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Abbreviations

$[Ca^{2+}]_i$,intracellular Ca^{2+} concentration

FFA, flufenamic acid

I-mGluR, group I metabotropic glutamate receptor

NSC, non-selective cation

oxo-M, oxotremorine M

PLC, phospholipase C

TTX, tetrodotoxin

Abstract

Signaling pathways involving phospholipase C (PLC) are involved in various neural functions. Understanding how these pathways are regulated will lead to a better understanding of their roles in neural functions. Previous studies demonstrated that receptor-driven PLCβ activation depends on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), suggesting the possibility that PLCβ-dependent cellular responses are basically Ca^{2+} dependent. To test this possibility, we examined whether modulations of ion channels driven by PLC-coupled metabotropic receptors are sensitive to $[Ca^{2+}]_i$ using cultured hippocampal neurons. Muscarinic activation triggered an inward current at -100 mV (the equilibrium potential for K^+) in a subpopulation of neurons. This current response was suppressed by pirenzepine (an M_1–preferring antagonist), PLC inhibitor, non-selective cation channel blocker, and lowering $[Ca^{2+}]_i$. Using the neurons showing no response at -100 mV, effects of muscarinic activation on K^+ channels were examined at -40 mV. Muscarinic activation induced a transient decrease of the holding outward current. This current response was mimicked and occluded by XE991, an M-current K^+ channel blocker, suppressed by pirenzepine, PLC inhibitor and lowering $[Ca^{2+}]_i$, and enhanced by elevating $[Ca^{2+}]_i$. Similar results were obtained when group I metabotropic glutamate receptors were activated instead of muscarinic receptors. These results clearly show that ion channel modulations driven by PLC-coupled metabotropic receptors are dependent on $[Ca^{2+}]_i$, supporting the hypothesis that cellular responses induced by receptor-driven

PLCβ activation are basically Ca^{2+} dependent.

Key Words: calcium dependence, channel modulation, phospholipase C, hippocampal

neuron, K$^+$ channel, non-selective cation channel

1. Introduction

Phosphoinositide-specific phospholipase C (PLC) enzymes are involved in a number of

cellular responses to receptor activation (Rebecchi and Pentyala, 2000; Rhee, 2001; Suh

et al., 2008). Among six families of PLC enzymes (β, γ, δ, ϵ, ζ and η), PLCβ family

members (PLCβ1-4) are driven by a wide range of G$_{q/11}$-coupled receptors including

M$_1$/M$_3$ muscarinic acetylcholine receptors, group I metabotropic glutamate receptors

(I-mGluRs), α_1 adrenergic receptors, and the receptors for cholecystokinin, bradykinin,

vasopressin, angiotensin II and thromboxane A$_2$ (Oude Weernink et al., 2007; Rebecchi

and Pentyala, 2000; Rhee, 2001; Werry et al., 2003). The PLCβ enzymes play critical

roles in neural functions, and PLCβ1- and PLCβ4-knockout mice develop epilepsy and

ataxia, respectively (Kim et al., 1997). The G$_{q/11}$-coupled receptor-PLCβ signaling

pathways contribute to neuronal functions through multiple second messengers that cause

modulation of ion channels (Anwyl, 1999; Hernandez et al., 2008; Hughes et al., 2007;

Suh and Hille, 2005), Ca^{2+} release from internal stores (Ross et al., 2005),

endocannabinoid release (Kano et al., 2009) and activation of enzymes (Amadio et al., 2006; Delmas et al., 2004). Understanding how these signaling pathways are regulated will undoubtedly lead to a better understanding of their roles in brain functions. We previously found that $G_{q/11}$-coupled receptor-driven endocannabinoid release, which requires PLCβ activity, was influenced by intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Hashimotodani et al., 2005; Maejima et al., 2005), and that this Ca^{2+} dependence was attributable to the Ca^{2+} dependence of receptor-driven PLCβ activation (Hashimotodani et al., 2005). These observations have raised the question of whether cellular responses elicited by receptor-driven PLCβ activation are all Ca^{2+} dependent, which has not been fully elucidated.

Activation of $G_{q/11}$-coupled receptors induces activation of non-selective cation (NSC) channels and inhibition of K^+ channels (Delmas et al., 2004; Delmas and Brown, 2005; Moran et al., 2004), both of which contribute to depolarization, in various types of neurons including hippocampal neurons (Chiang et al., 2010; Congar et al., 1997; Gee et al., 2003). In the present study, we used these channel modulations as examples of cellular responses elicited by receptor-driven PLCβ activation, and examined whether they are Ca^{2+} dependent. Using cultured hippocampal neurons, we observed that ion channel modulations driven by muscarinic receptors and I-mGluRs are dependent on $[Ca^{2+}]_i$. Importantly, the Ca^{2+} dependence of muscarinic inhibition of K^+ channels was similar to that of muscarinic receptor-mediated PLCβ activation, indicating that the Ca^{2+}

depencence of K$^+$ channel inhibition can be explained by the Ca^{2+} depencence of PLCβ

activation. These data are consistent with the hypothesis that cellular responses induced

by receptor-driven PLCβ activation are basically Ca^{2+} dependent.

2. Results

2.1. PLC dependence of muscarinic depolarization

First we examined effects of muscarinic receptor activation on the resting membrane

potential in cultured hippocampal neurons. In the presence of the Na$^+$ channel blocker

tetrodotoxin (TTX), the resting membrane potential was -64.0 ± 0.9 mV (n = 21). When

10 μM oxotremorine M (oxo-M), a muscarinic agonist, was locally applied for 10 s,

depolarization was induced in almost all neurons tested (Fig. lA, C). The averaged peak

amplitude of depolarization was 11.5 ± 2.0 mV (n = 21) (Fig. 1B). The oxo-M-induced

depolarization was markedly suppressed in the neurons that were pretreated with the PLC

inhibitor U73122 (10 μM, 10-30 min) (Fig. 1). In these neurons, the averaged amplitude

of depolarization was 1.3 ± 0.3 mV (n = 8) (Fig. 1B). As mechanisms of muscarinic

depolarization, potential candidates are inhibition of K$^+$ channels and activation of NSC

channels. In the following experiments, these two components were separately examined

by measuring current responses to oxo-M at -100 mV and -40 mV in voltage-clamp

mode.

2.2. PLC and Ca^{2+} dependence of the current response to oxo-M at -100 mV

To minimize the contribution of K^+ channel-derived component to current responses, the

membrane potential was clamped at -100 mV, which is close to the K^+ equilibrium

potential. At this potential, the holding current is expected to be insensitive to K^+

conductance change. Out of 13 neurons that were dialyzed with pCa 7-solution,

application of 3 μM oxo-M for 5 s induced an appreciable inward current (> 25 pA) in

only two neurons (Fig. 2C). At 10 μM, oxo-M application induced the inward current

more often (16 out of 26 cells). The amplitude of current responses varied from neuron to

neuron (Fig. 2C). Figure 2B shows the time course of current response to 10 μM oxo-M,

which was obtained by averaging ten current responses larger than 50 pA after

peak-normalization. The treatment with U73122, but not the inactive analog U73343,

almost completely abolished the current response to 10 μM oxo-M (Fig. 2D). The

M_1–preferring antagonist pirenzepine (1 μM) and NSC channel blocker flufenamic acid

(FFA) also markedly suppressed the current response (Fig. 2E-H). These results indicate

that activation of muscarinic receptors sensitive to 1 μM pirenzepine causes opening of

NSC channels in a PLC-dependent manner.

Next we examined the Ca^{2+} dependency of this process, by using pCa 7- and

pCa 9-pipette solutions. In this series of experiments, oxo-M was applied in the absence

of external Ca^{2+} in order to minimize possible Ca^{2+} influx through Ca^{2+}-permeable NSC

channels. In the neurons dialyzed with pCa 7-solution, application of 3 μM oxo-M in the absence of Ca^{2+} induced an appreciable inward current much more often (Fig. 3; pCa7) than in the presence of 2 mM Ca^{2+} (Fig. 2C; 3 oxo). This enhancement of current responses in the absence of external Ca^{2+} is the feature suggestive of NSC channels, as it is well known that the inward current through NSC channels is augmented when external Ca^{2+} ions are removed (Ishibashi et al., 2003; Lintschinger et al., 2000; Tsujino et al., 2005). In the neurons dialyzed with pCa 9-solution, no appreciable currents were induced by application of 3 μM oxo-M in the absence of Ca^{2+} (Fig. 3). These results indicate that the muscarinic activation of NSC channels requires intracellular Ca^{2+} ions. Further experiments were not performed to study the Ca^{2+} dependence of this process, because NSC channels are a diverse group of proteins including transient receptor potential (TRP) superfamily members and Ca^{2+} ions have been reported to exert variable effects on them (Blair et al., 2009; Lintschinger et al., 2000; Moran et al., 2004), which will make interpretation of experimental data difficult.

2.3. PLC and Ca^{2+} dependence of the current response to oxo-M at -40 mV

Muscarinic effects on outward K^+ currents were examined at a holding potential of -40 mV. To minimize contamination by current responses arising from NSC channels, neurons showing appreciable current responses (> 25 pA) to oxo-M application at -100 mV were not used for this series of experiments. In the absence of oxo-M, the outward

holding current at -40 mV was 171.6 ± 18.2 pA (n = 19). When 3 μM oxo-M was applied

for 5 s, the outward current was transiently decreased in most neurons (Fig. 4). In rare

cases (< 10 %), however, an increase in the outward current was observed. Because of

this rarity, it was difficult to examine this phenomenon satisfactorily and the data from the

neurons showing an increase in outward current more than 10 pA by oxo-M application

were excluded from analysis of the present study. In the neurons used for analysis,

application of 3 μM oxo-M decreased the outward current, on average, by 92.4 ± 14.2 pA

(n = 19). The time course of this current response (Fig. 4B) was slower than that observed

at -100 mV (Fig. 2B). Application of 10 μM oxo-M decreased the outward current by

118.4 ± 31.1 pA (n = 5), which was not significantly different from the value obtained

with 3 μM oxo-M. Pretreatment of neurons with U73122, but not the inactive analog

U73343, significantly suppressed the current response to 1 μM oxo-M (Fig. 4D). In this

set of experiments, we used a low dose (1 μM) of oxo-M in the hope of making the

response more sensitive to PLC inhibition. We also confirmed that the current response at

-40 mV was suppressed by 1 μM pirenzepine (Fig. 4E, F). A lower dose of pirenzepine

(0.1 μM) was less effective, and decreased the amplitude of current response by 31.9 ±

5.3 % (n = 21).

M-current is known to be regulated by $G_{q/11}$-coupled receptors including

muscarinic receptors and partly active at -40 mV. Molecular correlates of M-current,

Kv7/KCNQ subunits (M-channels), are widely expressed in the brain, and M-current has

been recorded in various types of neurons including hippocampal neurons (Brown and Adams, 1980; Brown and Passmore, 2009; Halliwell and Adams, 1982; Lawrence et al., 2006). Thus, it is most likely that muscarinic inhibition of M-channels is responsible for the current response observed at -40 mV. We examined whether the M-current blocker XE991 can mimic and occlude the effect of oxo-M on the outward holding current. In each neuron, we recorded current responses to each of oxo-M (3 μM) and XE991 (50 μM), and compared them (Fig. 5A, B). The amplitudes of the responses to these drugs were quite variable from neuron to neuron, but there was a good correlation between them (Fig. 5B). When oxo-M was applied in the presence of 50 μM XE991, no or minimal current responses were induced even if marked responses were induced in the absence of XE991 in the same neurons (Fig. 5C, D). These results strongly suggest that activation of muscarinic receptors causes the inhibition of XE991-sensitive K^+ channels, most likely M-channels, in a PLC-dependent manner in these neurons.

Using pipette solutions containing different Ca^{2+} concentrations, we examined the Ca^{2+} dependence of the K^+ channel inhibition (Fig. 6, 7). For comparison between the neurons dialyzed with pCa 7- and pCa 9-solutions, 3 μM oxo-M was applied in either the presence (2 mM) or absence of external Ca^{2+}. The current response to oxo-M was significantly attenuated by lowering the Ca^{2+} concentration of pipette solution from pCa 7 to pCa 9 (Fig. 6). In the presence of 2 mM Ca^{2+}, an appreciable current response (> 25 pA) was observed in 19 out of 19 neurons dialyzed with pCa 7-solution (the same data as

Fig. 4C), and five of 10 neurons with pCa 9-solution (Fig. 6B). On average, the amplitude

of the current response was decreased from 92.4 ± 14.2 pA (n = 19) to 31.2 ± 14.3 pA (n =

10). In the absence of external Ca^{2+}, the current response was observed in four out of six

neurons with pCa 7, but none of 14 neurons with pCa 9. On average, the amplitude was

decreased from 50.3 ± 15.9 pA (n = 6) to 7.0 ± 2.0 pA (n = 14).

In the next series of experiments, effects of raising $[Ca^{2+}]_i$ to pCa 6.5, which is

higher than the resting level, on current responses were examined by using a lower

concentration of oxo-M. In these experiments, 0.3 μM oxo-M was applied for 1 or 5 s in

the presence of 2 mM Ca^{2+}. The averaged amplitude of the current response to oxo-M was

significantly larger in the neurons dialyzed with pCa 6.5-soluton than in those with pCa

7.5-solution (Fig. 7). When oxo-M was applied for 1 s, the response (> 25 pA) was

observed in none of 10 neurons with pCa 7.5, and five of 10 neurons with pCa 6.5. When

applied for 5 s, the response was observed in two of 10 neurons with pCa 7.5, and seven

of 10 neurons with pCa 6.5 (Fig. 7B). On average, elevating the Ca^{2+} concentration from

pCa 7.5 to pCa 6.5 increased the amplitude of the current response from 7.1 ± 1.8 pA (n =

10) to 29.9 ± 9.7 pA (n = 10) for 1 s application, and from 13.1 ± 4.2 pA (n = 10) to $47.9 \pm$

12.7 pA (n = 10) for 5 s application. This increase in amplitude of the current response can

be simply interpreted as enhancement of the muscarinic inhibition of K^+ channels.

However, it is also possible that small conductance Ca^{2+}-activated K^+ (SK) channels

become active at pCa 6.5 and contribute to the current response. To test this possibility,

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effects of the SK channel inhibitor apamin were examined in the neurons dialyzed with

pCa 6.5-solution. Neither the outward holding current at -40 mV (P = 0.30) nor the

current response to oxo-M (P = 0.70 for 1 s; P = 0.78 for 5 s) was significangly changed

by treatment with 1 μM apamin (Fig. 7B, right), indicating that contribution of SK

channels to the enhanced response in pCa 6.5-neurons is negligible. This ineffectiveness

of apamin under our conditions is consistent with the previous study reporting that SK

channels are almost closed at pCa 6.5 (Maylie et al., 2004). Taken together, our data

clearly show that muscarinic inhibition of K^+ channels is highly sensitive to $[Ca^{2+}]_i$

within the physiological range.

2.4. Ca^{2+} dependence of the current response to DHPG at -100 mV and -40 mV

Similar experiments were performed with the I-mGluR agonist

(RS)-3,5-dihydroxyphenylglycine (DHPG) instead of oxo-M . In five out of 20 neurons,

application of 50 μM DHPG for 5 s induced an appreciable inward current (> 25 pA) at

-100 mV (Fig. 8A, B). On average, the amplitude of current response to DHPG was 43.3

± 22.7 pA (n = 20). In the neurons showing no appreciable responses at -100 mV, effects

of DHPG on the holding outward current at -40 mV were examined. Application of 50

μM DHPG decreased the outward current by 29.7 ± 5.4 pA (n = 15) (Fig. 8A, B).

Next, the Ca^{2+} dependence of the current response at -100 mV was examined.

In this series of experiments, 50 μM DHPG was applied in the absence of external Ca^{2+}.

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As observed for the response to oxo-M, the current response to DHPG was larger in the absence of external Ca^{2+} (Fig. 8C, D; pCa 7) than in the presence of 2 mM Ca^{2+} (Fig. 8A, B; -100 mV). The current response (> 25 pA) was observed in eight of 13 neurons. When the Ca^{2+} concentration of the pipette solution was changed from pCa 7 to pCa 9, the response to DHPG was dramatically attenuated (Fig. 8C, D). The amplitude of the current response was 462.1 ± 181.1 pA (n = 13) in the neurons dialyzed with pCa 7-solution, and 43.0 ± 23.9 pA (n = 12) in those with pCa 9.

The Ca^{2+} dependence of the current response at -40 mV was examined by changing $[Ca^{2+}]_i$. Because of methodological problems resulting from dominant coupling to NSC channels and distal distribution of I-mGluRs, we conducted experiments only with pCa 7.5 and pCa 6.5. First, the current response to 50 μM DHPG was compared between the neurons dialyzed with pCa 7.5- and pCa 6.5-solutions. The amplitude of the current response was 14.4 ± 2.2 pA (n = 9) in the neurons dialyzed with pCa 7.5-solution, and 35.6 ±11.9 pA (n = 8) in those with pCa 6.5. The mean amplitude was larger with pCa 6.5, but the difference was not significant. Considering the possibility that 50 μM DHPG might induce full response even at pCa 7.5, and the possibility that local Ca^{2+} concentration might be insufficiently clamped because of Ca^{2+} pump and store function, we decreased the DHPG concentration to 5 μM and pretreated the neurons with thapsigargin (1 μM, > 60 min) to deplete Ca^{2+} stores. The current response to 5 μM DHPG at -40 mV was slightly larger in the neurons dialyzed with pCa 6.5 than in those

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with pCa 7.5, but this difference was again not statistically significant (Fig. 8E, F).

Because I-mGluRs are enriched in dendritic spines (Lujan et al., 1996), it seems likely

that local $[Ca^{2+}]_i$ just beneath the membrane containing I-mGluRs is not sufficiently

elevated even if neurons are dialyzed with pCa 6.5-solution. In order to elevate local

$[Ca^{2+}]_i$, therefore, a voltage pulse from -80 mV to 0 mV for 3 s was applied just before

DHPG application. In this series of experiments, neurons were pretreated with 1 μM

apamin and dialyzed with pCa 7-solution. The DHPG concentration (1 or 5 μM) and

application duration (1 or 5 s) were adjusted to induce a small response (< 30 pA) when

DHPG was applied alone. Figure 8G shows an example of the experiments. In this neuron,

the current responses to DHPG application (1 μM, 1 s) alone (9.9 pA) and to

depolarization alone (10.6 pA) were small, but the response to DHPG application just

after depolarization (29.3 pA) was larger than the former two responses and even larger

than the sum of them (20.5 pA). To cancel the effect of depolarization itself on the

holding current, the current response to depolarization alone was removed from the

response to the combination of depolarization and DHPG application in each neuron (Fig.

8H; "3-2"), which will reflect the current response to DHPG at elevated $[Ca^{2+}]_i$. This

value was significantly larger than the current response to DHPG application alone (Fig.

8H). These results strongly suggest that the channel modulation driven by I-mGluRs as

well as muscarinic receptors is enhanced by elevation of $[Ca^{2+}]_i$.

3. Discussion

In the present study, we investigated whether channel modulations triggered by activation of muscarinic receptors and I-mGluRs are sensitive to $[Ca^{2+}]_i$ in cultured hippocampal neurons. First we identified two types of channel modulations upon muscarinic activation. One is activation of NSC channels, and the other is inhibition of K^+ channels. Both of them were confirmed to be blocked by a PLC inhibitor. We then demonstrated that these channel modulations are sensitive to $[Ca^{2+}]_i$, by using patch pipette solutions with different Ca^{2+} concentrations. Finally, we examined channel modulations induced by I-mGluR activation and found that they are also Ca^{2+} dependent. These findings support the hypothesis that cellular responses induced by receptor-driven PLCβ activation are basically Ca^{2+} dependent.

In the present study, we used U73122 as a PLC inhibitor. U73122 is widely used for identification of PLC involvement, but it has been shown to be highly non-specific (Hashimotodani et al., 2008; Horowitz et al., 2005; Won et al., 2009). Therefore, we performed control experiments using the inactive analog U73343. Our data showing the clear difference in effects between U73122 and U73343 strongly suggest the involvement of PLC. Previously, we used PLCβ1-, M_1-, M_3- and M_1/M_3-knockout mice, and demonstrated that in cultured hippocampal neurons PLCβ1 and M_1/M_3 muscarinic receptors play critical roles in muscarinic responses including endocannabinoid release

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and exogenous TRPC6 activation (Fukudome et al., 2004; Hashimotodani et al., 2005; Ohno-Shosaku et al., 2003). Therefore, it is most likely that these molecules are also involved in channel modulations by muscarinic activation. Experiments with knock-out mice will be needed to verify involvement of these molecules.

The Ca^{2+} dependency of receptor-driven PLCβ activation has been supported by several lines of evidence. Biochemical studies have shown that the activity of PLC enzymes including the PLCβ family is Ca^{2+} dependent (Biddlecome et al., 1996; Rebecchi and Pentyala, 2000). In rat cultured cerebellar granule cells, carbachol-stimulated inositol phosphate accumulation, which reflects PLC activity, was shown to be highly sensitive to small changes in $[Ca^{2+}]_i$ (del Rio et al., 1994). An electrophysiological study using exogenous TRPC6 channel as a biosensor for the PLC product diacylglycerol in our laboratory demonstrated that PLCβ1 activation driven by muscarinic receptors (Fig. 9A) and I-mGluRs is sensitive to $[Ca^{2+}]_i$ within the physiological range in rat culured hippocampal neurons (Hashimotodani et al., 2005). The studies using fluorescent probes for membrane lipids have also reported that muscarinic receptor-mediated activation of PLC is sensitive to $[Ca^{2+}]_i$ within the physiological range in human embryonic kidney tsA-201 cells (Horowitz et al., 2005) and neuroblastoma SH-SY5Y cells (Nelson et al., 2008).

The Ca^{2+} dependence of PLCβ-mediated cellular responses has also been reported. In Chinese hamster ovary cells stably expressing noradrenergic α_{1B} and M_3

receptors, receptor-mediated IP_3 production was shown to be sensitive to changes in

$[Ca^{2+}]_i$ between basal and 200 nM (Young et al., 2003). Endocannabinoid release driven

by PLCβ-coupled receptors including M_1/M_3 muscarinic receptors and I-mGluRs has

been demonstrated to be Ca^{2+} dependent and enhanced by elevation of $[Ca^{2+}]_i$ in many

types of neurons (Kano et al., 2009) including hippocampal neurons (Hashimotodani et

al., 2005) and cerebellar Purkinje cells (Maejima et al., 2005). The Ca^{2+} dependence of

muscarinic inhibition of M-channels has also been reported (Beech et al., 1991; Horowitz

et al., 2005; Shapiro et al., 2000; Yu et al., 1994). In rat sympathetic neurons, intracellular

11-12 mM BAPTA reduced the M-current inhibition by 10 μM oxo-M from 82 % to 15 %

(Beech et al., 1991). In bullfrog sympathetic neurons, application of 1 μM and 10 μM

muscarine inhibited the M-current by 29 % and 38 %, respectively, at 0 Ca^{2+} (no added

Ca^{2+}), and by 69 % and 77 % at 120 nM Ca^{2+} (Yu et al., 1994). In these studies, the

authors suggested that the muscarinic inhibition of M-current has Ca^{2+} -dependent steps,

although they were not identified. In tsA-201 cells expressing M_1 receptors and the

M-channel subunits KCNQ2 and KCNQ3, muscarinic inhibition of M-channels was

shown to be Ca^{2+} dependent (Horowitz et al., 2005; Shapiro et al., 2000). The data

showed that M-channel inhibition requires some free Ca^{2+} , is induced to some degree at

the resting $[Ca^{2+}]_i$ level, and is potentiated when $[Ca^{2+}]_i$ is elevated above the resting

level. In the present study, we used hippocampal neurons and demonstrated that

muscarinic inhibition of K^+ channels, most likely M-channels, is dependent on $[Ca^{2+}]_i$

within the physiological range. The Ca^{2+} dependence of K^+ channel inhibition observed here appears similar to that of muscarinic activation of PLCβ1 reported previously (Hashimotodani et al., 2005) (Fig. 9B), indicating that the Ca^{2+} dependence of K^+ channel inhibition can be explained by the Ca^{2+} depencence of PLCβ activation. Furthermore, we examined whether channel modulations driven by other PLC-coupled receptors are also Ca^{2+} dependent. We found that the activation of I-mGluRs, like muscarinic activation, induced the activation of NSC-like channels and inhibition of K^+ channels, and that these channel modulations were influenced by changing $[Ca^{2+}]_i$. Our electrophysiological data so far obtained with cultured hippocampal neurons are all consistent with the hypothesis that cellular responses induced by receptor-driven PLCβ activation are intrinsically dependent on $[Ca^{2+}]_i$.

Many studies have shown the Ca^{2+}-dependent property of NSC channel activation (Blair et al., 2009; Congar et al., 1997; Fraser and MacVicar, 1996; Gee et al., 2003; Magistretti et al., 2004; Yan et al., 2009; Zhang et al., 2011), and it has been mostly interpreted as the Ca^{2+} dependence of NSC channel itself. Our data do not negate the Ca^{2+} dependence of NSC channel itself, but suggest that caution must be exercised in interpreting experimental data (Blair et al., 2009). Further studies are needed to analyze multiple Ca^{2+}-dependent processes separately.

The functional significance of the Ca^{2+} dependent nature of receptor-driven PLCβ activation remains to be clarified, but one can speculate that it serves to make the

response regenerative and activity dependent (Hashimotodani, 2005, Horowitz, 2005).

Activation of PLCβ induces an elevation of Ca^{2+} through activation of Ca^{2+}-permeable

NSC channels or IP_3 receptors, which in turn facilitates PLCβ activation. This positive

feedback loop enables the regenerative activation of the signaling pathway, if PLCβ and

NSC channels or IP_3 receptors are located close together. The existence of this feedback

loop is supported by experimental evidence showing attenuation of cellular responses to

$G_{q/11}$-coupled receptor activation by prior emptying of intracellular Ca^{2+} stores with

thapsigargin. For example, thapsigargin-treatment reduced muscarinic receptor-driven

PLC activation and M-channel inhibition in tsA-201 cells (Horowitz et al., 2005),

endocannabinoid release driven by protease-activated receptor 1, which is a

$G_{q/11}$-coupled receptor and stimulates PLCβ (McLaughlin et al., 2005; Ossovskaya and

Bunnett, 2004), in cultured hippocampal neurons (Hashimotodani et al., 2011), and NSC

channel activation driven by α_1 adrenergic receptors in parasympathetic neurons

(Ishibashi et al., 2003), and slowed the M-channel inhibition driven by histamine H_1

receptors in human embryonic kidney HEK293 cells (Liu et al., 2008). These results

suggest that cellular responses driven by $G_{q/11}$-coupled receptors might be potentiated by

Ca^{2+} ions released from Ca^{2+} stores under physiological conditions. If PLCβ-dependent

cellular responses are induced in a regenerative manner through positive feedback loop,

this will make it difficult to quantitatively analyze its Ca^{2+} dependence under

physiological conditions. Increasing Ca^{2+} buffering capacity, removing external Ca^{2+} ions

and depleting Ca^{2+} stores would be helpful to disrupt the feedback loop. In the present study, using the former two treatments we successfully demonstrated the Ca^{2+} dependence of channel modulations. The Ca^{2+} dependency of PLCβ activation also enables the receptor-$G_{q/11}$-PLCβ signaling pathway to be activity dependent. Subthreshold activation of $G_{q/11}$-coupled receptors could induce PLCβ-dependent cellular responses only when it coincides with postsynaptic activity leading to Ca^{2+} elevation. The PLCβ-dependent endocannabinoid release driven by muscarinic receptors was shown to be actually activity dependent, and enhanced when combined with depolarization in hippocampal neurons (Hashimotodani et al., 2005). Therefore, we propose that cellular responses driven by $G_{q/11}$-coupled receptors may be potentially regenerative and activity dependent by virtue of the Ca^{2+} dependency of PLCβ activation, which remains to be tested in future studies.

4. Experimental Procedure

4.1. Preparation of neurons

All experiments were performed according to the guidelines laid down by the animal welfare committee of Kanazawa University. Cultured hippocampal neurons were prepared from newborn Sprague-Dawley rats, as described previously (Ohno-Shosaku et al., 2001). Briefly, following ether anesthesia, rats were decapitated and cells were mechanically dissociated from the hippocampi and plated onto culture dishes (35 mm)

pretreated with poly L-ornithine (0.01 %). The cultures were kept at 36 °C in 5 % CO_2 for

8-17 days before use.

4.2. Electrophysiology

Neurons were whole-cell clamped using patch pipettes (3 –5 MΩ) filled with an internal

solution. In current-clamp recordings, the pipettes were filled with the solution containing

(in mM) 120 K-gluconate, 15 KCl, 6 $MgCl_2$, 10 HEPES, 5 EGTA, 20 KOH, 5 Na_2ATP

and 0.2 Na_2GTP (pH 7.3, adjusted with KOH). In voltage-clamp recordings, we used

pipette solutions adjusted to < pCa 9 and pCa 7 with 10 mM EGTA or pCa 7.5 and pCa

6.5 with 10 mM BAPTA. The solution adjusted to < pCa 9 is referred to simply as pCa

9-solution. These solutions contained (in mM) 100 K-gluconate, 15 KCl, 6 or 5 $MgCl_2$,

10 HEPES, 10 EGTA or BAPTA, 5 Na_2ATP, 0.2 Na_2GTP and appropriate concentrations

of $CaCl_2$ (0 for pCa 9, 4.65 for pCa7, 1.24 for pCa 7.5, 5.87 for pCa 6.5). The pCa value

was calculated using a computer program (WinMAXC v2.45). The pH of each solution

was adjusted to 7.3 with KOH and the osmolarity was maintained at 290-300 mOsm with

K-gluconate. Resting membrane potentials and whole-cell currents were recorded at the

current- and voltage-clamp mode, respectively, with a patch-clamp amplifier (EPC9/3,

HEKA Electronics, Germany).

A standard external solution contained (in mM) 140 NaCl, 2.5 KCl, 2 $CaCl_2$, 1

$MgCl_2$, 10 HEPES and 10 glucose (pH 7.3, adjusted with NaOH). In some experiments,

we used a Ca^{2+}-free external solution in which $CaCl_2$ was replaced with $MgCl_2$. To suppress the generation of action potentials and spontaneous synaptic currents, $0.1\ \mu M$ TTX was added to the standard external solution. The recording chamber was perfused with the TTX-containing external solution at a flow rate of 1-3 ml/min. The muscarinic agonist oxo-M and the I-mGluR agonist DHPG were added to the TTX-containing external solution with or without 2 mM $CaCl_2$ and locally applied for 1-10 s through a capillary tube (250 μm inner diameter) using a perfusion valve controller (VC-6M, Warner Instruments, CT). The applied agonist was rapidly washed out by continuous bath perfusion. All experiments were performed at room temperature.

4.3. Drugs

Apamin, DHPG, oxo-M, pirenzepine and XE991 were purchased from Tocris Cookson, FFA and thapsigargin were from Sigma-Aldrich, and U73122 and U73343 were from Calbiochem. Pirenzepine, thapsigargin, U73122 and U73343 were dissolved in DMSO as stock solutions.

4.4. Data analysis

Two groups of data were compared using Student's paired or unpaired *t*-test or Welch's *t*-test and statistical significance was concluded if $P < 0.05$. Single, double and triple asterisks in figures indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. All data are

expressed as mean ± SEM.

Acknowledgements

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Figure Legends

Figure 1. Muscarinic activation induces a depolarization in a PLC-dependent manner.

(A) Representative examples showing depolarization induced by oxo-M application in control and U73122-treated neurons. (B) Averaged data for peak amplitudes of depolarization induced by 10 μM oxo-M in control and U73122-treated neurons. (C) Individual (open circles) and averaged (closed circles) data for resting membrane potential before and at the peak of depolarization induced by oxo-M application in control (left) and U73122-treated neurons (right).

Figure 2. Activation of muscarinic receptors causes the opening of NSC channels in a PLC-dependent manner.

(A) Representative examples showing current responses to 3 or 10 μM oxo-M at -100 mV. (B) Inward currents induced by 10 μM oxo-M were averaged after normalization to their peak values. (C) Individual (left) and averaged (right) data for the amplitude of oxo-M-induced inward currents at each condition. (D) Averaged data for the amplitude of 10 μM oxo-M-induced inward currents in neurons treated with U73122 or U73343. (E) Representative current responses to 10 μM oxo-M before and after addition of 1 μM pirenzepine. (F) Individual (left) and averaged (right) data for the amplitude of the current

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responses in these two conditions. Data from the same neurons are connected with lines.

(G) Representative current responses to 10 μM oxo-M before and after addition of 100

μM FFA. (H) Individual (left) and averaged (right) data for the amplitude of the current

responses in these two conditions. Data from the same neurons are connected with lines.

Figure 3. Suppression of the current response to muscarinic activation at -100 mV

by lowering $[Ca^{2+}]_i$.

(A) Representative examples of the current responses to 3 μM oxo-M in the neurons

dialyzed with pCa 7- and pCa 9-solutions. (B) Individual (left) and averaged (right) data

for the amplitude of oxo-M-induced inward currents in these neurons.

Figure 4. Activation of muscarinic receptors decreases the outward holding current

at -40 mV in a PLC-dependent manner.

(A) Representative examples of the current responses to 3 or 10 μM oxo-M. (B) The

current responses to 3 μM oxo-M were averaged after normalization to their peak values.

(C) Individual (left) and averaged (right) data for the amplitude of current responses at

each condition. (D) Averaged data for the amplitude of current responses to 1 μM oxo-M

in neurons treated with U73122 or U73343. (E) Representative current responses to 3 μM

oxo-M before and after addition of 1 μM pirenzepine. (F) Individual (left) and averaged

(right) data for the amplitude of current responses in these two conditions. Data from the

same neurons are connected with lines.

Figure 5. The M-current blocker XE991 mimics and occludes the current response to muscarinic activation at -40 mV.

(A) Representative current responses to 3 μM oxo-M and 50 μM XE991. (B) In each neuron, effects of oxo-M and XE991 were measured. There was a good correlation between these effects. (C) Representative current responses to oxo-M before and after addition of 50 μM XE991. (D) Individual (left) and averaged (right) data for the amplitude of current response to oxo-M in these two conditions. Data from the same neurons are connected with lines.

Figure 6. Suppression of the current response to muscarinic activation at -40 mV by lowering $[Ca^{2+}]_i$.

(A) Representative current responses to 3 μM oxo-M in the presence (2 mM) or absence of external Ca^{2+} recorded from the neurons dialyzed with pCa 7- and pCa 9-solutions. (B) Individual (left) and averaged (right) data for the amplitude of current responses to oxo-M in these conditions.

Figure 7. Enhancement of the current response to muscarinic activation at -40 mV by elevating $[Ca^{2+}]_i$.

(A) Representative current responses to application of 0.3 μM oxo-M for 1 or 5 s in the

neurons dialyzed with pCa 7.5- and pCa 6.5-solutions. (B) Individual (left) and averaged

(right) data for the amplitude of current responses at these conditions in control and

apamin-treated (black bars) neurons.

Figure 8. The Ca^{2+} dependence of current responses to activation of I-mGluRs at

-100 mV and -40 mV.

(A) Representative current responses to application of 50 μM DHPG for 5 s in the

neurons dialyzed with pCa 7-solution. (B) Individual (left) and averaged (right) data for

the amplitude of current responses at -100 mV and -40 mV. (C) Representative current

responses to 50 μM DHPG in the absence of external Ca^{2+} at -100 mV in the neurons

dialyzed with pCa 7- and pCa 9-solutions. (D) Individual (left) and averaged (right) data

for the amplitude of the current responses in these neurons. (E) Representative current

responses to application of 5 μM DHPG in the thapsigargin-treated neurons dialyzed

with pCa 7.5- and pCa 6.5-solutions. (F) Individual (left) and averaged (right) data for the

amplitude of current responses in these neurons. (G) Representative current responses at

-40 mV to application of 1 μM DHPG for 1 s (upper), a voltage pulse from -80 mV to 0

mV for 3 s (middle), and the combination of these two protocols (lower) in an

apamin-treated neuron. (H) In each neuron, the amplitude of current response to

depolarization alone was digitally subtracted from the amplitude of current response to

the combination of depolarization and DHPG application ("3-2"). The obtained value was compared with the amplitude of current response to DHPG alone ("1"). Individual (left) and averaged (right) data for these values.

Figure 9. A comparison between the present and our previous studies regarding the Ca^{2+} dependence of cellular responses to muscarinic activation in cultured hippocampal neurons.

(A) The Ca^{2+} dependence of PLCβ1 activation, which was monitored by TRPC6 channel activity (Hashimotodani et al., 2005). (B) The data showing the Ca^{2+} dependence of K$^+$ channel inhibition (Fig. 6B and Fig. 7B) obtained in the present study (closed circles) are superimposed on the Ca^{2+} dependence of PLCβ1 activation (open circles) shown in (A). The mean values of K$^+$ channel inhibition for pCa 7 and pCa 6.5 were adjusted to fit the curve for the Ca^{2+} dependence of PLCβ1 activation, and the values for pCa 9 and pCa 7.5 were calculated, respectively.
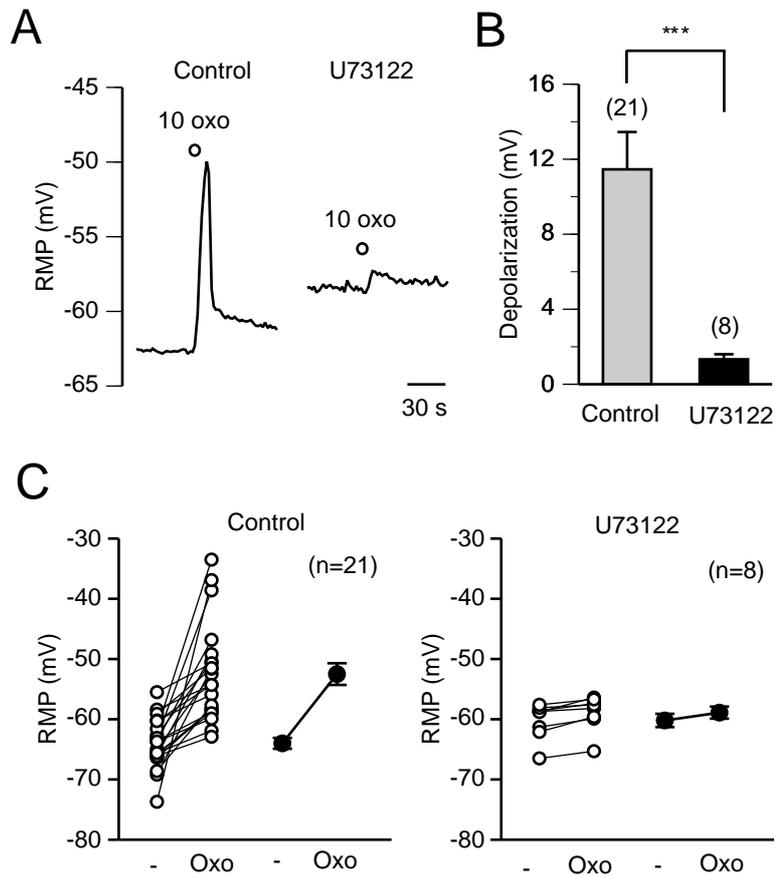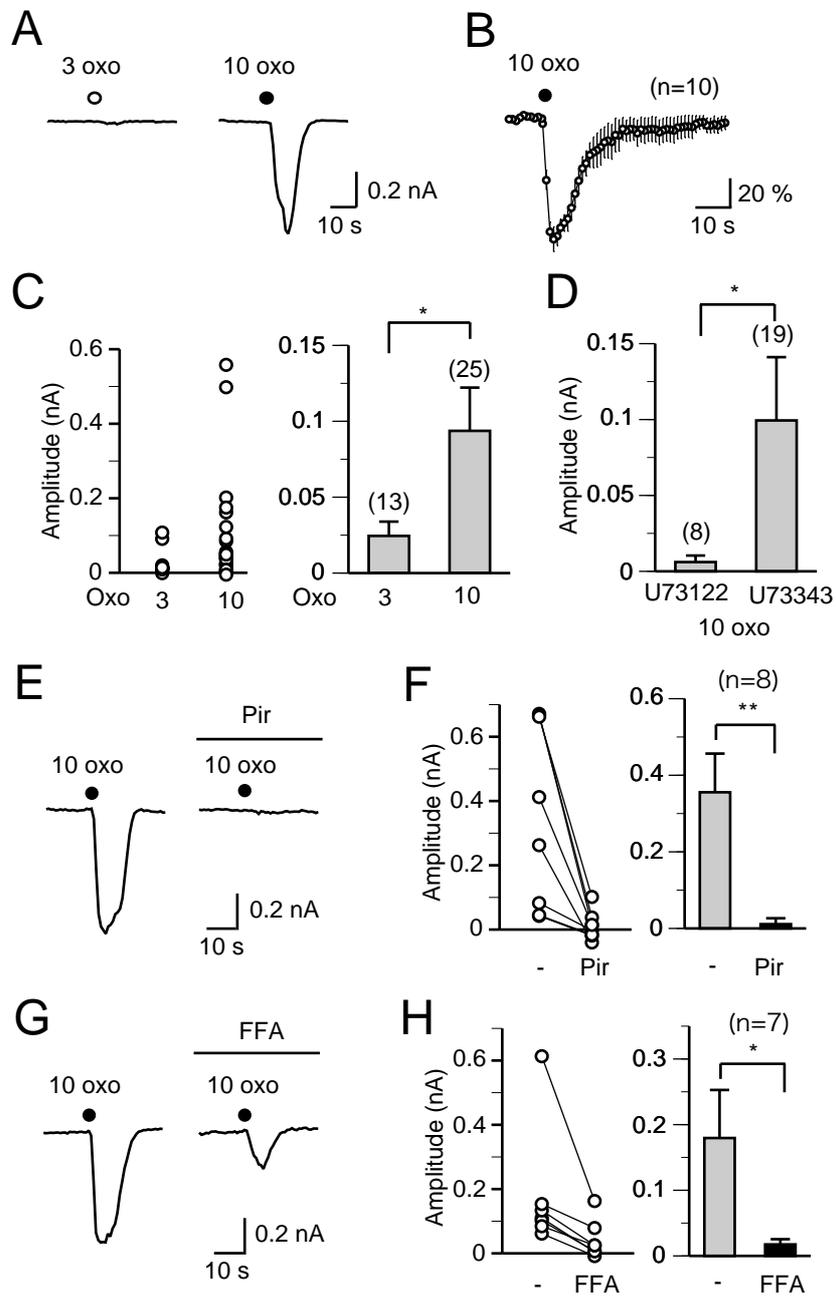
Figure 1 (Y. Sugawara et al.)

Figure 2 (Y. Sugawara et al.)

A

pCa7   pCa9
3 oxo (Ca-free)   3 oxo (Ca-free)

50 pA
10 s

B

Int   pCa7   pCa9
Ext   0 Ca

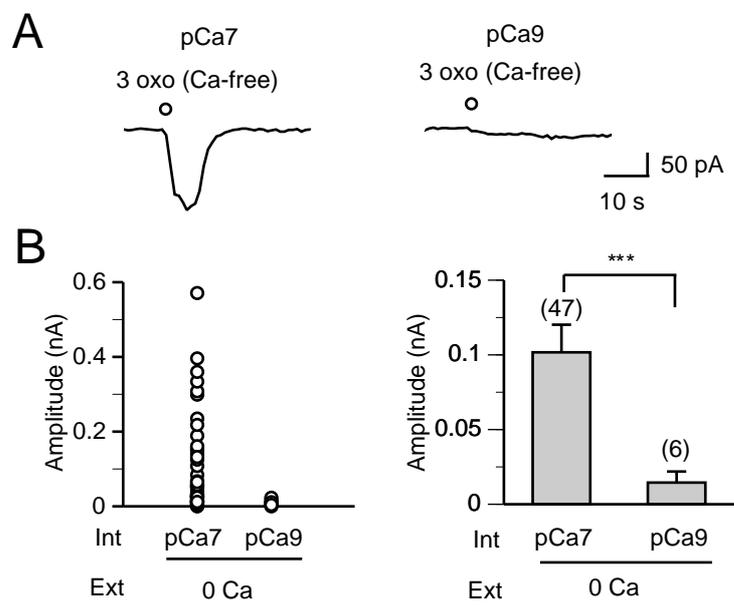Int   pCa7   pCa9
Ext   0 Ca
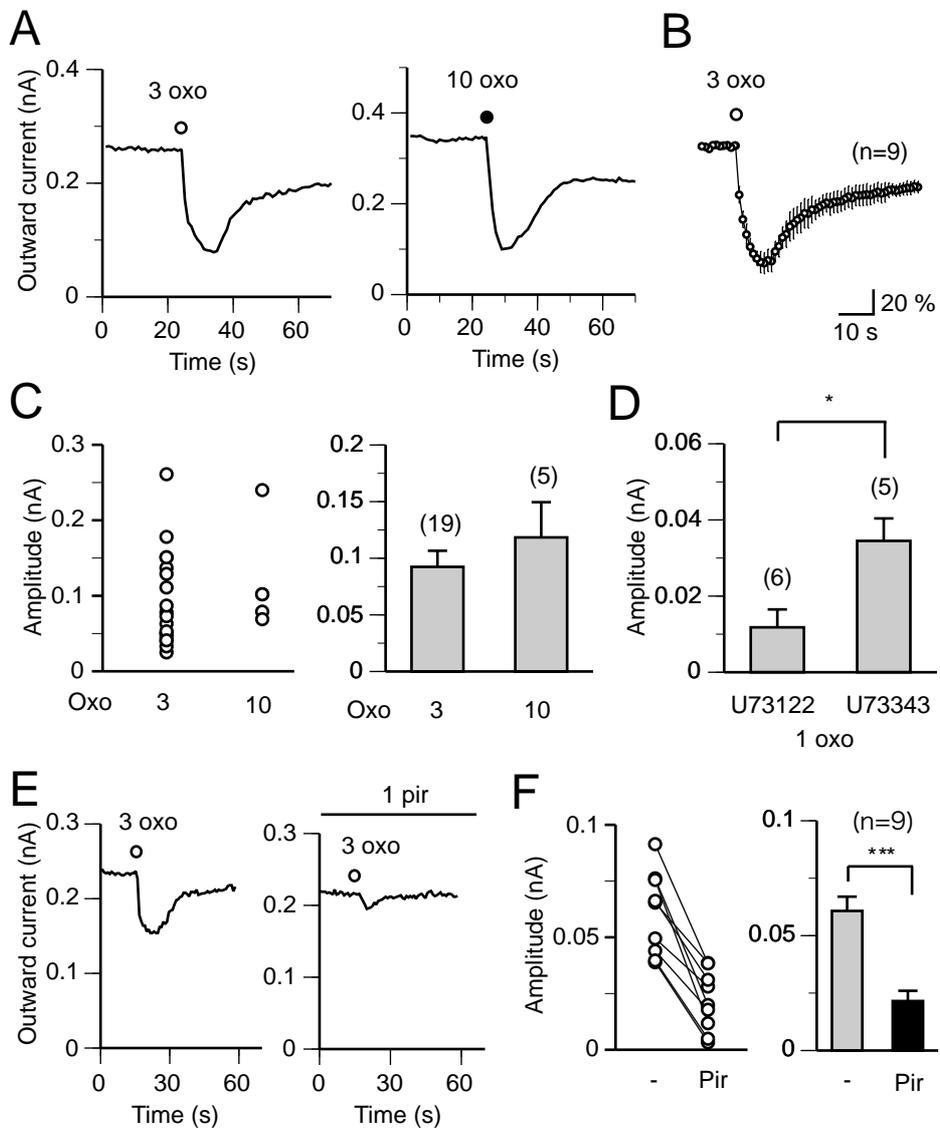
(47)
(6)
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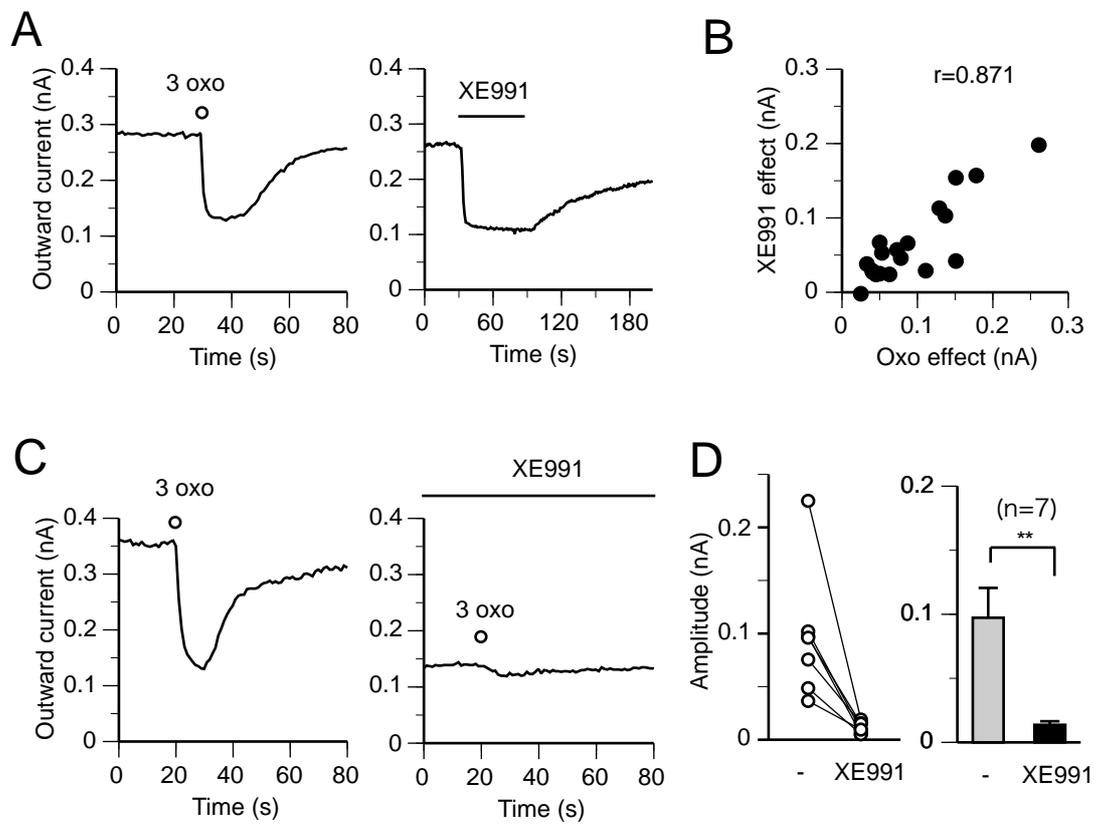Figure 3 (Y. Sugawara et al.)

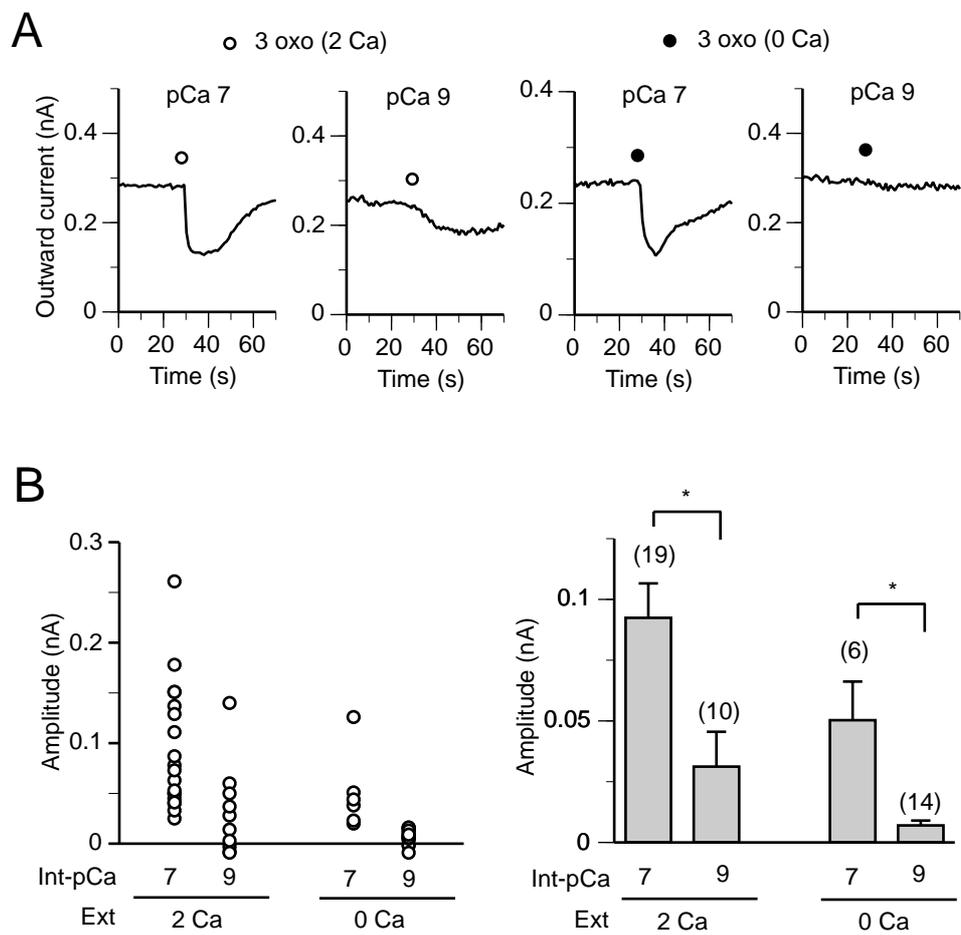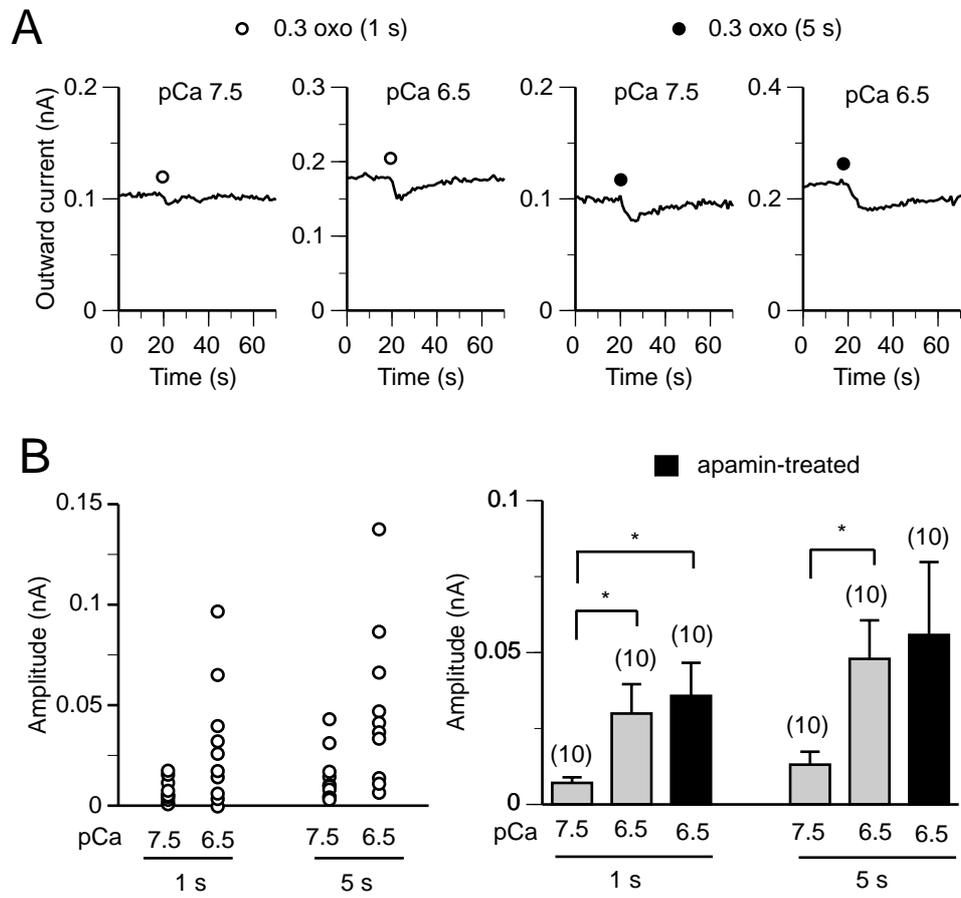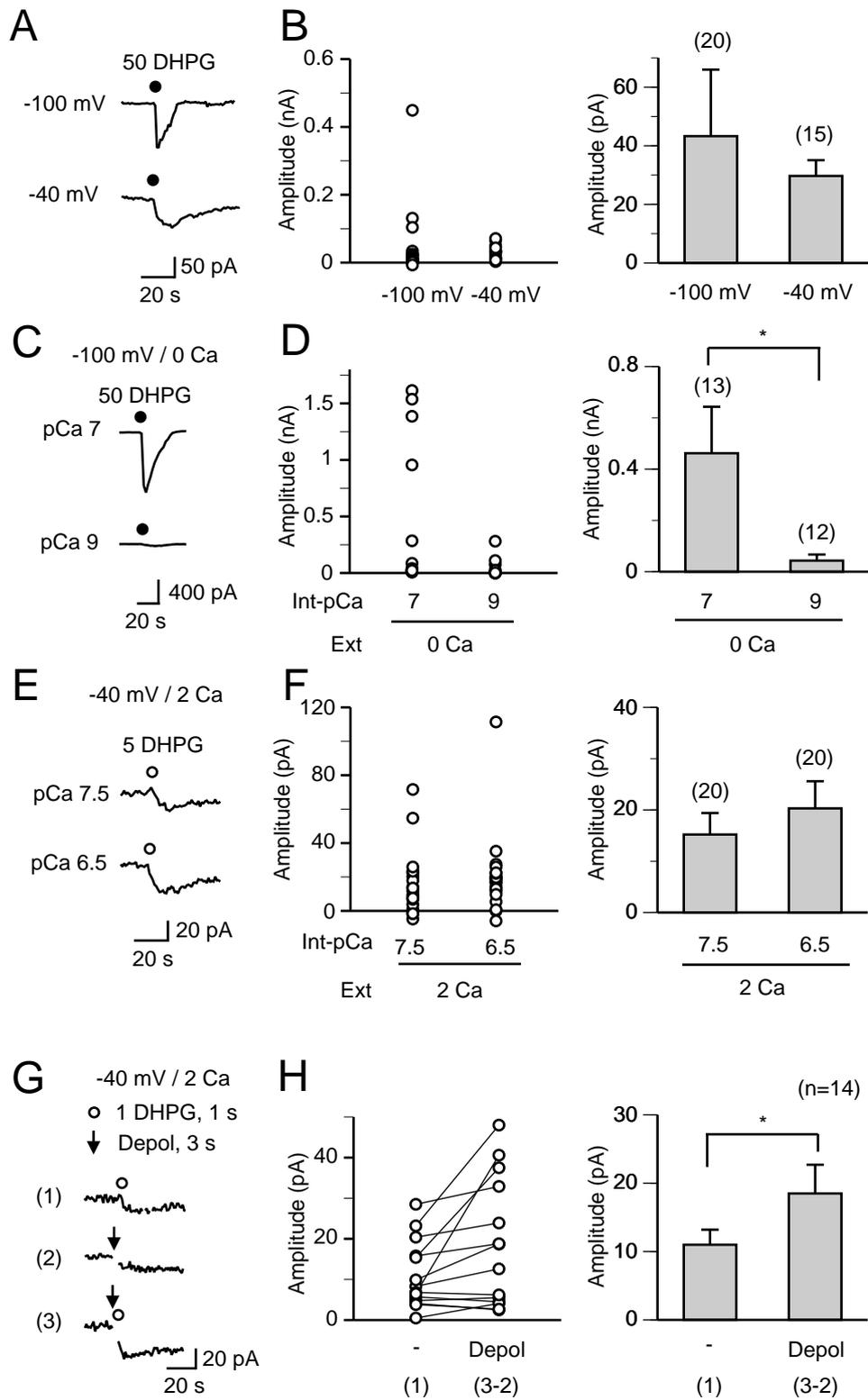Figure 4 (Y. Sugawara et al.)

Figure 5 (Y. Sugawara et al.)

Figure 6 (Y. Sugawara et al.)

Figure 7 (Y. Sugawara et al.)

Figure 8 (Y. Sugawara et al.)

A

Relative amplitude (%)

○ PLC activity
(Hashimotodani et al. 2005)

125
100
75
50
25
0
-25

pCa of pipette solution
9 8 7 6

B

Relative amplitude (%)

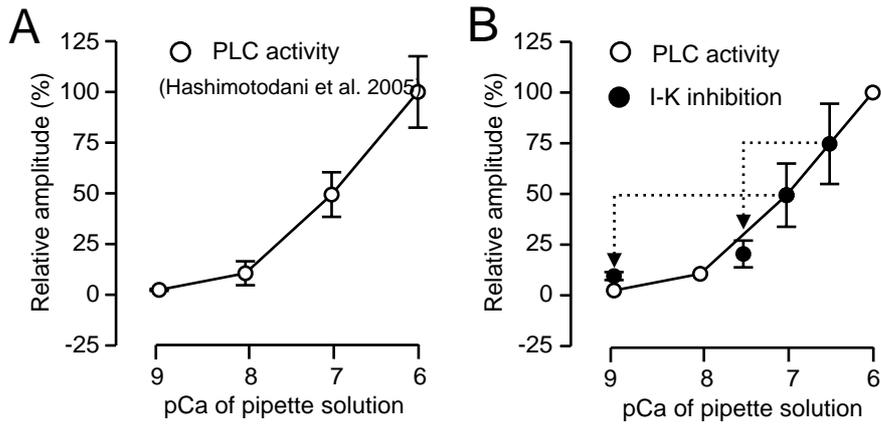○ PLC activity
● I-K inhibition

125
100
75
50
25
0
-25

pCa of pipette solution
9 8 7 6

Figure 9 (Y. Sugawara et al.)