The 2-Aminoethoxydiphenyl Borate Analog Dpb161 Blocks Storeoperated Ca 2+ Entry In Acutely Dissociated Rat Submandibular Cells

Kunkun Xia
Zegang Ma
Jianxin Shen
Menghan Li
Baoke Hou

See next page for additional authors

Follow this and additional works at: https://scholar.barrowneo.org/neurobiology

Recommended Citation
Xia, Kunkun; Ma, Zegang; Shen, Jianxin; Li, Menghan; Hou, Baoke; Gao, Ming; Zhang, Shuijun; and Wu, Jie, "The 2-Aminoethoxydiphenyl Borate Analog Dpb161 Blocks Storeoperated Ca 2+ Entry In Acutely Dissociated Rat Submandibular Cells" (2017). Neurobiology. 469. https://scholar.barrowneo.org/neurobiology/469

This Article is brought to you for free and open access by Barrow - St. Joseph's Scholarly Commons. It has been accepted for inclusion in Neurobiology by an authorized administrator of Barrow - St. Joseph's Scholarly Commons. For more information, please contact molly.harrington@dignityhealth.org, andrew.wachtel@dignityhealth.org.
Authors
Kunkun Xia, Zegang Ma, Jianxin Shen, Menghan Li, Baoke Hou, Ming Gao, Shuijun Zhang, and Jie Wu
The 2-aminoethoxydiphenyl borate analog, DPB161 blocks store-operated Ca\(^{2+}\) entry in acutely dissociated rat submandibular cells

Kunkun Xia\(^{1,2,*}\), Zegang Ma\(^{3,2,*}\), Jianxin Shen\(^4\), Menghan Li\(^4\), Baoke Hou\(^2\), Ming Gao\(^2\), Shuijun Zhang\(^1\) and Jie Wu\(^{1,2,3,4}\)

1Department of Hepatobiliary and Pancreatic Surgery, First Affiliated Hospital of Zhengzhou University, Zhengzhou, China
2Department of Neurobiology, Barrow Neurological Institute, St. Joseph’s Hospital and Medical Center, Phoenix, AZ, USA
3Department of Physiology, Shandong Provincial Key Laboratory of Pathogenesis and Prevention of Neurological Disorders, Shandong Provincial Collaborative Innovation Center for Neurodegenerative Disorders and State Key Disciplines, Physiology, Medical College of Qingdao University, Qingdao, China
4Department of Physiology, Shantou University Medical College, Shantou, China

*These authors have contributed equally to this work

Correspondence to: Jie Wu, email: jie.wu@dignityhealth.org

Keywords: 2-aminoethoxydiphenyl borate, store-operated Ca\(^{2+}\) entry (SOCE), DPB161, Ca\(^{2+}\) oscillations, rat submandibular cells

Received: February 04, 2017  Accepted: May 06, 2017  Published: June 27, 2017

Copyright: Xia et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License 3.0 (CC BY 3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

ABSTRACT

Cellular Ca\(^{2+}\) signals play a critical role in cell physiology and pathology. In most non-excitable cells, store-operated Ca\(^{2+}\) entry (SOCE) is an important mechanism by which intracellular Ca\(^{2+}\) signaling is regulated. However, few drugs can selectively modulate SOCE. 2-Aminoethoxydiphenyl borate (2APB) and its analogs (DPB162 and DPB163) have been reported to inhibit SOCE. Here, we examined the effects of another 2-APB analog, DPB161 on SOCE in acutely-isolated rat submandibular cells. Both patch-clamp recordings and Ca\(^{2+}\) imaging showed that upon removal of extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_o\)=0), rat submandibular cells were unable to maintain ACh-induced Ca\(^{2+}\) oscillations, but restoration of [Ca\(^{2+}\)]\(_o\) to refill Ca\(^{2+}\) stores enable recovery of these Ca\(^{2+}\) oscillations. However, addition of 50 μM DPB161 with [Ca\(^{2+}\)]\(_o\) to extracellular solution prevented the refilling of Ca\(^{2+}\) store. Fura-2 Ca\(^{2+}\) imaging showed that DPB161 inhibited SOCE in a concentration-dependent manner. After depleting Ca\(^{2+}\) stores by thapsigargin treatment, bath perfusion of 1 mM Ca\(^{2+}\) induced [Ca\(^{2+}\)]\(_i\) elevation in a manner that was prevented by DPB161. Collectively, these results show that the 2-APB analog DPB161 blocks SOCE in rat submandibular cells, suggesting that this compound can be developed as a pharmacological tool for the study of SOCE function and as a new therapeutic agent for treating SOCE-associated disorders.

INTRODUCTION

Intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) signals play critical roles in the modulation of cellular physiology and pathophysiology. In many non-excitable cells, intracellular Ca\(^{2+}\) signals respond to extracellular agonist stimulation in an oscillatory, rather than sustained, manner [1–3]. It is well accepted that two distinct intracellular Ca\(^{2+}\) pools, the inositol-1, 4, 5-trisphosphate (InsP\(_3\))-sensitive and ryanodine-sensitive pools, participate in the genesis of oscillatory Ca\(^{2+}\) signals [4]. In 1986, Putney presented a model for capacitive calcium (Ca\(^{2+}\)) entry conveying that depletion of endoplasmic reticulum-stored Ca\(^{2+}\) leads to activation of plasma membrane Ca\(^{2+}\) channels that mediate influx of Ca\(^{2+}\) from the extracellular space into cells [5], in a process called store-operated Ca\(^{2+}\) entry (SOCE). Recent molecular studies have identified that stromal interaction molecule (STIM), the Ca\(^{2+}\) sensor of the intracellular
compartments, together with Orai, the subunits of Ca\(^{2+}\) permeable channels on the plasma membrane [6], cooperate in regulating multiple cellular functions as diverse as proliferation, differentiation, migration and gene expression, demonstrating that SOC channels are important targets for normal cell function and a variety of diseases [7]. Unfortunately, there are few selective blockers for SOCE. There is a need to develop new compounds for SOCE as pharmacological tools for investigation and as therapeutic agents for diseases.

The membrane-penetrable compound, 2-aminodiphenyl borinate (2-APB) was initially developed as membrane-permeable InsP\(_3\) receptor antagonist since 2-APB produced concentration-dependent inhibition of InsP\(_3\)-induced Ca\(^{2+}\) release from mouse cerebellar membranes without affecting InsP\(_3\) binding [8]. After this initial report, numerous studies have reported that 2-APB antagonizes SOCE in different cell types [9, 10]. Thereafter, Mikoshiba’s group further developed 2-APB analogs and demonstrated that 2-APB analogs exhibit more selective inhibition of SOCE compared to inhibition of InsP\(_3\)-induced Ca\(^{2+}\) release, in either transfected cell lines or DT40 cells [11, 12]. In the present study, we used patch-clamp recording and Ca\(^{2+}\) imaging to test the effects of a novel 2-APB analog, DPB161 (Figure 1) on SOCE in rat submandibular cells. We found that DPB161 inhibited SOCE in a concentration-dependent manner. Thus, this study provides evidence that a 2-APB analog, DPB161 serves as a SOCE blocker to modulate cell Ca\(^{2+}\) signals in rat submandibular cells.

**RESULTS**

**Rat submandibular cells model of SOCE**

To open SOC channels, intracellular Ca\(^{2+}\) store need to be depleted. Initial experiments were designed to compare two types of cells (mouse pancreatic acinar cells and rat submandibular cells) for agonist-induced emptying of Ca\(^{2+}\) stores. Under free extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_o\)=0) conditions, continuous bath-application of ACh (20 nM) induced persistent Ca\(^{2+}\) oscillatory responses as measured by the Ca\(^{2+}\)-dependent Cl\(^{-}\) current using patch-clamp whole-cell recordings in mouse pancreatic acinar cells (Figure 2A). However, in rat submandibular cells under the same experimental conditions, ACh-induced Ca\(^{2+}\) oscillatory responses stopped within minutes, and after washout of ACh with [Ca\(^{2+}\)]\(_o\)=0 solution for minutes, ACh still failed to induce any oscillatory responses (Figure 2B), suggesting an emptied intracellular Ca\(^{2+}\) store that was not refilled with extracellular Ca\(^{2+}\) in a SOCE-dependent manner. These results demonstrate that compared to pancreatic acinar cells, the response of rat submandibular cells to ACh stimulation is more dependent on Ca\(^{2+}\) entering through SOC channels to refill the Ca\(^{2+}\) store. Therefore, we used rat submandibular cells for all further experiments.

**DPB161 blocks Ca\(^{2+}\) store refilling following ACh-induced depletion of intracellular Ca\(^{2+}\) stores**

In these experiments, we characterized the effects of DPB161 on SOCE-dependent intracellular Ca\(^{2+}\) store refilling in rat submandibular cells. Under [Ca\(^{2+}\)]\(_o\)=0 conditions, bath-application of 20 nM ACh for 3-5 min induced an oscillatory response, which likely caused complete release of Ca\(^{2+}\) from the intracellular Ca\(^{2+}\) store since after washout of ACh for one min, reaplication of ACh failed to induce any oscillatory responses. Then, 1 mM Ca\(^{2+}\) was bath-applied to refill the Ca\(^{2+}\) store. Thereafter, we applied ACh again under [Ca\(^{2+}\)]\(_o\)=0 conditions and induced a Ca\(^{2+}\) response, suggesting that under [Ca\(^{2+}\)]\(_o\)=0 condition, the initial 20 nM ACh application emptied the Ca\(^{2+}\) store, and the subsequent bath-perfusion of 1 mM [Ca\(^{2+}\)]\(_o\) successfully refilled Ca\(^{2+}\).

![Figure 1: Chemical structure of DPB-161.](image-url)
store (Figure 3A). When 50 μM DPB161 was included in the bath-perfusion with 1 mM [Ca$^{2+}$], refilling of the Ca$^{2+}$ store as prevented. Similar experimental results were obtained from 6 cells tested. These results suggest that DPB161 blocks SOCE (Figure 3B).

**DPB161 blocks Ca$^{2+}$ store refilling measured by fura-2 Ca$^{2+}$ imaging**

To further evaluate the role of DPB161 in inhibiting SOCE, we measured intracellular Ca$^{2+}$ concentrations ([Ca$^{2+}$])$_i$ using Fura-2 imaging in isolated rat submandibular cells. Application of high concentration (1 μM) of ACh resulted in a spike in [Ca$^{2+}$]$_i$, that declined much faster under [Ca$^{2+}$]$_o$=0 conditions (Figure 4A) than under 1 mM [Ca$^{2+}$]$_o$ conditions (Figure 4B), suggesting that ACh empties Ca$^{2+}$ stores and opens SOCE to refill the Ca$^{2+}$ stores. However, when the bath solution contained DPB161 (50 μM), 1 μM ACh induced [Ca$^{2+}$]$_i$ spike, with the same rise and decline, regardless of whether the bath solutions contained Ca$^{2+}$ or not (Figure 4C, 4D). Statistical analysis indicated that the ACh-induced current areas (between 4 – 12 min) were 4504.8 ± 409.4 under 1 mM [Ca$^{2+}$]$_o$ condition (n=12, A) and 3542.4 ± 231.4 for the [Ca$^{2+}$]$_o$=0 conditions (n=13, B). The difference was significant (p<0.05, unpaired t-test). In the presence of 50 μM DPB161, the ACh-induced current areas under 1 mM [Ca$^{2+}$]$_o$ (n=11, C) and [Ca$^{2+}$]$_o$=0 conditions (n=16, D) were 1575.4 ± 80.2 and 1377.8 ± 190.8 (p>0.05, unpaired t-test). These results support the patch-clamp finding that DPB161 blocks the refilling of the Ca$^{2+}$ store through SOCE channels.

**DPB161 blocks Ca$^{2+}$ store refilling in a concentration-dependent manner**

To compare the effects of different concentrations of DPB161 on SOCE, we quantitatively measured ACh (1 μM)-induced elevation of [Ca$^{2+}$]$_i$ twice with 5-10 min intervals with or without external Ca$^{2+}$. As shown in Figure 5A, under [Ca$^{2+}$]$_o$=0 without external Ca$^{2+}$ refilling, the second ACh-induced elevation of [Ca$^{2+}$]$_i$ was eliminated, suggesting that the first ACh exposure (1 μM for ~4 min) has already depleted the Ca$^{2+}$ store. However, with [Ca$^{2+}$]$_o$ perfusion (1 mM for ~ 4 min), the Ca$^{2+}$ store was able to refill as the second ACh exposure induced [Ca$^{2+}$]$_i$ elevation to a similar extent (Figure 5B). Thereafter, we used this protocol to compare the ratio of the second [Ca$^{2+}$]$_i$ response (S2) and first [Ca$^{2+}$]$_i$ response with different concentrations of DPB161 under conditions of 1 mM [Ca$^{2+}$]$_o$ perfusion. One-way ANOVA showed a statistical difference in S2/S1 ratio after perfusion of DPB161 (F(4)=56.1, p<0.001). Tukey analysis showed little effect of 5 μM DPB161 in prevention of [Ca$^{2+}$]$_o$ entry compared to 0 μM DPB161.

![Figure 2: Comparison of ACh-induced Ca2+ oscillations between pancreatic acinar cells and submandibular cells using patch-clamp recordings.](image-url)

Under [Ca$^{2+}$]$_o$ = 0 condition, ACh (20 nM) induced a long-lasting Ca$^{2+}$ oscillatory response in mouse pancreatic acinar cells (A) but induced a short-lasting Ca$^{2+}$ oscillatory response in rat submandibular cells (B). Typical traces from 6 cells tested are presented in A (mouse pancreatic acinar cells) and B (rat submandibular cells), respectively.
(without DPB161, the S2/S1 ratio was normalized as 100%, the 5 μM DPB161 was 87.6±7.1%, \(p>0.05\), \(n=8\), Figure 5C), but 20 μM DPB161 reduced S2/S1 ratio to 64.6±7.0% \((p<0.001, n=8)\), and 50 μM DPB161 reduced S2/S1 ratio to 23.0±4.8% \((p<0.001, n=15, \text{Figure 4D})\). These data demonstrate that DPB161 prevents SOCE in a concentration-dependent manner in rat submandibular cells (Figure 5E).

DPB161 blocks SOC channel-dependent \([\text{Ca}^{2+}]_\text{i}\) elevation

Data presented thus far demonstrates that DPB161 blocks ACh-triggered SOCE in rat submandibular cells. Finally, we directly measured SOC channel-mediated \([\text{Ca}^{2+}]_\text{i}\) elevation under conditions of DPB161 treatment. To measure SOC channel-mediated \([\text{Ca}^{2+}]_\text{i}\) elevation, we depleted intracellular \(\text{Ca}^{2+}\) store by pre-treating cells with thapsigargin (TG) under \([\text{Ca}^{2+}]_\text{o}=0\) conditions. Depletion of \(\text{Ca}^{2+}\) stores was demonstrated by the inability of ACh application to induce \([\text{Ca}^{2+}]_\text{i}\) elevation (Figure 6A). After depletion of the \(\text{Ca}^{2+}\) store, SOC channels were opened, and we were able to record 1 mM external \(\text{Ca}^{2+}\)-induced \([\text{Ca}^{2+}]_\text{i}\) elevation through SOC channels (Figure 6B). Then, we tried to refill the \(\text{Ca}^{2+}\) store with 1 mM \([\text{Ca}^{2+}]_\text{o}\) in the presence of 5 μM (Figure 6C) or 50 μM DPB161 (Figure 6D). At 50 μM, but not 5 μM, DPB161 prevented the SOC channel-mediated \([\text{Ca}^{2+}]_\text{i}\) elevation.

**DISCUSSION**

In the present study, we employed both patch-clamp recordings and Fura-2 \(\text{Ca}^{2+}\) imaging to examine the effects of a 2-APB analog, DPB161 on SOCE in freshly isolated rat submandibular cells. We demonstrate that DPB161 blocks rat submandibular cell SOCE in a concentration-dependent manner, suggesting that this 2-APB analog can serve as a pharmacological tool to investigate intracellular \(\text{Ca}^{2+}\) signaling mechanisms and provide a new therapeutic strategy for treatment of SOCE-associated diseases.

**Figure 3:** Effects of DPB-161 on \([\text{Ca}^{2+}]_\text{i}\) responses to ACh measured by patch-clamp recordings in rat submandibular cells. (A) Under \([\text{Ca}^{2+}]_\text{o}=0\) conditions, bath-application of 20 nM ACh induced \(\text{Ca}^{2+}\) oscillatory response. Bath-application of 1 mM \(\text{Ca}^{2+}\) refills the \(\text{Ca}^{2+}\) stores through opened SOC channels. (B) Using the same experimental protocol but refilling \(\text{Ca}^{2+}\) stores with 1 mM \(\text{Ca}^{2+}\) and 50 μM DPB161 prevented the refilling of \(\text{Ca}^{2+}\) stores, suggesting that DPB161 blocks SOCE. Typical traces in A and B are presented from 6 submandibular cells tested, respectively.
Figure 4: Effects of DPB-161 on 
\[Ca^{2+}\] responses to ACh measured by Ca^{2+} imaging in rat submandibular cells. (A) In the presence of extracellular Ca^{2+}, the cell was stimulated with 1 μM ACh (4 min). (B) ACh was applied to the cell in a Ca^{2+}-free solution. (C) Before (1 min) and during stimulation with ACh in the Ca^{2+}-free solution, 50 μM DPB-161 was applied. (D) Before (1 min) and during stimulation with ACh in the Ca^{2+}-containing solution, 50 μM DPB-161 was applied. The representative trace of 11-16 experiments is shown in each panel.
Figure 5: Effects of DPB-161 on Ca\textsuperscript{2+} store refilling following ACh-induced Ca\textsuperscript{2+} store depletion in rat submandibular cells. 

(A) In Ca\textsuperscript{2+}-free solution, the cell was stimulated by 1 μM ACh (4 min). After the first challenge of ACh, a second ACh challenge (4 min) was performed. 

(B) Between the two ACh challenges, Ca\textsuperscript{2+}-containing solution was applied for 2 min. 

(C) Before (1 min) and during addition of extracellular Ca\textsuperscript{2+}, 5 μM DPB-161 was applied. 

(D) Before (1 min) and during addition of extracellular Ca\textsuperscript{2+}, 50 μM DPB-161 was applied. The representative trace of 5-19 experiments is shown in each panel. 

(E) Concentration-response relationships for DPB-161 inhibition of store-operated Ca\textsuperscript{2+} entry. Relative sizes of the second ACh responses to the first ones are shown, measured by the area under the curve. Values are means of 5, 8, 8, and 15 cells tested for 0, 5, 20 and 50 μM DPB-161, respectively. Vertical bars indicate SE. Statistical significance was obtained by one-way ANOVA. *: \( p < 0.05 \), **: \( p < 0.01 \), ***: \( p < 0.001 \).
of ACh (20 nM) induces a short-lasting oscillatory response (measured Ca²⁺-dependent Cl⁻ currents) under [Ca²⁺]₀=0 conditions. This is due to the ACh has released all releasable Ca²⁺ from Ca²⁺ stores without extracellular Ca²⁺ refilling. After emptying Ca²⁺ stores, exposure to 1 mM external Ca²⁺ enables the refilling of Ca²⁺ stores and recovery of the 20 nM ACh-induced oscillatory responses (Figure 3A). We used this experimental protocol to examine the effect of DPB161 on Ca²⁺ refilling, and found that co-application of 50 μM DPB161 in the presence of 1 mM external Ca²⁺ prevents the refilling of Ca²⁺ stores as judged by the lack of a detectable ACh-induced response after attempting to refill Ca²⁺ stores in the presence of 50 μM DPB161 (Figure 3B). These data suggest that 50 μM DPB161 completely blocks SOCE in rat submandibular cells. Ca²⁺ imaging experiments comparing [Ca²⁺], elevation by ACh in the presence and absence of external Ca²⁺ (1 mM) indicate that the duration of the [Ca²⁺], response is remarkably short in the absence of extracellular Ca²⁺, suggesting that during ACh depletion of Ca²⁺ stores, the refilling of Ca²⁺ stores requires the presence of external Ca²⁺ (Figure 4A, 4B). Using the same protocol, but comparing 1 mM external Ca²⁺ plus 50 μM DPB161 perfusion vs. perfusion in the absence of external Ca²⁺, we find ACh induces similar waveforms of [Ca²⁺], elevation when perfused with 50 μM DPB161 plus 1 mM external Ca²⁺, suggesting that DPB161 blocks SOCE. To qualitatively compare the concentration dependence of DPB161 on SOCE, we applied ACh twice under conditions with and without external Ca²⁺. Without external Ca²⁺, the second Ca²⁺ response is dramatically reduced (Figure 5A), whereas in the presence of external Ca²⁺, sequential

Figure 6: Effects of DPB-161 on the Ca²⁺ entry following thapsigargin-induced Ca²⁺ store depletion in rat submandibular gland acinar cells. (A) In Ca²⁺-free solution, 1 μM thapsigargin (TG) was first applied (4 min), followed by 1 μM ACh. (B) After TG treatment (4 min), the Ca²⁺-containing solution was applied (2 min). (C) Before (1 min) and during addition of extracellular Ca²⁺, 5 μM DPB-161 was applied. (D) Before (1 min) and during addition of extracellular Ca²⁺, 50 μM DPB-161 was applied. (E) Statistical analysis of 14-21 experiments are shown in panels B, C, D.
applications of ACh (interval > 10 min) induces [Ca\textsuperscript{2+}i] elevations of similar size (Figure 5B). In the presence of different concentrations of DPB161 during the second ACh application, the concentration-dependent reduction in the ratio of the second to the first peak amplitude of ACh-induced [Ca\textsuperscript{2+}i] elevations under free extracellular Ca\textsuperscript{2+} conditions demonstrates that DPB161 blocks SOCE in a concentration-dependent manner (Figure 5E). Under [Ca\textsuperscript{2+}]o = 0 conditions, thapsigargin depletes Ca\textsuperscript{2+} stores and opens SOC channels enabling bath perfusion with 1 mM Ca\textsuperscript{2+} to increase [Ca\textsuperscript{2+}i] through SOC channels. Co-application of DPB161 with 1 mM Ca\textsuperscript{2+} prevents SOCE-mediated [Ca\textsuperscript{2+}i] elevation. Taken together, these results suggest that the 2APB analog, DPB161 inhibits SOCE in rat submandibular cells.

Previous work demonstrates that the 2APB analogs DPB162 and DPB163 potently block SOCE in cell lines, in which these 2-APB analogs show high affinity for blocking the SOCE [12, 20, 21]. We further demonstrate that another 2APB analog, DPB161 also inhibits SOCE in rat submandibular cells. However, the potency of SOCE inhibition by DPB161 in rat submandibular cells is lower. For example, 5 μM DPB161 fails to block SOCE elicited by ACh- or thapsigargin-induced depletion of Ca\textsuperscript{2+} stores. This suggests that the structure difference between DPB161 and the other 2APB analogs may partially explain the difference in its potency to block the SOCE. The IC\textsubscript{50} values for SOCE inhibition by DPB161 are about 50-fold higher than DPB163 (1.7x10\textsuperscript{-3} vs. 5.9x10\textsuperscript{-2} μM) in InsP\textsubscript{3} receptor-deficient DT40 cells [11], suggesting a lower affinity of DPB161 compared to DPB163 for inhibition of SOCE, which is consistent with the findings in the present study. However, the affinity of DPB161 for inhibition of the SOCE in native rat submandibular cells is much lower than that in InsP\textsubscript{3} receptor-deficient DT40 cells. These lines of evidence suggest a diversity of SOCE in different cell types.

**MATERIALS AND METHODS**

All experiments were performed in accordance with approved guidelines set and all experimental protocols were approved by the First Affiliated Hospital of Zhengzhou University, the Barrow Neurological Institute, and the Shantou University Medical College Ethical Committees.

**Preparation of single rat submandibular cells**

Acute dissociation of rat submandibular cells was performed as described in our previous publication [19]. In brief, after isoflurane anesthesia, the fragments from adult Wistar rats (both genders) submandibular glands were minced and digested with collagenase (100 units/ml) for 15 min at 37°C. At the end of the collagenase digestion, the cell suspension was gently pipetted to obtain further separation of the cells. The cell suspension was then filtered through a 100 pm platinum mesh, washed twice with the extracellular solution containing 0.2% bovine serum albumin (BSA), and kept in the solution until use. A small amount of the cell suspension was added to a 2 ml-capacity experimental bath on a stage of an inverted microscope (Olympus IX7, Japan). The isolated cells usually adhered to the bottom within 15–20 min and were used for recording within 3 h after preparation. All experiments were performed at room temperature (22 ± 1°C). For the experiments, single cells were usually chosen, but in some cases, small clusters of three to five cells were also used.

**Patch-clamp whole-cell recordings**

Conventional whole-cell, patch-clamp recording was used to record the Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents for monitoring intracellular Ca\textsuperscript{2+} signal oscillations, as reported previously [13, 17, 19]. The recording pipettes, made from borosilicate glass capillaries (Narishige, Japan), were pulled in two stages by a vertical microelectrode puller (P10, Narishige, Japan) to make patch-clamp electrodes. The tip resistance of the electrodes ranged from 4-6 MΩ when filled with 140 mM KC1-containing internal solution. After reaching a high seal resistance (> 2 GΩ), the whole-cell configuration was established by applying a brief negative suction. Transmembrane currents were recorded with a patch-clamp amplifier (Axopatch 200B; Molecular Devices; Sunnyvale, CA USA) at a holding potential (V\textsubscript{h}) of −60 mV. No series resistance was compensated in these experiments.

**Measurement of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}i])**

Isolated cells were preloaded with a standard external solution containing 2 μM Fura-2/AM (Invitrogen, Oregon, USA) for 40 min at 37°C. Ca\textsuperscript{2+} images were captured using an inverted fluorescence microscope (Olympus IX70, Japan) and a silicon intensifier target camera (Cool SNAPfx, Roper Scientific), and recorded on a fluorescence-imaging system (LAMBAD 10-2, Sutter Instrument Co, USA). For measurement of [Ca\textsuperscript{2+}i], with Fura-2, the excitation wavelengths were 340 nm and 380 nm, a dichroic mirror of 440 nm and an emission filter of 510 nm were used. [Ca\textsuperscript{2+}i] was calculated from the ratio (R) of the two fluorescence intensities, F340/F380 [22]. The change in [Ca\textsuperscript{2+}i] was calculated by subtracting [Ca\textsuperscript{2+}i] at the peak response level from that at the pre-stimulated level.

**Experimental solutions**

The standard extracellular (bath) solution contained (in mM) NaCl 130, KCl 14.7, CaCl\textsubscript{2} 1.0, MgCl\textsubscript{2} 1.13, KH2PO4 1.2, KH\textsubscript{2}PO4 0.4, NaHCO\textsubscript{3} 25, Ca\textsubscript{2+}Cl\textsubscript{2} 1.0, Mg\textsubscript{2+}Cl\textsubscript{2} 1.13, and the standard intracellular (internal) solution contained (in mM) CsCl 120, MgCl\textsubscript{2} 5, CsSO\textsubscript{4} 5, EGTA 10, HEPES 10, and the internal solution was adjusted to pH 7.4.
HEPES 10, and glucose 10, titrated with NaOH to pH 7.2. To make a Ca²⁺-free external solution ([Ca²⁺]ₒ = 0 mM), CaCl₂ was removed from and 1 mM EGTA was routinely added to the standard extracellular solution. The standard intracellular (pipette) solution contained (in mM) KCl 140, MgCl₂ 1.13, HEPES 10, glucose 10, ATP 1.0, and EGTA 0.25, titrated with KOH to pH 7.2.

**Application of drugs**

Cells were perfused continuously with a stream of the extracellular solution (~0.5 ml/min). A computer-controlled U-tube system was used for drug application (Huang et al., 2016). The reagents used for the present study were acetylcholine and thapsigargin (Sigma-Aldrich, St. Louis, MO), collagenase (Wako Chemicals, Japan), and Fura-2/AM (Invitrogen, Oregon, USA). The 2-APB analog DPB161 (Figure 1) was a gift from Professor Katsuhiko Mikoshiba.

**Data analysis and statistics**

For patch-clamp experiments, the Ca²⁺-activated Cl⁻ current responses were presented as net charge (current area/Cm/min), then the drug-induced changes were normalized to the current response (induced by ACh) before testing drug (e.g., DPB161) exposure (Baseline). If data were obtained from the same recorded cell and the changes of ACh response were compared prior to, during and after testing drug exposure, a paired t-test was used. To analyze multiple effects (e.g., different concentrations), one-way ANOVA with Tukey’s post hoc tests were used.

**CONFLICTS OF INTEREST**

The authors declare no conflicts of interest.

**ACKNOWLEDGMENTS**

Authors thank Professor Stanley Lin for his comments and carefully correction and edition of English.

**REFERENCES**


