CCh), an agonist of both nAChRs and mAChRs in the perforated patch clamp configuration. The long-lasting depolarizing hump was suppressed by application of nAChR antagonist mecamylamine (Mec) and FFA (part 1).

Although we showed that the FFA-sensitive current was induced by Ca^{2+}-influx through the nAChRs, the intracellular signaling pathway for activation of the current is not yet clear. In this study, we examined the involvement of Ca^{2+}-activated second messenger pathways in the induction of the FFA-sensitive current. Ca^{2+} increase in cytosol induces various physiological effects and Ca^{2+}-binding proteins serve as transducers of the cytosolic Ca^{2+} signal (Ghosh and Greenberg, 1995; Rathouz et al., 1996; Berridge, 1998). Calmodulin (CaM) is known as one of the most ubiquitous Ca^{2+}-binding

proteins (Lisman et al., 2002). When activated by Ca^{2+}, its conformational change is induced and the resulting Ca^{2+}/CaM complex can interact with target proteins, such as Ca^{2+}/CaM-dependent kinase (CaMK), which modulates wide range of cellular functions (Sola et al., 2001) including synaptic transmission. In this study, we tested a possible involvement of Ca^{2+}/CaM and CaMKII in the induction of the FFA-sensitive current in DA neurons.

Results

The DA neurons were identified by their location in the SNc or VTA and their morphological and electrophysiological properties as described in our previous paper (Part 1).

FFA-sensitive current depends on intracellular calmodulin

As shown in our previous paper, a brief pressure application of 1 mM ACh induced a fast inward current in DA neurons at a holding potential (Vh) of -60 mV, which was completely suppressed 10 μM mecamylamine (Mec), a broad antagonist of nAChRs (Alkondon and Albuquerque, 1993). This result has suggested that the inward current

depends on activation of nAChRs. The inward current was reduced by about 30% in the solution containing 200 μM fulfenamic acid (FFA; Fig. 1a) or 50 μM phenytoin (data not shown), which are antagonists of a subgroup of Ca^{2+}-activated nonselective cation currents (I_{CAN}) (Haj-Dahmane and Andrade, 1999; Di Prisco et al., 2000). The inward FFA-sensitive current was activated by the Ca^{2+}-influx through nAChRs (part 1; Yamashita and Isa, 2003a).

As described in Introduction, we examined whether Ca^{2+}/CaM was involved in activation of the FFA-sensitive current. To test this possibility, we examined the FFA sensitivity of the ACh-induced current in the solution containing a CaM antagonist, N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride (W-7; 1 μM) or trifluoperazine (TFP; 1.5 μM) or calmidazolium (calmida; 100 nM) (Fig. 1b and c) (Kleene, 1994; Perrier et al., 2000) respectively. Under each condition 200 μM FFA did not induce any effect on the ACh-induced current, which indicated that suppression of Ca^{2+}/CaM eliminated the inward FFA-sensitive current. These results strongly suggested that Ca^{2+}/CaM was involved in induction of the FFA-sensitive current.

I-V relationship of W-7 or TFP-sensitive current

In part 1, we showed that the FFA-sensitive current in DA neurons exhibited negative slope conductance between -80 and -40 mV, which was different from the current-voltage relationship of the nAChRs studied in ganglion cells (Yawo, 1989; Mathie et al., 1990). To confirm whether the CaM antagonists described above actually suppressed the same FFA-sensitive current, we examined the I-V relationship of the current component that was suppressed by the application of each of W-7 and TFP. If the CaM antagonist suppressed the same FFA-sensitive current, the inward current suppressed by these drugs should exhibit negative slope conductance as the FFA-sensitive current. To obtain the I-V curve of the W-7-sensitive current, we first applied ACh to the DA neurons at a holding potential of -60 mV and then after the peak of the current, applied voltage steps to -80 mV, -60 mV (unchanged), and -40 mV (Fig. 2a). Subtraction of the current responses after W-7 application from the control response yielded the I-V relationship of the W-7 sensitive current. As shown in Fig. 2b and c, the inward current sensitive to W-7 also exhibited negative slope conductance between -80 to -40 mV. We also examined the current-voltage relationship of the TFP-sensitive current (Fig.3). In this case, we did not test the TFP-sensitive current above -40 mV, because

TFP suppressed the Ca^{2+}-activated K^+ channel as reported by Ikemoto et al. (1992) and accordingly the TFP-sensitive current measured at -40 mV included considerable amount of Ca^{2+}-activated K^+ currents. In this case, the TFP-sensitive current exhibited negative slope conductance from -90 to -50 mV (n = 4). These results strongly suggested that both W-7 and TFP suppressed the same inward current as the FFA-sensitive current shown in our preceding study. All these results suggest that Ca^{2+}/CaM is involved in activation of the FFA-sensitive current, presumably I_{CAN}.

CaM kinase II is essential for activation of the FFA-sensitive current

The next question is how the Ca^{2+}/CaM activates the FFA-sensitive current. Among the various CaM-binding proteins, we examined the possible involvement of Ca^{2+}/CaM-dependent protein kinase II (CaMKII), since CaMKII has been reported to be ubiquitous in the CNS (Giese et al., 1998; Soderling et al., 2001). To test this possibility, we applied 50 μM KN93, an antagonist CaMKII to the internal solution and examined the effect of FFA on the ACh-induced current. After formation of the whole cell patch clamp configuration, the amplitude of ACh-induced current gradually decreased and then became stable in 3-4 min. After the stable recording was acquired, we examined

the effect of 200 μM FFA on the ACh-induced current. As shown in Fig. 4a and c, FFA had little effect on the ACh-induced current. As a control, we tested the effect of 50 μM KN92, an inactive analogue of KN93, in the internal solution. In this case, 200 μM FFA reduced the ACh-induced current by about 25% (Fig. 4b and c). These results showed that suppression of CaMKII activity eliminated the FFA-sensitive current, which suggested that CaMKII was involved in activation of the FFA-sensitive current.

Carbachol-induced long lasting depolarizing hump depended on Ca^{2+}/CaM

In our previous study, we showed that bath application of carbachol (CCh; 30-50 μM), an agonist of nicotinic and muscarinic AChRs, markedly enhanced the depolarizing response of DA neurons induced by a brief pressure application of glutamate (Glu; 0.5-1 mM, frequency; 1/30 s) in a perforated patch clamp configuration (part 1, Yamashita and Isa, 2003a). The Glu-induced depolarizing responses were changed to a long-lasting depolarizing hump. The long-lasting depolarizing hump was supposed to be induced by two major factors; induction of the FFA-sensitive current that exhibits negative slope conductance via nAChR activation, and suppression of Ca^{2+}-activated K^+ channels via muscarinic ACh receptor activation (Scroggs et al., 2001). Based on

these findings, we tested the effects of the CaM antagonists (13 μM W-7 and 30 μM TFP) on the CCh-activated long-lasting depolarizing hump. We first applied 0.5 – 1.0 mM Glu with a brief pressure (10 ms duration, 1/30s) and induced depolarizing and firing responses (Fig. 5a1 and b1). Bath application of 30-50 μM CCh markedly enhanced the Glu-induced depolarizing responses and came to induce a long-lasting depolarizing hump (Fig. 5a2 and b2). In this case, the application of CCh induced the depolarization of the base-line membrane potential by 5 - 10 mV. To compare the effect at the same baseline level, we usually applied constant hyperpolarizing current to set the baseline membrane potential to the control level and investigated the effect of Glu application. Then additional applications of 13 μM W-7 and 30 μM TFP both reversibly suppressed the CCh-activated long-lasting depolarizing hump (Fig. 5a3, a4 and b3, b4). All these results gave further support to our hypothesis that Ca^{2+}/CaM was involved in activation of the FFA-sensitive current, which markedly enhanced the depolarizing responses of the DA neurons.

Another CaM antagonist, calmidazolium (100 nM), was not tested in this experiment. It was because it took much longer (> 10 min) for this drug to be effective than TFP and

W-7 (2 - 3 min) (see Methods).

Discussion

Ca^{2+}/calmodulin-CaMKII pathway involved in activation of the FFA-sensitive current

As described in Introduction, we tested whether Ca^{2+}/CaM-CaMKII pathway would be involved in activating this FFA-sensitive current. In this study, we found that CaM antagonists, TFP, W-7 and calmidazolium and CaMKII antagonist, KN93 eliminated the FFA-sensitive current.

Further, we examined the I-V relationship of the CaM antagonist-sensitive current. Both the W-7 sensitive and TFP-sensitive currents showed a negative slope conductance. These results further support that CaM antagonists did not suppress nAChRs themselves but Ca^{2+}/CaM is involved in generating the FFA-sensitive current.

Moreover, we examined the effect of continuous bath application of CaM antagonists on the burst firing and long lasting depolarizing hump induced by combination of transient application of Glu and continuous application of CCh. The CCh-induced burst firing was also suppressed by W-7 or TFP. These results further suggested that

Ca^{2+}/CaM was involved in the induction of the CCh-induced burst firing and long lasting depolarizing hump via activation of the FFA-sensitive current.

We showed that in addition to Ca^{2+}/CaM, subsequent activation of CaMKII were essential to induce the FFA-sensitive current. Then a question may arise as to which process the Ca^{2+}/CaM-CaMKII pathway regulates for the induction of the FFA-sensitive current. Elevation of intracellular Ca^{2+} has a potential role to act at many sites and to regulate a variety of cellular events. Rapid removal and buffering of elevated free Ca^{2+} helps to restrict the spatial distribution of Ca^{2+} (Ghosh and Greenberg, 1995; Berridge, 1998). It has been shown that the clusters of α7 type nAChRs are found within lipids rafts, which are reported to function as membrane platforms for the assembly of signaling complexes including CaM (Anderson, 1998; Bruses et al., 2001). Thus, if nAChRs and CaM are located close to each other, Ca^{2+}-influx through nAChRs can be quickly bound to CaM. In our previous study, we described that the very early component of the FFA-sensitive current occurred around 9 ms after the onset of the inward current mediated via nAChRs (part 1). Thus one of the possible role of the Ca^{2+}/CaM-CaMKII pathway is to mediate the Ca^{2+}-signal via entry through the nAChRs

to the induction of the early component of the FFA-sensitive current. Alternative possibility is that the constitutive activation of the Ca^{2+}/CaM-CaM KII is essential for the FFA-sensitive current. This question remains open at this stage.

Coincidence detection in the dopamine neurons

DA neurons receive glutamatergic inputs from the prefrontal cortex (Sesack and Pickel, 1992; Naito and Kita, 1994) and subthalamic nucleus (Kita and Kitai, 1987) and also glutamatergic neurons in the PPTN (Takakusaki et al., 1996). In addition, DA neurons receive cholinergic inputs from the PPTN (Inglis and Winn, 1995; Takakusaki et al., 1996). DA neurons exhibit both low frequency tonic firing and phasic burst firing (White, 1996; Overton and Clark, 1997). It has been reported the dopamine release in the forebrain is much enhanced following the burst firing compared with tonic firings (Gonon and Buda, 1985; Chergui et al., 1994; Garris and Wightman, 1994). DA neurons exhibited low rate of firing in response to transient application of glutamate because of huge afterhyperpolarization caused by SK type Ca^{2+}-activated potassium channels (Scroggs et al. 2001). However, when CCh was bath applied, which mimics diffuse transmission of ACh (Descarries et al., 1997), the firing response of the DA

neurons was switched to bursts because activation of muscarinic receptors suppressed the SK channels (Scroggs et al., 2001) and activation of nAChRs induced the FFA-sensitive current (part 1; Yamashita and Isa, 2003a). Thus the glutamatergic or cholinergic inputs alone is not sufficient to induce burst, but coincident inputs from the two systems may be necessary for the DA neurons to exhibit burst discharges. Our present results may represent a part of the molecular mechanism underlying such coincidence detection in DA neurons. The molecular process found in this study may endow the DA neuron system with constraints on the time window for association of the glutamatergic and cholinergic inputs.

Fig. 1

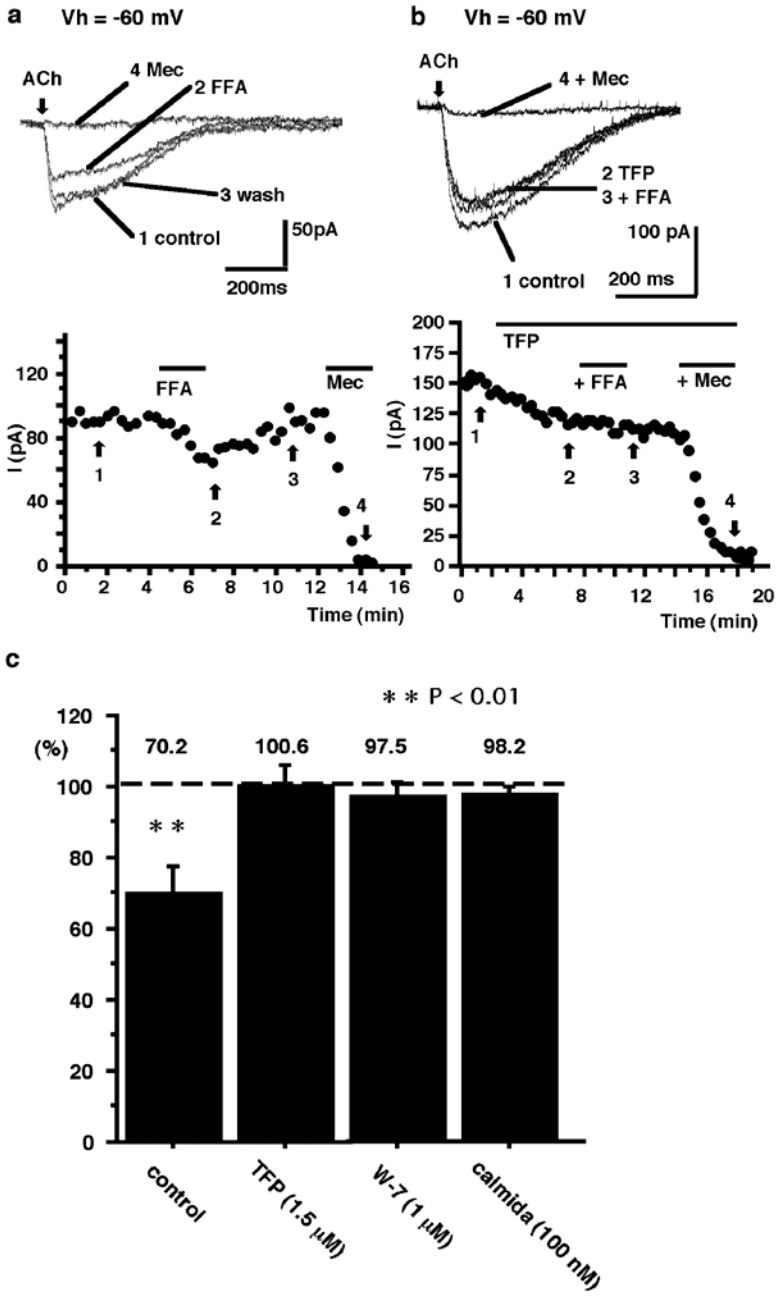

a
Vh = -60 mV
ACh
4 Mec
2 FFA
3 wash
1 control
50pA
200ms
b
Vh = -60 mV
ACh
4 + Mec
2 TFP
3 + FFA
100 pA
1 control
200 ms
TFP
+ FFA
+ Mec
FFA
Mec
I (pA)
Time (min)
c
** P < 0.01
(%)
70.2
100.6
97.5
98.2
control
TFP (1.5 μM)
W-7 (1 μM)
calmida (100 nM)

Fig. 1 **a**, The effect of fulfenamic acid (FFA; 200 μM) and mecamylamine (Mec; 10 μM) on the ACh-induced current. The ACh-induced current was almost completely blocked by Mec and partially blocked by FFA. Cells were recorded in voltage-clamp mode at a holding potential (Vh) of -60 mV. Superimposed sample waveforms (top panel; an average of 3 records) were recorded under respective conditions at the timing indicated by arrows with the corresponding numerals in the bottom panel. **b,** The effect of 200 μM FFA and 10 μM Mec on the ACh-induced current under the extracellular solution containing an antagonist of calmodulin (CaM), trifluoperazine (TFP; 1.5 μM). FFA was ineffective in this case. Other details as in Fig. 1 **a**. **c,** Pharmacology of the FFA-sensitive current. Average effects of FFA on the ACh-induced current in voltage clamp recordings (Vh = -60 mV) in control solution (n = 9), or in the solution containing a CaM antagonist, 1 μM W-7 (n = 5), 1.5 μM TFP (n = 9) and 100 nM calmidazolium (n = 7), respectively. In the case of CaM antagonists, FFA had little effect on the ACh-induced current. The vertical bars indicate standard deviation. Statistical significance was examined by Student's t-test (** $P < 0.01$).

Fig. 2

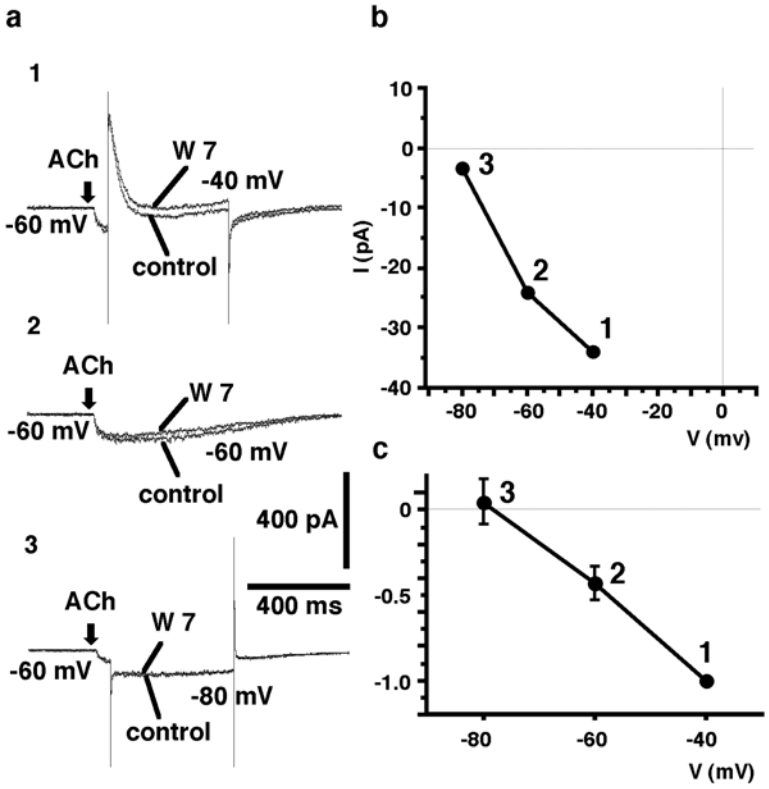

a
b
1
ACh
W 7
-40 mV
-60 mV
control
2
ACh
W 7
-60 mV
-60 mV
control
400 pA
3
400 ms
ACh
W 7
-60 mV
-80 mV
control
I (pA)
V (mv)
c
V (mV)

Fig. 2 I-V relationship of the W-7 (1 μM)-sensitive current. **a**, The membrane voltage was stepped from -60 mV to -80 mV (**3**) or -40 mV (**1**) or left unchanged at -60 mV (**2**) 100 ms after application of ACh. The responses recorded in the control solution and in the presence of W-7 are superimposed. **b**, I-V plots of the W-7-sensitive currents obtained by subtracting the current responses in the W-7-containing solution from those obtained in the control solution in **a**. **c**, The average (±S.D.) of the normalized I-V relationship of 5 DA neurons (The current records at -40 mV were normalized to be -1.0). Note that the W-7-sensitive current exhibited a negative slope conductance between -80 and -40 mV.

Fig. 3

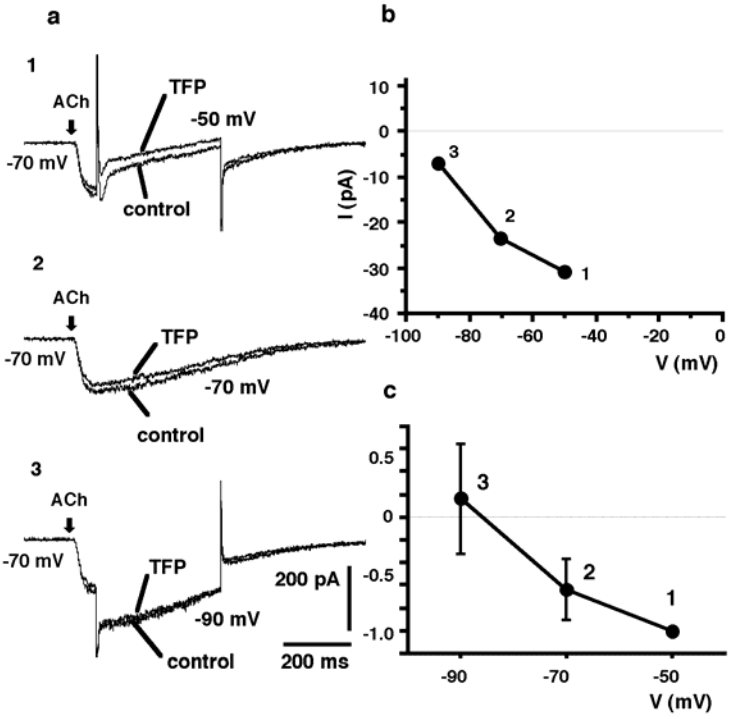

a
b
1
ACh
TFP
-50 mV
-70 mV
control
2
ACh
TFP
-70 mV
-70 mV
control
3
ACh
-70 mV
TFP
-90 mV
control
200 pA
200 ms
I (pA)
V (mV)
c
V (mV)

Fig. 3 I-V relationship of the TFP (1.5 μM)-sensitive current. **a**, The membrane voltage was stepped from -70 mV to -90 mV (**3**) or -50 mV (**1**) or left unchanged at -70 mV (**2**) 100 ms after application of ACh. The responses recorded in the control solution and in the presence of TFP are superimposed. **b**, I-V plots of the TFP-sensitive currents obtained by subtracting the current responses in the TFP-containing solution from those obtained in the control solution in **a**. **c**, The average (±S.D.) of the normalized I-V relationship of 4 DA neurons (The current records at -50 mV were normalized to be -1.0). Note that the TFP-sensitive current exhibited a negative slope conductance between -90 and -50 mV.

Fig. 4

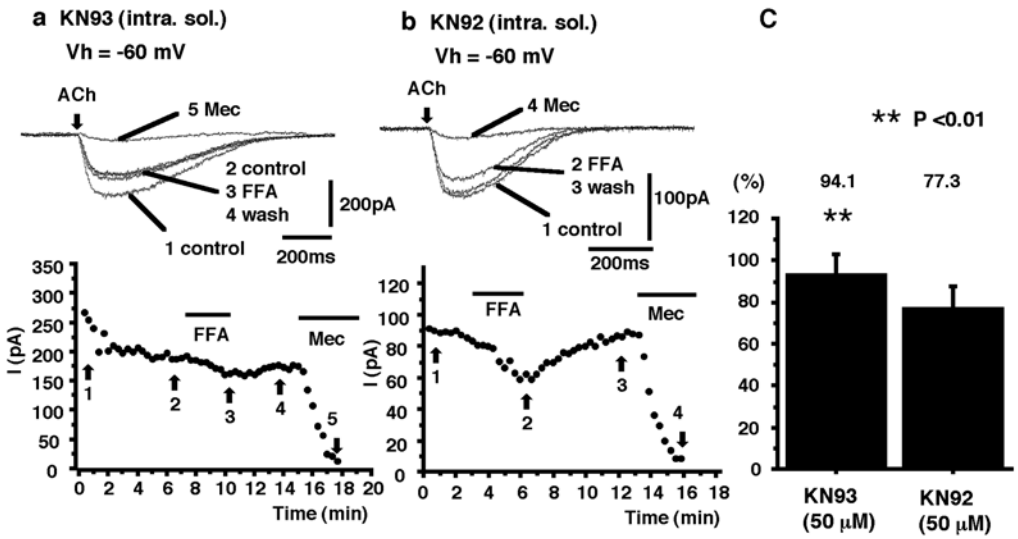

a KN93 (intra. sol.)
Vh = -60 mV
ACh
5 Mec
2 control
3 FFA
4 wash
1 control
200pA
200ms
I (pA)
FFA
Mec
Time (min)
b KN92 (intra. sol.)
Vh = -60 mV
ACh
4 Mec
2 FFA
3 wash
1 control
100pA
200ms
FFA
Mec
I (pA)
Time (min)
C
** P <0.01
(%)
94.1
77.3
**
KN93
(50 μM)
KN92
(50 μM)

Fig. 4 **a, b** The effect of 200 μM FFA and 10 μM Mec on the ACh-induced current with the intracellular solution containing an antagonist of Ca^{2+}/CaM-dependent protein kinases II (CaM-KII), KN93 (50 μM) or KN92 (50 μM), an inactive analogue of KN93. Cells were recorded in the voltage-clamp mode at a holding potential (Vh) of -60 mV. FFA was virtually ineffective in the cases of KN93-containing intracellular solution but effective in the case of KN92-containing intracellular solution. Other details as in Fig. 1 **a**. **c**, Pharmacology of FFA-sensitive current. Average effects of FFA on ACh-induced current in intracellular solution containing an antagonist of CaM-KII, KN93 (50 μM) or KN92 (50 μM). In the case of KN92, FFA normally suppressed ACh-induced current (n = 12) but in the case of KN93, FFA had little effect on the ACh-induced current (n = 11). The vertical bars indicate standard deviation. Statistical significance was examined by Student's t-test (** $P < 0.01$).

Fig. 5

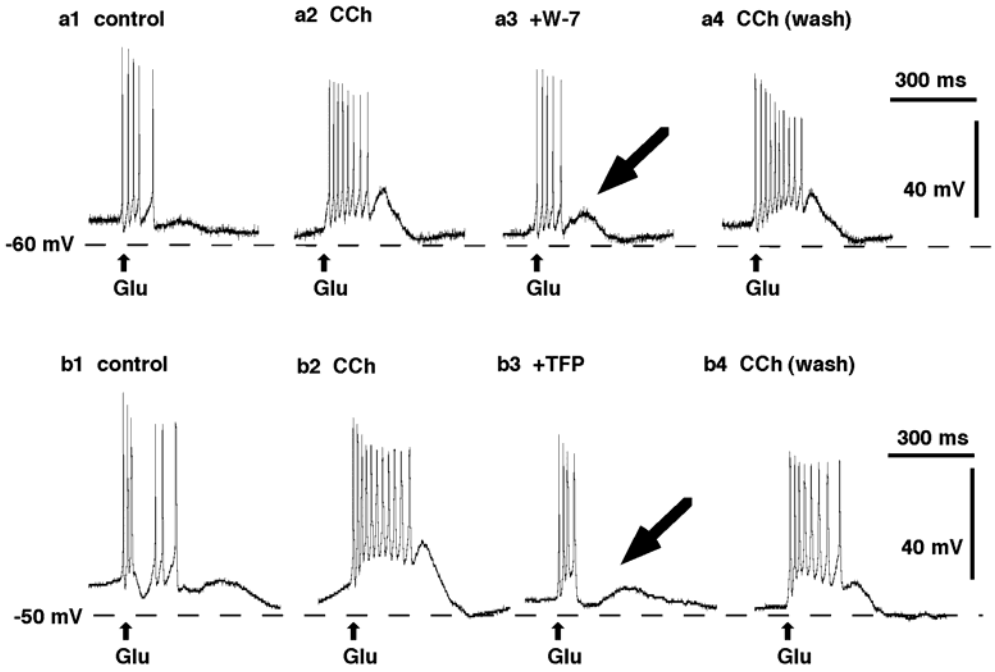

a1 control
a2 CCh
a3 +W-7
a4 CCh (wash)
300 ms
40 mV
-60 mV
Glu
Glu
Glu
Glu
b1 control
b2 CCh
b3 +TFP
b4 CCh (wash)
300 ms
40 mV
-50 mV
Glu
Glu
Glu
Glu

Fig. 5 The effect of glutamate-induced depolarising response of DA neurons investigated in current clamp recording mode with perforated patch clamp technique. **a1**. Control response to a brief application of glutamate (Glu 0.5 -1 mM, 10 ms). **a2**. During application of 30-50 μM carbachol (CCh), the Glu application induced high frequency burst firing overriding the long lasting depolarizing hump. **a3**. Effect of calmodulin antagonist of W-7 (13 μM) on the CCh-induced depolarization. (n = 4) **b**. Effect of another calmodulin antagonist of 30 μM TFP (**b3**) on the CCh depolarisation. (n = 4) Note these calmodulin antagonists significantly suppressed the CCh-induced long lasting depolarizing hump.

6. Part 3

Enhancement of excitatory postsynaptic potential by coincident cholinergic input in dopamine neurons

Summary

In previous studies, we reported that the firing patterns of dopamine (DA) neurons are modulated by a fulfenamic acid sensitive, Ca^{2+}-dependent inward current, presumably the Ca^{2+}-activated nonselective cation current, I_{CAN}. This current is activated by Ca^{2+} influx through nicotinic acetylcholine receptors and has a negative slope conductance between -80 mV and -40 mV. The activation of this current leads to a drastic change in responses to glutamatergic transient inputs from tonic single spikes to multiple spike burst discharges. The glutamatergic inputs were mimicked by transient pressure application of glutammate in the previous papers, thus it remained unknown whether the excitatory postsynaptic potentials are modulated by this current.

In order to examine the effect of the cholinergic inputs on EPSPs, we applied ACh (1 mM) by air pressure just before the onset of electrical stimulation that induces EPSPs. The application of ACh enhanced the EPSPs at an interval of 0.8 – 2.0 s, but the effect was not observed at an interval of 3 s. In addition, this enhancement was critically dependent on the intracellular Ca^{2+} and the membrane potentials of the postsynaptic cell. Furthermore, the effect of the enhancement caused by ACh application was sensitive to

FFA and phenytoin. Together, these results suggest that the Ca^{2+} influx caused by cholinergic inputs enhances the EPSPs via the activation of the FFA- and phenytoin-sensitive current, presumably I_{CAN}.

Introduction

Mesencephalic dopamine (DA) neurons received attention because of their physiological and patho-physiological roles, such as in reinforcement learning (Dani and Heinemann, 1996; Schultz, 1998) and in the control of muscle tone and movement (Olanow and Tatton, 1999) . Their firing patterns range from tonic single spikes to multiple spike bursting discharges, and the physiological significance of their change in firing pattern has been discussed for many years (White, 1996; Overton and Clark, 1997; Kitai et al., 1999). Anatomical reports have shown that DA neurons receive inputs from the cerebral cortex (Sesack and Pickel, 1992; Naito and Kita, 1994), subthalamic nucleus (Kita and Kitai, 1987) and pedunculopontine tegmental nucleus (PPTN) (Takakusaki et al., 1996) that are mediated by excitatory amino acids and inhibitory inputs from the globus pallidus and substantia nigra pars reticulata that are

mediated by gamma-aminobutyric acid (GABA) (Celada et al., 1999). A number of studies suggest that the burst firings of DA neurons depend on the activation of N-methyl-D-aspartate receptors (NMDARs), but that the activation of NMDARs does not explain the entire mechanism of the induction of bursting activity (Kitai et al., 1999).

Recent studies have examined the role of cholinergic inputs from the PPTN (Takakusaki et al., 1996; Inglis and Winn, 1995). Physiological studies have shown that presynaptic nicotinic acetylcholine receptors (nAChRs) on glutamatergic and GABAergic terminals modulate the neurotransmitter release from these terminals (Mansvelder and McGehee, 2000; Mansvelder et al., 2002). Moreover, Scrogg et al. (2001) reported that the activation of postsynaptic muscarinic acetylcholine receptors (mAChRs) reduced the amplitude of the slow afterhyperpolarization (sAHP), which may facilitate the burst firing response to glutamatergic inputs in DA neurons. Moreover, the DA neurons express abundant functional nAChRs on their somatodendritic membrane (Dani and Heinemann, 1996; Pidoplichko et al., 1997; Picciotto et al., 1998). In addition to the depolarizing effects caused by the inward current through the nAChRs, we reported recently that Ca^{2+} influx through nAChRs

subsequently activates a fulfenamic acid (FFA)- and phenytoin-sensitive inward current, presumably the Ca^{2+}-activated nonselective cation current, I_{CAN} (Yellen, 1982; Partridge and Swandulla, 1988; Partridge et al., 1994; Perrier and Hounsgaard, 1999; Yamashita and Isa, 2003a; Part 1). Furthermore, we showed that the activation of the FFA- and phenytoin-sensitive current depends not only on Ca^{2+} influx through the nAChRs, but also on the Ca^{2+}/calmodulin (Ca^{2+}/CaM)-CaM dependent kinase II (CaMKII) pathway (Yamashita and Isa, 2003b; Part 2). A continuous application of carbachol markedly enhanced the depolarizing responses of the DA neurons induced by a transient pressure application of glutamate (Glu; 1 mM), suggesting that FFA- and phenytoin-sensitive current contributes to the induction of burst firing (Yamashita and Isa, 2003a, 2003b; Part 1 and Part 2). Together, our results suggest that the depolarizing responses to glutamatergic input is enormously enhanced when the input coincides with the cholinergic input. Thus, a coincidence detection may occur in the DA neurons between the cholinergic and the glutamatergic inputs (Engel et al., 2001).

However, in our previous reports, glutamatergic synaptic inputs were mimicked by a transient pressure application of Glu. Therefore, an investigation as to whether FFA-

and phenytoin-sensitive current enhances the glutamanergic synaptic inputs to DA neurons is required. To examine the effect of acetylcholine (ACh) on excitatory postsynaptic potentials (EPSPs), ACh (1 mM) was transiently applied to the soma of DA neurons by air pressure prior to the electrical stimulation of the afferent fibers. We found that the preceding ACh application markedly enhanced the following EPSPs in the DA neurons. We examined the possibility that the enhancement of the EPSPs in this case was caused by the FFA- and phenytoin-sensitive current subsequently induced by the nAChR activation.

Results

Whole-cell patch clamp recordings were made from 76 DA neurons. The DA neurons were identified by their location in the SNc and their morphological and electrophysiological properties, as described in part 1.

Glutamatergic synaptic inputs were enhanced by a preceding application of ACh

In the solution containing PTX (50 μM), an antagonist of $GABA_A$ receptors, electrical stimulation induced EPSPs, which were almost completely suppressed by the glutamate

receptor antagonists, CNQX (10 μM) and APV (50 μM), suggesting that the EPSPs observed under the present experimental conditions were mediated predominately by AMPA and NMDA type glutamate receptors (Fig. 1 A). When the electrical stimulation was preceded by a transient application of ACh at a short time interval (0.8 – 1.7 s), the EPSPs were markedly enhanced (Fig. 1 B; red arrows 2 and 3). As shown in Figure 3, the integrated amplitude of the EPSPs was enhanced by 3.44 times by the preceding application of ACh (n = 6). Occasionally, the effect of this enhancement persisted 20 seconds after the ACh application (Fig. 1 B; see the trial next to the ACh application indicated by the red arrow 3).

In part 1 and part 2, the Ca^{2+} influx through nAChRs in the DA neurons activates an inward current component that is sensitive to FFA and phenytoin, antagonists of I_{CAN}. This FFA- and phenytoin-sensitive current exhibited a negative slope conductance between -40 to -80 mV, which was presumed to enhance the depolarizing response and induce the burst firing of DA neurons. Therefore, we conducted further experiments to examine the possibility that the FFA- and phenytoin-sensitive current contributes to the enhancement of EPSPs by ACh.

Time window of the effect of a preceding ACh application on EPSPs

We investigated the time window of the effectiveness of a preceding ACh application in enhancing subsequent EPSPs by changing the interval of application. As shown above, when ACh was applied at "short interval" (1.4 – 2.0 s., n = 5), the EPSPs were enhanced to 181.8% of the control response (n = 5, Fig. 2A red record and 2B). In contrast, as shown in Figure 2A (blue record), when the interval was set to "the long interval" (3.0 s, n = 5), the preceding ACh application was not effective (97.7 % of the control response, Fig.2B). These results suggest that cholinergic inputs can enhance the EPSPs that are induced in the DA neurons a short time later, but are not able to enhance the excitatory inputs that arrive after a longer interval. Further, the time window for such enhancement is less than 3 s.

The effect of preceding ACh application on EPSPs depends on intracellular Ca^{2+}

To examine whether the enhancement of EPSPs by preceding application of ACh depends on intracellular Ca^{2+}, we applied 10 mM BAPTA, a Ca^{2+} chelator, to the intracellular solution. In the presence of BAPTA, the preceding ACh application did not enhance the EPSPs (Fig. 3A). On average, the EPSPs with ACh application were

decreased by about 15% by BAPTA compared to that of the control (n = 6, Fig. 4). The mechanism of this reduction is unknown. These results showed that internal Ca^{2+} is essential for the enhancement of EPSPs by a preceding ACh application. However, Ca^{2+} influx through voltage dependent Ca^{2+} channels alone could induce the enhancement of EPSPs. To address this possibility, we examined whether the repetitive firing of DA neurons induced by depolarizing current pulses (500 ms duration and 200 pA in amplitude) could enhance the succeeding EPSPs. As shown in Figures 2B and 3, the repetitive spike firings of induced by the injection of current pulses were not effective in enhancing succeeding EPSPs (n = 5), suggesting that the Ca^{2+}-influx through the voltage gated Ca^{2+} channels activated by the depolarization induced by ACh application is not involved in the enhancement of succeeding EPSPs (Wolfart and Roeper, 2002).

The mechanisms to enhance the EPSPs by preceding ACh application

As shown in our previous studies (part 1 and part 2; Yamashita and Isa, 2003a, 2003b), one of the possible mechanisms to enhance the EPSPs by a preceding ACh application is through the activation of the FFA- and phenytoin-sensitive current by Ca^{2+}-influx

through the nAChRs, which enhances EPSPs by their property of negative slope conductance. An alternative possibility is that either the NMDARs or low threshold type voltage dependent Ca^{2+} channels (T-type) on the DA neurons are enhanced by a preceding ACh application, which would result in a marked enhancement of the EPSPs. In order to test these possibilities, we examined the effect of a preceding ACh application on the EPSPs in the solution containing 50 μM APV, an antagonist of NMDARs (n = 6, Figs. 3C and 4) or 100 μM NiCl, an antagonist of T-type Ca^{2+} channels (n = 6, Figs. 3D and 4). In both cases, the effect of a preceding ACh application on EPSPs remained 218.7% and 174.4% of the control response, respectively (Fig. 4). Together, these results suggest that the enhancement of NMDARs or T-type Ca^{2+} channels (Wolfart and Roeper, 2002) are not essential factors for the enhancement of succeeding EPSPs by ACh application.

Previous results showed that the FFA-sensitive current was linked to the activation of nAChRs and that the activation of mAChRs or the Ca^{2+} release from internal stores were not essential for the induction of the current (part 1; Yamashita and Isa, 2003a). In line with these results, we examined the effect of a preceding ACh application on

EPSPs when mAChRs were blocked by atropine (1 μM) (n = 6, Figs. 3E and 4) or when Ca^{2+} stores were depleted by 10 minutes of preincubation with thapsigargin (1 μM), a SERCA (smooth ER Ca^{2+} ATPase) pump blocker (Berridge, 1998) (n = 5, Figs. 3F and 4). In both cases, the effect of a preceding ACh application on the EPSPs remained 166.4 % and 184.4 % of the control response, respectively. These results suggest that a preceding ACh application can enhance EPSPs without the excitation of mAChRs or Ca^{2+} release from internal stores.

Finally, to examine whether FFA- and phenytoin-sensitive current contributes to the effect of a preceding ACh application on EPSPs, we examined the enhancement in a solution containing 200 μM FFA (n = 7) or 50 μM phenytoin (n = 8) (Figs. 3 E and F). In both cases, the effect of a preceding ACh application on EPSPs was not observed (95.7 % and 101.7 % of the control response, respectively) (Fig. 4). Taken together, these results suggest that FFA- and phenytoin-sensitive current, presumably I_{CAN}, contributes to the enhancement of EPSPs.

The effect of a preceding ACh application on EPSPs depends on the membrane potential (Vm)

During the process of examining the ACh effect, we maintained the membrane potential of the recorded cell at approximately -60 mV, and gradually depolarized the membrane potential by varying the injected current. We noticed that when the membrane potential exceeded a particular level, the enhancement of EPSPs appeared suddenly. The threshold to induce the enhancement was -54.5 +/- 1.8 mV (mean +/- S.D., n = 6). The enhancement of EPSPs disappeared when the membrane potential (Vm) was hyperpolarized by just 2 mV below the threshold. As shown in Figure 5A, the control EPSPs were induced at Vm of -55 mV (Fig. 5 A arrows 1), and the transient pressure application of ACh enhanced EPSPs (Fig. 5A, red arrows 2, 3 and 5). However, when Vm was hyperpolarized by 2 mV to -57 mV, the enhancement disappeared (Fig. 5A, blue arrow 4). These results demonstrate that the enhancement of EPSPs depends critically on Vm of the postsynaptic neuron. As shown in the population data in Figure 5B, the hyperpolarization by 2 mV across the threshold reduced the enhancement effect from 292.8 % to 118.9% (n = 6) of controls. These results support our current hypothesis that channels with the property of negative slope conductance, such as the FFA- and phenytoin-sensitive current, are involved in the process of the EPSP

enhancement. A possibility remains that the pressure application of ACh induced the presynaptic facilitation of glutamate release to DA neurons and that such effects contributed to enhancement of the EPSPs. However, considering that the effect of a preceding ACh application was critically influenced by a slight change (2 mV) in the Vm of the postsynaptic neuron, the possibility of such a presynaptic mechanism can be excluded safely.

Discussion

Cholinergic postsynaptic inputs to DA neurons and their downward cascade of Ca^{2+} signalling

As shown in Figure 1A, most of the EPSPs in DA neurons were completely suppressed by the bath application of glutamate receptor antagonists. However, the presence of strong cholinergic inputs to DA neurons from the PPTN has been generally accepted (Clarke et al., 1987; Takakusaki et al., 1996), and functional nAChRs are on DA neurons, which may be involved in the self-administration of nicotine (Picciotto et al., 1998). Furthermore, using light and electron microscopy with immunohistochemistry,

postsynaptic nicotinic receptors were found on mesencephalic DA neurons in several studies (Sorenson et al., 1998; Arroyo-Jim nez et al., 1999). In addition, cholinergic EPSPs have been reported on DA neurons in an electrophysiological study (Futami et al., 1995). Although positive data exist that show cholinergic postsynaptic inputs to DA neurons, some reports suggest that only very small nicotinic receptor-mediated EPSPs are present in DA neurons (Fiorillo and Williams, 2000). In fact, cholinergic EPSPs have been reported less frequently in the CNS (Role and Berg, 1996). Of those reported, the amplitude is very small (Roerig et al., 1997; Hefft et al., 1999).

Recent immuno-electronmicroscopic studies showed that a low frequency of synaptic configurations are observed on ACh (ChAT-immunostained) axon terminals (boutons or varicosities) in the CNS (Umbriaco et al., 1995; Descarries et al., 1997). These results suggest that rather than fast synaptic transmissions, diffuse (or volume) transmission might prevail in the CNS. In fact, we have induced burst firing of DA neurons when we used the bath application of carbachol, an agonist of nAChRs and mAChRs (part 1; Yamashita and Isa, 2003a). This condition may be similar to the ways ACh actually acts on the DA neurons.

It is speculated that both types of transmission may function in the cholinergic system in the CNS. In the case of the point-to-point synaptic transmission, the modulation would primarily depend on various properties of nAChR subtypes (Klink et al., 2001) such as affinity for agonists (Mansvelder et al., 2002; Fenster et al., 1997) and desensitisation kinetics (Pidoplichko et al., 1997; Wooltorton et al., 2003). In addition, receptor location has been considered to be an important factor for the synaptic effect (Shoop et al., 2001; Sargent and Pang, 1989; Berg and Conroy, 2002). These modulations may contribute to the spatio-temporal patterns of the Ca^{2+} signaling succeeding the receptor activation (Albuquerque et al., 1995; Rathouz et al., 1996).

On the other hand, in case of diffuse transmission, the extracellular environment may play more important roles. For example, glial cells have been reported to affect the composition and volume of the extracellular space and to modulate the volume transmission (Sykova and Chvatal, 2000). Furthermore, recent reports suggest that extracellular matrix molecules derived from neurons and glia may modulate synaptic transmission by imposing diffusion constraints for neurotransmitters and trophic factors (Dityatev and Schachner, 2003). Other than the point-to-point synaptic transmission, a

more complicated and dynamic mechanism has to be elucidated.

Cholinergic inputs may modulate the firing patterns of DA neurons

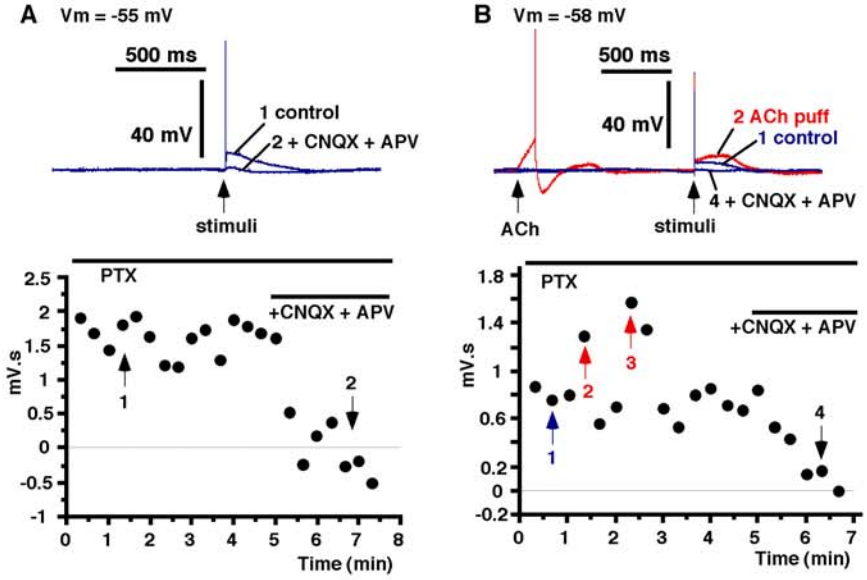
DA neurons in the SNc and VTA exhibit a continuum of patterned activity that ranges from tonic single spikes to multiple spike bursting discharges (Grace and Bunney, 1984). A switch from a single spiking mode to a burst-firing mode may be important in regulating the DA release at the projection sites (Reynolds et al., 2001). Due to their physiological and patho-physiological importance, the mechanism of modulating DA firing patterns has attracted the interest of many researchers and remains controversial (White, 1996; Overton and Clark, 1997; Kitai et al., 1999).

Our results suggest that the excitation of postsynaptic nAChRs enhances glutamatergic EPSPs on DA neurons through FFA- and phenytoin-sensitive current, presumably I_{CAN}. Our methods do not completely exclude the involvement of a presynaptic effect of AChRs, but the effect of a preceding ACh application on EPSPs was quite sensitive to the holding potential of postsynaptic cells (Figs. 5A,B) and disappeared when the intracellular solution contained BAPTA (Figs. 3A and 4). These results strongly suggest that the action of ACh observed in the present study can not be explained by the

presynaptic action of ACh.

The FFA- and phenytoin-sensitive current is unique in modulating the firing pattern of DA neurons. This current is distinct because of its feed forward effect on the Ca^{2+} influx from extracellular spaces. Many of the Ca^{2+} permeable channels are suppressed by Ca^{2+}/CaM after their activation and are regulated by the feedback effects of the Ca^{2+} influx (Levitan, 1999). However, in the present case, the Ca^{2+} influx through nAChRs subsequently activates the FFA- and phenytoin-sensitive current, which induces a greater depolarization and a larger Ca^{2+} influx caused due to its negative slope conductance in conjunction with the coactivation of mAChRs that suppress SK type K^{+} channels (Scroggs et al. 2001). These events lead to a regenerative cycle that produces a dramatic change in the membrane potential and switches the firing pattern from tonic single spikes to multiple bursting spikes (part 1 and part 2; Yamashita and Isa, 2003a, 2003b). Taken together, these results suggest that somatodendritic nAChRs have a significant role in modulating DA neuron activities by the subsequent activation of the current, presumably I_{CAN}.

Fig. 1



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Enhancement of excitatory postsynaptic potential by coincident cholinergic input in dopamine neurons
Fig. 1

Fig. 1 **A**, Effect of CNQX (10 μM) and APV (50 μM) on the electrically induced EPSPs in DA neurons tested in the solution containing 50 μM PTX. Recordings were performed in current-clamp mode at a membrane potential (Vm) of –55 mV. Superimposed sample waveforms (top panel; average of 3 records) were recorded under the control condition at the timing indicated by the corresponding numerals in the bottom panel (n = 5). The integrated value of the EPSPs are plotted in the vertical axis of the bottom panel. **B,** The effects of preceding ACh application (1 mM) on the EPSPs. Recordings were performed in current-clamp mode at a membrane potential (Vm) of –58 mV. Sample waveforms were superimposed in the top panel. In the case of the control record (blue line), three records were averaged, and other records were from individual trials. The waveforms in the top panel were recorded at the timing indicated by the corresponding numerals in the bottom panel. ACh was applied by air pressure in the way the excitatory effect of ACh came to the end just before the onset of electrical stimuli of afferent fibers. Note that the enhancement of EPSP also occurred (in the following trial of red arrow 3). In this case, the effect of enhancement remained even 20 seconds after the previous challenge of ACh.

Fig. 2

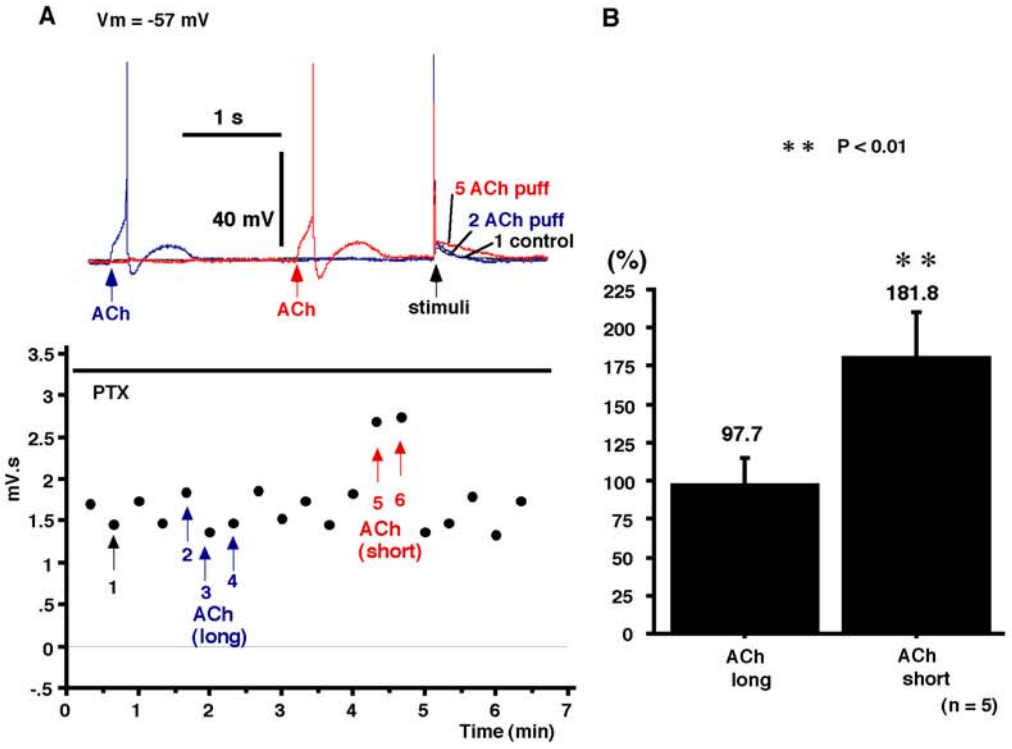

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Fig. 2

Figure 2 The time window of the effect of a preceding ACh application on the EPSPs. A. When ACh was applied at a long time interval (3 s, n = 5) before the onset of stimuli, the amplitude of the EPSPs was not enhanced (blue line in the top panel and blue arrows 2, 3, and 4 in the bottom panel). In contrast, when ACh was applied at a short time interval (1.4 s in this case) before the electrical stimuli of the afferent fibers, the enhancement was observed (red line in the top panel and red arrows 5 and 6 in the bottom panel). The control record of the EPSP without ACh application is indicated as a blue line (black arrow 1 in the bottom panel). Other details as in Figure 1B. B. The ratio of the integrated amplitude of the EPSPs following the preceding ACh application divided by that of the EPSPs in the control records without the preceding ACh application. The averaged data of 5 cells. Statistical significance was examined by Student's t-test (**; $p < 0.01$).

Fig. 3

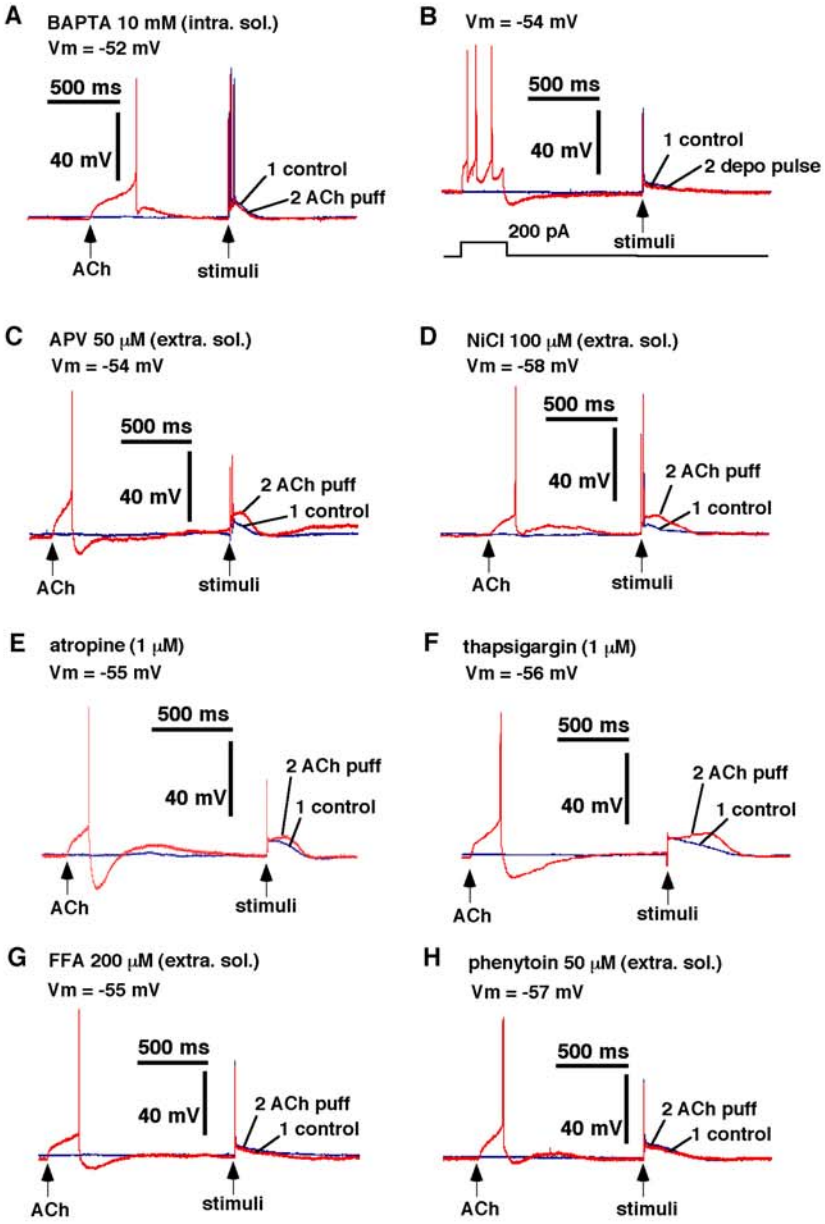

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Fig. 3

Fig. 3 The effects of preceding ACh application (A,C-H) and injection of a current pulse on the EPSPs. Sample waveforms were superimposed. Three records were averaged for the control records without ACh application or the current injection, while single sample record is illustrated for the records with ACh application or current injection under each pharmacological condition. The onset of the ACh application and current pulses were set in the way the excitatory effect came to end just before the electrical stimulation of the afferent fibers. **A,** Effect of BAPTA (10 mM) in the internal solution. **B,** The effect of current injection (200 pA in amplitude and 500 ms duration), which induced 3 successive spike firings. **C,** The effect of APV (50 μM). **D,** The effect of NiCl (100 μM). **E,** The effect of atropine (1 μM). **F,** The effect of pre-incubation in the solution containing thapsigargin (1 μM) for 10 min. **G and H,** The effect of FFA (200 μM; n = 7) or phenytoin (50 μM; n = 8) respectively.

Fig. 4

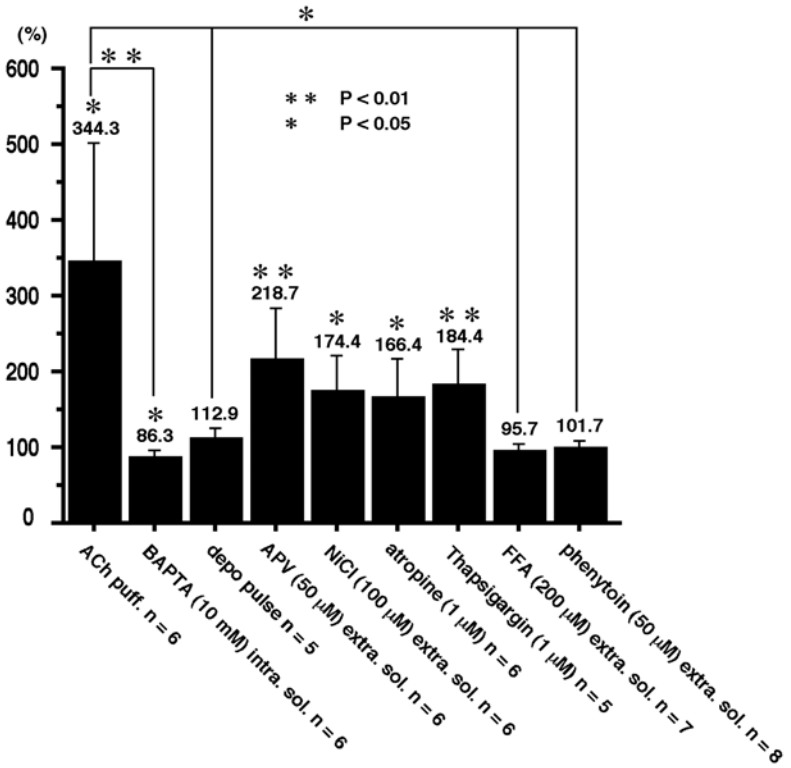

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Fig. 4

Fig. 4 Average ratio of the integrated amplitude of the EPSPs relative to the control EPSPs under various pharmacological manipulations preceded by ACh application or preceded by current pulses shown in Fig. 3. The control response was obtained by averaging three EPSPs without the ACh application or the current injection. Statistical significance was examined by Student's t-test (**; $p < 0.01$, *; $p<0.05$).

Fig. 5

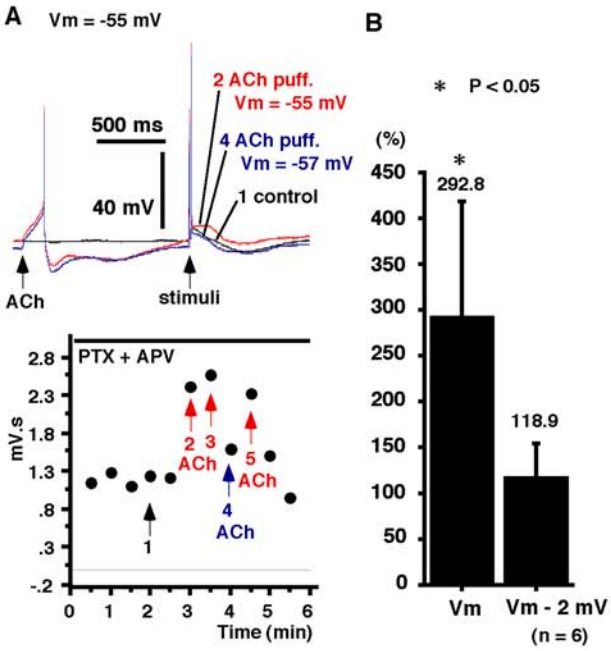

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Fig. 5 The voltage dependency of the effect of preceding ACh application on EPSPs. **A.** When ACh was applied at the membrane potential (Vm) of -55 mV, the amplitude of EPSPs was enhanced (red line in the top panel and red arrows 2, 3 and 5). In contrast, when ACh was applied at the membrane potential of -57 mV, the enhancement was not observed (blue line in the top panel and blue arrow 4 in the bottom panel). Other details as in Fig. 1**B**. Control record without ACh application (black arrow 1 in the bottom panel) is indicated as a black line in the top panel. **B.** The averaged ratio of the integrated amplitude of the EPSPs relative to the control EPSPs recorded without the preceding ACh application. Average effect of the preceding ACh application on the EPSPs recorded at the threshold level of the enhancement effect (recorded at -54.5 +/- 1.8 mV, mean +/- S.D. mV, n = 6) and recorded 2 mV hyperpolarized to the threshold level (Vm-2mV, n = 6). Statistical significance was examined by Student's t-test (*; $p<0.05$).

7. General Discussion

In the present series of studies, we have clarified the cellular mechanism for activation of the FFA- and phenytoin sensitive current and its role in modulation of the DA neuron activities. However, we have to admit that we have not proven that this FFA- and phenytoin-sensitive current is I_{CAN} because we could not investigate the ion selectivity of this channel due to technical difficulties in recording its reversal potential. In addition, FFA and phenytoin are not selective antagonists of I_{CAN}. They affect various cellular processes in addition to antagonistic action to the channels. Based on these concerns, it may be claimed that the negative slope conductance of FFA- and phenytoin-sensitive current is not that of I_{CAN}. However, in this regard, several other results supported our hypothesis. First, the current-voltage relationship of the FFA- and phenytoin-sensitive current is completely different from that of nAChRs (Yawo, 1989; Mathie et al., 1990). Moreover, FFA did not suppress the nAChRs in the Ca^{2+}-free extracellular solution. These results suggested that FFA or phenytoin did not suppress nAChRs themselves (part 1, Fig. 5 and Fig. 6). Next, it could be supposed that the negative slope conductance may be caused by the effect of FFA and phenytoin on the Ca^{2+} activated K^{+} channels. However, the negative slope conductance of the FFA- and

phenytoin-sensitive current was detected under the condition that the intracellular solution contained Cs^+ and the extracellular solution contained apamine, an antagonist of Ca^{2+} activated K^+ channels, suggesting the negative slope conductance was not induced by the enhancement of Ca^{2+} activated K^+ conductance (SK type) (part 1, See discussion).

On the other hand, coactivation of cholinergic and glutamatergic inputs to DA neurons would induce a dramatic change from tonic single spikes to multiple burst spikes, and this process is sensitive to FFA, suggesting that the negative slope conductance of the FFA-sensitive current would contribute to the generation of burst firing (part 1 and part 2). Finally, it may be possible that the current-voltage relationship of low threshold type Ca^{2+} channel (T type) might contribute to such negative slope conductance. But we have shown that the enhancement of EPSPs by ACh application could be observed after T type Ca^{2+} channels were blocked by NiCl (part 3). These results have suggested that the negative slope conductance of FFA- and phenytoin-sensitive current is attributed to I_{CAN}.

The present series of studies would make a contribution to the understanding of the

functions of DA neurons by elucidating three aspects described below. First, mesopontine DA neurons showed a continuum of patterned activity that ranges from tonic single spikes to multiple bursting spike discharge (Grace and Bunney, 1984). The mechanism of burst firing has attracted a keen interest of many researchers because of the physiological and patho-physiological importance of DA neurons (Dani and Heinemann, 1996; Schultz, 1998; Olanow and Tatton, 1999). But it remained elusive (White, 1996; Overton and Clark, 1997; Kitai et al., 1999). Our results have suggested that FFA- and phenytoin-sensitive current might induce the burst firing of DA neurons by its negative slope conductance (part 1 and part 2). Thus, cholinergic inputs to DA neurons might modulate the activity of DA neurons by the activation of the FFA- and phenytoin-sensitive current, presumably I_{CAN}. Second, other report suggested that I_{CAN} would be activated by CaM (Perrier et al., 2000). And we have reported that FFA- and phenytoin-sensitive current might be activated by Ca^{2+}/CaM-CaMKII pathway (part 2). But our report might be the first one that suggested the I_{CAN}-like current depends not only on Ca^{2+} and CaM but also CaMKII. Thus, we showed that the FFA- and phenytoin-sensitive current might be a novel substrate of CaMKII, in addition to

Glutamate receptors. Third, it is generally known that cholinergic EPSPs have been less frequently reported in CNS (Role and Burg, 1996). Of those reported, the amplitude is very small (Roerig et al., 1997; Hefft et al., 1999). In fact, we could not record cholinergic EPSPs in DA neurons (part 3, See discussion). Our results suggested that cholinergic input might function by way of diffuse transmission, which was mimicked by a bath application of its agonist (part 1 and part 2). Coactivation of transient glutamatergic inputs and bath application of carbachol could lead to the burst firing of DA neurons by the negative slope conductance of FFA- and phenytoin-sensitive current. These results have suggested that the "diffuse" cholinergic system exerts a dynamic modulatory action on the "point-to-point" glutamatergic system.

Taken together, our reports have clarified several novel aspects related to the following issues; the cellular mechanism of burst firing of DA neurons, the downward cascade of cholinergic inputs, I_{CAN} as a new target of CaMKII and the diffuse transmission of ACh. Many problems have remained unsolved, but we believe a series of our reports would be a milestone on the way of studying such conundrums.

Conclusion

The modulation of firing pattern of DA neurons has attracted interests of many researchers and has intensively been investigated for many years (White, 1996; Overton and Clark, 1997; Kitai et al., 1999). The series of our three reports has tried to clarify such problem. Our reports have suggested that the Ca^{2+} influx through somatodendritic nAChRs subsequently activate the FFA- and phenytoin-sensitive current, presumably I_{CAN}. This current could induce the burst firing of DA neurons by its property of negative slope conductance. In addition, the activation of this current would be activated not only Ca^{2+} but also Ca^{2+} /CaM-CaMKII pathway. Furthermore cholinergic input to DA neurons are supposed to modulate glutamatergic EPSPs by the coincidental activation of this current. Taken together, our results have suggested the burst firing of DA neurons might be induced by the activation of I_{CAN} caused by their cholinergic inputs.

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