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**Short communication**

**A non-selective cationic channel activated by diacylglycerol in mouse intestinal myocytes.**

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## **Abstract**

Application of 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), an analogue of the second messenger diacylglycerol (DAG) formed via M<sub>3</sub> muscarinic receptors, produced inward currents in symmetrical Cs<sup>+</sup> solutions in mouse intestinal myocytes voltage-clamped at -50 mV. Outside-out patches from the myocytes responded to OAG with openings of 115-pS cationic channels characterized by a mean open time ( $O_{\tau}$ ) of 0.15 ms. M<sub>3</sub> receptor stimulation is reportedly capable of causing brief openings ( $O_{\tau}$ =0.23 ms) of 120-pS cationic channels in intestinal myocytes, thus the present results strongly support the idea that the M<sub>3</sub>-mediated 120-pS channel opening is brought about via DAG-dependent mechanisms.

**Keywords:** muscarinic receptor; M<sub>3</sub> subtype; diacylglycerol; cationic channels; intestinal smooth muscle.

## **1. Introduction**

In gastrointestinal smooth muscles M<sub>2</sub> and M<sub>3</sub> muscarinic receptors are expressed, mediating various effects elicited by the neurotransmitter acetylcholine or other muscarinic agonists (see Unno et al., 2006). A primary, typical muscarinic effect is to produce cationic currents, which cause depolarization and accelerated action potential discharges, in turn elevating intracellular Ca<sup>2+</sup> concentration ( $[Ca^{2+}]_i$ ), resulting in contraction. Recent single channel analyses in guinea-pig or murine intestinal myocytes have resulted in the identification of three types of muscarinic cationic channels characterized by unitary conductances of 10-20, 60-70, and 120-140 pS, respectively, in nearly symmetrical Cs<sup>+</sup> solutions and intracellular Ca<sup>2+</sup> concentration ( $[Ca^{2+}]_i$ ) buffered to 100 nM (Zholos et al., 2004; Dresviannikov et al., 2006; Sakamoto et al., 2007). Furthermore, the use of mice lacking certain subtypes of muscarinic receptors has led to

the discovery of three distinct muscarinic pathways leading to cationic channel activation in gut myocytes. One of these is initiated by M<sub>3</sub> receptor stimulation for the opening of the 60-70 pS and the 120-140 pS channels concurrently (Sakamoto et al., 2007). Yet, the mechanisms underlying the channel opening remain to be elucidated.

The M<sub>3</sub> muscarinic receptor preferentially interacts with G<sub>q/11</sub> type G-proteins, stimulating phospholipase C $\beta$  (PLC $\beta$ ) and hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>), which results in the formation of inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and DAG (Ehlert et al., 1999). It is highly probable that DAG plays a pivotal role in the generation of cationic currents mediated by  $\alpha$ 1-adrenoceptors in vascular myocytes (Helliwell and Large, 1997; Albert and Large, 2006). Therefore, we have attempted to study cationic currents evoked by OAG in mouse intestinal myocytes, using whole-cell or outside-out patch clamp techniques. OAG has been used as a membrane-permeable analogue of DAG in various cells including vascular and visceral myocytes (Helliwell and Large, 1997; Okamoto et al., 2004).

## **2. Methods**

All procedures described below were performed according to the guidelines approved by the local animal ethics committee of the Faculty of Applied Biological Sciences, Gifu University.

### *2.1. Cell preparation*

Mice (a hybrid of 129J1 and CF1) of either sex, weighing 30-40 g, were killed by cervical dislocation. A segment of the small intestine was removed from a region over the jejunum and ileum, from which the longitudinal muscle layer was peeled off. Single myocytes were isolated from the muscle layers enzymatically, as described elsewhere (Sakamoto et al., 2006), and they were then suspended in physiological salt solution

(PSS) containing 0.5 mM CaCl<sub>2</sub>, placed on coverslips in small aliquots, and stored at 4°C until being used on the same day.

## 2.2. Electrophysiological recordings

Membrane current recordings were made at room temperature (22-26 °C) using conventional whole-cell or outside-out patch clamp techniques (Sakamoto et al., 2007). The external solution in which whole-cell or outside-out patch currents were recorded had the following composition (mM): CsCl 120, glucose 12, HEPES 10, and pH was adjusted to 7.4 with CsOH (Cs<sup>+</sup> = 124 mM in total). Patch pipettes (3-6 MΩ) were filled with the following composition (mM): CsCl 80, MgATP 1.0, Na<sub>2</sub>GTP 1.0, creatine 5, glucose 20, HEPES 10, BAPTA 10, CaCl<sub>2</sub> 4.6 (calculated free calcium = ~100 nM) and pH was adjusted to 7.4 with CsOH (Cs<sup>+</sup> = 140 mM in total). Such symmetrical Cs<sup>+</sup>-rich solutions have been employed as optimal conditions for the recording of muscarinic receptor-operated cationic currents in gut myocytes (Zholos et al., 2004; Sakamoto et al., 2006; 2007). Whole-cell current recording was made with a CEZ-2300 voltage-clamp amplifier (Nihon Kohden, Tokyo, Japan) and current signals were filtered at 1 kHz and captured at a sampling rate of 4 kHz using an analog-digital converter (DIGIDATA 1322A; Axon Instruments Inc., Union city, CA, USA) interfaced to a computer (IMC-P642400, Inter Medical Co, Nagoya, Japan) running the pCLAMP program (version 9, Axon Instruments Inc.). For outside-out patch recording, an Axopatch 200B voltage-clamp amplifier (Axon Instruments Inc.) was used, and single channel currents were filtered at 2 kHz and captured at a sampling rate of 50 kHz. For illustration, the current signals were filtered with a 0.5- or 1-kHz low-pass Gaussian filter.

When the effects of OAG or the muscarinic agonist carbachol (both from Sigma in St. Louis, MO, USA) on membrane potential were examined, single myocytes were bathed in PSS consisting of (mM) NaCl 126, KCl 6, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 1.2, glucose 14,

and HEPES 10.5 (adjusted to pH 7.2 with NaOH) and held under current clamp mode with patch pipettes filled with a solution consisting of (mM) KCl 134 and HEPES 10.5 (adjusted to pH 7.4 with KOH) and containing nystatin at 0.2 mg/ml. Changes in membrane potential were recorded with nystatin-perforated patch clamp techniques (Unno et al. 2000). Signals were stored in the same way as described for whole-cell current recordings.

### 2.3. Data analysis

Data analysis and plotting were done using the pCLAMP 9 (Axon Instruments Inc.) and Origin 7.5 software (Origin Lab., Northampton, MA, USA). Current-voltage (I–V) curves were obtained by application of a 5-sec ramp pulse from –110 to 50 mV followed by leakage subtraction (Sakamoto et al., 2007). Single-channel events in outside-out patches were detected on the basis of the half-amplitude threshold-crossing criterion, in which events < 0.16 ms (e.g., rise time for 2 kHz filter) were ignored in the constructing of amplitude and open-time histograms (Sakamoto et al., 2007). Fitted-levels amplitudes were plotted with 0.1 pA bins, and the amplitude histograms of a train of channel events were subjected to Gaussian fitting in order for single channel current amplitudes to be determined. Histograms of channel open-times were constructed with 0.1 ms bins and fitted conventionally with exponential functions by method of maximum likelihood. The open probability ( $P_o$ ) was calculated using pCLAMP 9. Because single channel events produced by OAG were very brief in open (Fig. 2B) and the time resolution for the event detection was not sufficient to follow all of the events, measured values for single channel activity were “apparent” values.

Values in the text are given as means  $\pm$  s.e.mean with the number of measurements. Statistical significance between two groups was assessed using Student’s unpaired *t*-test. When more than three groups were compared, one-way analysis of

variance (ANOVA) followed by a post-hoc Bonferroni-type multiple comparison test was used. Differences were considered significant when  $P < 0.05$ .

### 3. Results

#### 3.1. OAG-induced cationic currents

Under conditions optimal for activation and isolation of the muscarinic cationic current (nearly symmetrical  $\text{Cs}^+$  solutions and  $[\text{Ca}^{2+}]_i$  buffered to 100 nM: Zholos et al., 2004; Sakamoto et al., 2006; 2007), bath application of OAG at a holding potential of  $-50$  mV produced an inward current that persisted until OAG was washed away (Fig.1A). Fig.1B shows the average concentration-response curve for the OAG-induced current, which was obtained by cumulative applications of ascending OAG concentrations (0.1-100  $\mu\text{M}$ ). Current amplitude increased in a concentration-dependent manner with an  $EC_{50}$  value of  $5.8 \pm 0.2$   $\mu\text{M}$ , a maximum current ( $I_{max}$ ) of  $38.2 \pm 4.8$  pA and a Hill coefficient of  $0.97 \pm 0.01$  (n=10 for each).

In cells pretreated with a protein kinase C (PKC) inhibitor, calphostin C (500 nM), OAG (30  $\mu\text{M}$ ) still induced an inward current with a mean amplitude of  $20.1 \pm 4.2$  pA (n=4). As shown in Fig. 1 C, the value was comparable to that in control cells ( $26.9 \pm 10.0$  pA, n=7). However, in guinea pig ileal myocytes, OAG (3 or 10  $\mu\text{M}$ ) inhibited ATP-sensitive  $\text{K}^+$  channel current ( $I_{KATP}$ ), probably through the activation of PKC, and OAG's effect on  $I_{KATP}$  was eliminated after the treatment of calphostin C (500 nM) (Unno, Matsuyama and Komori, unpublished observation). These results suggest that PKC is unlikely to be involved in the OAG-induced inward current activation.

Fig.1D shows the averaged I–V curve for the OAG currents evoked in five different myocytes, in each of which 5-sec voltage ramps from  $+50$  to  $-110$  mV were applied in the absence and presence of 30  $\mu\text{M}$  OAG. After leakage subtraction (b – a:

see the inset in Fig.1D), the size of the net OAG current was normalized by taking it at  $-50$  mV as the unity. The I–V curve indicated that the reversal potential ( $E_{rev}$ ) for the OAG current is around  $0$  mV ( $3.8 \pm 1.6$  mV,  $n=5$ ). From  $E_{rev}=0$  mV to about  $-70$  mV, the OAG current increased in proportion to the electromotive force, but as negativity was further increased, it progressively declined or remained almost unaltered (Fig.1D). Carbachol-evoked cationic currents in gut myocytes from  $M_2$  receptor-knockout mice have been described as showing a similar shape in I–V curves (Sakamoto et al., 2007).

Inward  $Ca^{2+}$  currents were activated by depolarization steps from  $-50$  mV to  $0$  mV during the application of  $30$   $\mu$ M OAG under conditions where  $2$  mM  $Ca^{2+}$  was present outside the cell and the internal BAPTA/ $Ca^{2+}$  mixture was omitted. No appreciable tail current appeared upon repolarization following the depolarizing pulse that evoked the  $Ca^{2+}$  current ( $n=3$ ; data not shown), indicating that the OAG current is not readily potentiated by a rise in  $[Ca^{2+}]_i$ . A similar feature has been reported for the carbachol current in the  $M_2$  receptor-knockout myocytes (Sakamoto et al., 2007).

### 3.2. Changes in membrane potential by OAG

We have examined whether the activity of OAG in the inducing of inward cationic currents is physiologically relevant. Changes in membrane potential of single myocytes were measured with nystatin-perforated patch clamp techniques (Unno et al., 2000). The myocytes tested had a resting potential of  $-47.3 \pm 3.3$  mV ( $n=5$ ). An application of  $30$   $\mu$ M OAG produced a sustained and reversible depolarization, on which action potential discharges were usually superimposed (Fig.1E). The size of depolarization was  $7.7 \pm 0.7$  mV on average ( $n=5$ ) with cell-to-cell variations of  $5.7$  to  $10.0$  mV.

### 3.3. OAG-gated channels

Ion channels mediating the OAG-evoked currents were characterized in

outside-out patch clamp experiments. Every outside-out patch was excised from a different myocyte and then exposed to 30  $\mu\text{M}$  OAG at a holding potential of  $-40$  mV under the same ionic conditions as used for whole-cell current recordings. Six out of 33 patches excised displayed a significant channel activity without OAG (see Fig.2A), but the spontaneous channel opening was not plentiful enough for histogram analyses to determine unitary conductance, nor could mean open time ( $O_{\tau}$ ) of channels be allowed. However, visual inspections indicated that the channel activity depends at least partly on 70-pS channels, as previously reported (Sakamoto et al., 2007). In 21 of the 33 patches (64 %), regardless of their spontaneous activity, OAG (30  $\mu\text{M}$ ) caused no noticeable change in channel gating. As shown in Fig.2A, the spontaneous channel gating seemed to remain almost unchanged after OAG application (n=2). In fact, the probability of channel opening ( $P_o$ ) before and after OAG application was, on average, almost identical (0.00024 and 0.00018, respectively). In the remaining 12 patches (36 %), OAG produced an increased channel activity which consisted of brief open events at a high frequency, as exemplified in Fig. 2B (\*). Replacing external  $\text{Cs}^+$  with the impermeable cation N-methyl-D-glucamine (NMDG) eliminated the channel activity in a reversible manner (n=3), indicating that channel currents depended on the  $\text{Cs}^+$  but not the  $\text{Cl}^-$  gradient (data not shown).

The OAG-induced channel activity gradually declined with time and, in 8 of 12 active patches, the number of acquired opening events was insufficient for histogram analysis, though visual inspections described channel currents of 4.0 to 5.5 pA (corresponding to unitary conductance of 100 to 135 pS), at least partly accounting for the channel activity.

The four remaining patches could provide a number of data sufficient for fitting analysis of histograms for channel properties. Fig.2C shows histograms of current

amplitude for the channel activity in Fig.2B, which revealed a major peak at 4.4 pA. A unitary channel conductance estimated from the major peak current as  $E_{rev} = 0$  mV was 110 pS. Histograms of distribution of apparent open times are shown in Fig.2D, which followed a single exponential function with a time constant (i.e.,  $O_\tau$ ) of 0.17 ms. Similar results were also obtained in the three other patches. The average unitary conductance and  $O_\tau$  were estimated to be  $115.1 \pm 2.1$  pS and  $0.15 \pm 0.03$  ms (n=4), respectively, and the average probability of channel opening ( $P_o$ ) was estimated to be  $0.0021 \pm 0.0009$  (n=4).

#### 4. Discussion

Gastrointestinal smooth muscle cells seem likely to be endowed with multiple muscarinic signalling pathways, each of which leads to the generation of depolarizing membrane currents (Unno et al., 2006; Sakamoto et al., 2007). One of these, revealed in mouse gut myocytes, is initiated by  $M_3$  receptor stimulation, leading to concurrent openings of 70-pS and 120-pS cationic channels (Sakamoto et al., 2007), though channel activation mechanisms have yet to be clarified. The present results have shown that OAG is capable of activating 115-pS cationic channels and depolarizing the membrane in mouse gut myocytes. The OAG-induced channel gating was characterized by brief openings with a mean open state of 0.15 ms. The gating property closely resembled that described for the  $M_3$ -mediated 120-pS channel activity (a mean open time of 0.23 ms; Sakamoto et al., 2007). Therefore, it seems highly probable that 120-pS channel activation via the  $M_3$ -initiated pathway is brought about by DAG formed via PLC $\beta$  hydrolysis of PIP $_2$ . This idea is consistent with the findings that the channel activation by muscarinic stimulation was not commonly detected in outside-out patches compared with cases for cell-attached patches, suggesting possible involvement

of a diffusible factor in the channel activation (Zholos et al., 2004; Dresviannikov et al., 2006).

The present observation that OAG failed to open 70-pS channels or modulate their spontaneous gating (Fig. 2A) shows it is likely that these channels involved in the  $M_3$ -initiated pathway are gated via mechanisms independent of DAG. It is supposed that loss of  $PIP_2$  from the membrane is crucial for 70-pS channel opening. Recently, we consistently observed that the PLC inhibitor U73122 (1  $\mu$ M) blocked spontaneous gating of 70-pS channels or their increased activity by muscarinic receptor stimulation (data not published; Sakamoto, Unno, Matsuyama and Komori). Therefore, our suggestion at present is that in the  $M_3$ -initiated pathway for cationic channel activation, 70-pS channel opening arises due to the loss of  $PIP_2$  via the  $G_{q/11}/PLC\beta$  system, while 120-pS channel opening is due to the formation of DAG from  $PIP_2$  breakdown (see Fig.12 in Sakamoto et al., 2007).

DAG is generally believed to activate protein kinase C (PKC). However, in vascular and ileal myocytes, OAG-induced cationic current responses seem unlikely to involve PKC (Helliwell & Large, 1997; Albert and Large, 2006; present study). Although DAG plays a pivotal role in  $\alpha 1$ -adrenoceptor-mediated activation of cationic currents, full activation of the currents may require not only DAG but also the counterpart product,  $IP_3$  (Helliwell & Large, 1997; Albert & large, 2003). Further study is needed to investigate the roles of PKC and  $IP_3$  in the  $M_3$ -mediated activation of 120-pS channels in gut myocytes.

Recent evidence suggests that receptor-operated cationic channels in the smooth muscle are composed of transient receptor potential (TRP) channel proteins (for review, see Beech et al., 2004). It is also suggested that, of seven canonical TRP (TRPC) proteins, TRPC3, TRPC6 and TRPC7 are preferentially activated by DAG (Dietrich et

al., 2005). In vascular myocytes, TRPC6 has been identified as a component of native cationic channels activated by  $\alpha$ 1-adrenoceptor stimulation (Inoue et al., 2001). The heterologously-expressed TRPC6 channel or the native adrenergic cationic channel has a conductance of 20-35 pS in symmetrical  $\text{Cs}^+$  solutions or in external  $\text{Na}^+$ /internal  $\text{Cs}^+$  (Hofmann et al., 1999; Albert & Large, 2001). Therefore, the 120-pS channel activated by  $\text{M}_3$  receptor stimulation or OAG application in gut myocytes is possibly composed of some type of TRPC channel other than TRPC6. Its molecular basis, as well as that of the 70-pS channel, remains to be identified.

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### **Conflict of interest**

The authors state no conflict of interest.

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## **Legends**

### **Figure 1.**

Membrane current and potential responses induced by OAG in single myocytes isolated enzymatically from the longitudinal muscle layer of a mouse's small intestine. A: An inward current evoked by bath-application of OAG (30  $\mu$ M) in nearly symmetrical  $Cs^{+}$ -rich solutions outside and inside of the cell with intracellular free-ionized  $Ca^{2+}$

buffered to 100 nM and held under voltage-clamp at  $-50$  mV. B: An averaged concentration-response curve for OAG-evoked inward current obtained by cumulative applications of ascending OAG concentrations ( $n=10$ ). C: Current amplitudes induced by OAG ( $30 \mu\text{M}$ ) in the presence ( $n=4$ ) or absence (control,  $n=7$ ) of the PKC inhibitor calphostin C ( $500$  nM). D: Current-voltage (I-V) relationship for the OAG current that is normalized by taking it at  $-50$  mV as the unity ( $n=5$ ). A 5-sec negative-going ramp pulse from  $50$  to  $-110$  mV was applied before and during application of  $30 \mu\text{M}$  OAG, and the I-V curve constructed after leakage subtraction (see the inset). E: Changes in membrane potential of a single gut myocyte produced by  $30 \mu\text{M}$  OAG and, subsequently, by the muscarinic agonist carbachol (CCh;  $1 \mu\text{M}$ ) under equi-physiological ionic environments (high  $\text{Na}^+$  outside and high  $\text{K}^+$  inside). The potential changes were recorded by nystatin-perforated patch clamp techniques.

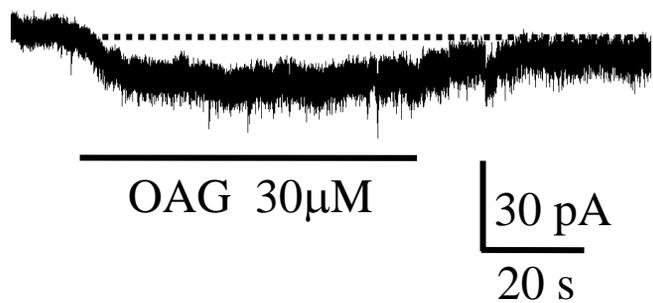
**Figure 2.**

OAG-induced single channel currents in outside-out patches held at  $-40$  mV under the same ionic environment as in Fig.1 A. A: OAG ( $30 \mu\text{M}$ ) had no significant effect on a spontaneously active patch generating brief currents, which were mediated at least partly by channels with a unitary conductance of about  $70$  pS, as determined by visual inspection. The time-expanded traces in the absence (control) and presence of OAG were also shown. These traces were not consecutive, but selected as  $70$  pS channel openings were obvious. B: OAG caused prominent generation of channel currents (\*) in a different patch. An underlined portion of the current recording trace (a) is shown with a faster time scale. Calibrations in A are applicable to current traces in B. C: Fitted levels amplitude histogram counted per  $0.1$  pA bins for the channel current in B, in which a peak amplitude of  $4.4$  pA was detected, implying the opening of single

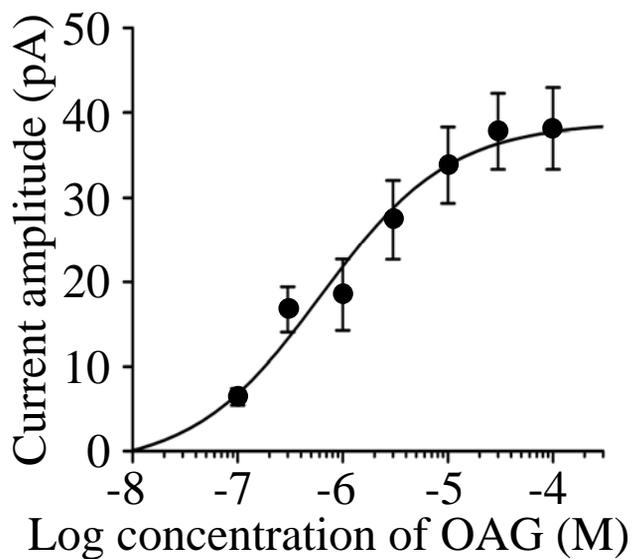
channels with a conductance of 110 pS. D: open time histograms for the channel. The open time distributions per 0.1 ms bins were fitted by a single exponential function with a time constant, or a mean open time ( $O_p$ ), of 0.17 ms.

**Figure 1**

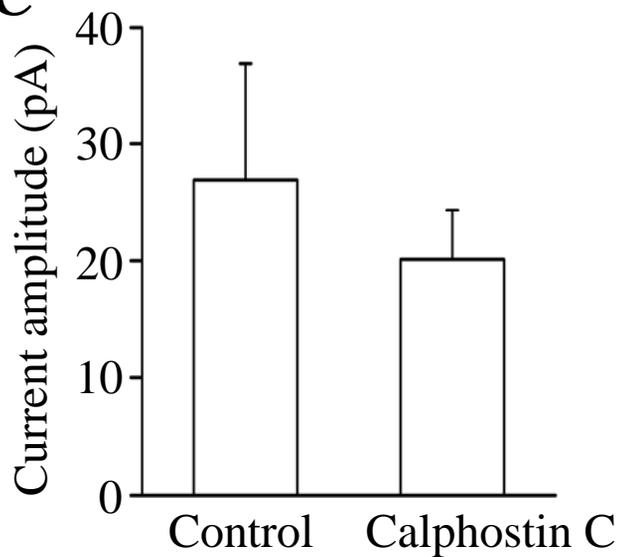
**A**



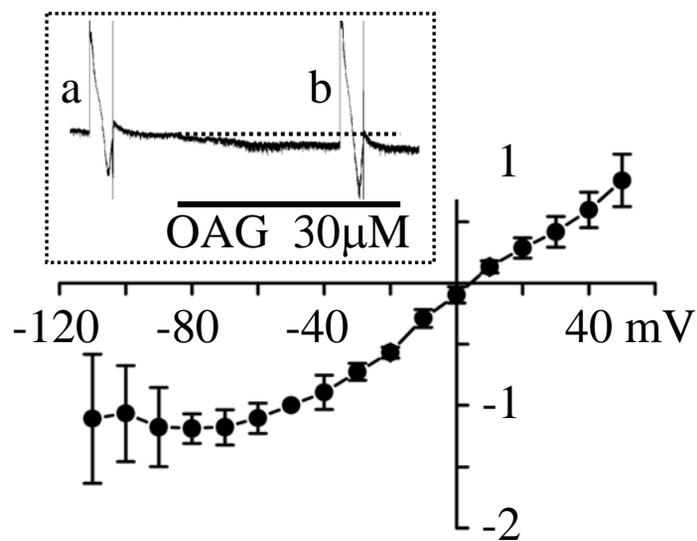
**B**



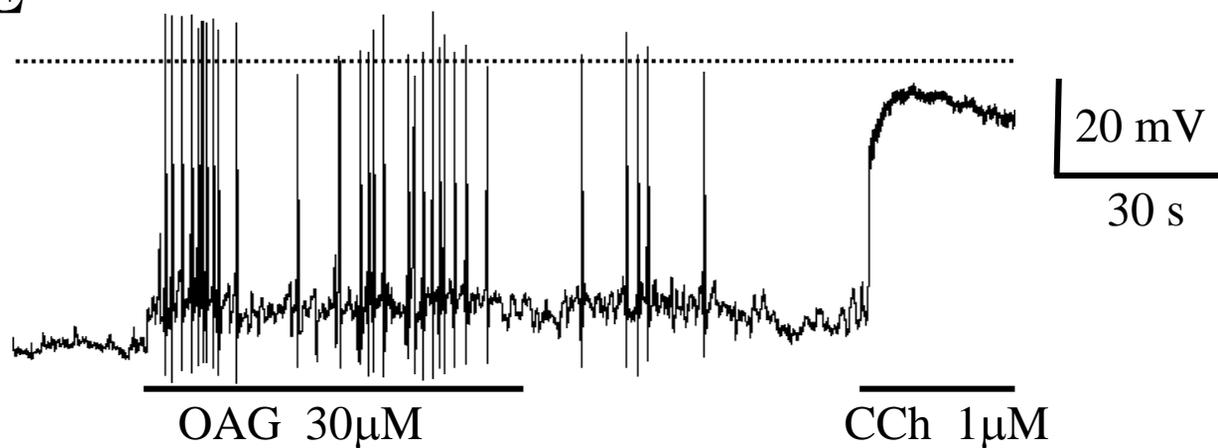
**C**



**D**



**E**



# Figure 2

