Development of selective blockers for Ca\(^{2+}\)-activated Cl\(^{-}\) channel using Xenopus laevis oocytes with an improved drug screening strategy

Soo-Jin Oh\(^{1,3}\), Jung Hwan Park\(^{2}\), Sungyu Han\(^{1}\), Jae Kyun Lee\(^{2}\), Eun Joo Roh\(^{2}\) and C Justin Lee*\(^{1}\)

Address: \(^{1}\)Center for Neural Science, Future Fusion Technology Laboratory, Korea Institute of Science and Technology (KIST), Seoul 136-791, Republic of Korea, \(^{2}\)Center for Chemoinformatics Research, Life Sciences Division, Korea Institute of Science and Technology (KIST), Seoul 136-791, Republic of Korea and \(^{3}\)Department of Cell and Developmental Biology, Dental Research Institute, School of Dentistry, Seoul National University, Seoul 110-740, Republic of Korea

Email: Soo-Jin Oh - osj@kist.re.kr; Jung Hwan Park - chemistpark@hanmail.net; Sungyu Han - hanpisa@hanmail.net; Jae Kyun Lee - jy602@kist.re.kr; Eun Joo Roh - r8636@kist.re.kr; C Justin Lee* - cjl@kist.re.kr

* Corresponding author

Abstract

Background: Ca\(^{2+}\)-activated Cl\(^{-}\) channels (CaCCs) participate in many important physiological processes. However, the lack of effective and selective blockers has hindered the study of these channels, mostly due to the lack of good assay system. Here, we have developed a reliable drug screening method for better blockers of CaCCs, using the endogenous CaCCs in Xenopus laevis oocytes and two-electrode voltage-clamp (TEVC) technique.

Results: Oocytes were prepared with a treatment of Ca\(^{2+}\) ionophore, which was followed by a treatment of thapsigargin which depletes Ca\(^{2+}\) stores to eliminate any contribution of Ca\(^{2+}\) release. TEVC was performed with micropipette containing chelerythrine to prevent PKC dependent run-up or run-down. Under these conditions, Ca\(^{2+}\)-activated Cl\(^{-}\) currents induced by bath application of Ca\(^{2+}\) to oocytes showed stable peak amplitude when repetitively activated, allowing us to test several concentrations of a test compound from one oocyte. Inhibitory activities of commercially available blockers and synthesized anthranilic acid derivatives were tested using this method. As a result, newly synthesized \(N\)-(4-trifluoromethylphenyl)anthranilic acid with trifluoromethyl group (-CF\(_{3}\)) at para position on the benzene ring showed the lowest IC\(_{50}\).

Conclusion: Our results provide an optimal drug screening strategy suitable for high throughput screening, and propose \(N\)-(4-trifluoromethylphenyl)anthranilic acid as an improved CaCC blocker.

Background

Ca\(^{2+}\)-activated Cl\(^{-}\) channels (CaCCs) are anion-selective channels that can be activated by an increase in cytosolic Ca\(^{2+}\). CaCCs serve a number of important physiological roles in a variety of cell types. These functions include vascular tone regulation, cardiac excitability, smooth muscle contraction, fast block of polyspermy in certain eggs [1]. CaCCs are also known to regulate epithelial secretion of electrolytes and water in kidneys, airways, intestines, pancreas and salivary glands [1]. In addition, CaCCs appear to
participate in signal processing of olfactory transduction, photo receptor light response, gustation and somaesthetic sensation by regulating neuronal cell excitability. CaCC currents in non-sensory neurons of the spinal cord and the autonomic nervous system were also reported, and further investigation may prove an even more extensive expression in the nervous system [2]. Despite this physiological importance of CaCC, the channel remains poorly understood at the molecular, biophysical and pharmacological level, owing to the lack of specific pharmacologic tools with high potent and selectivity. Currently available blockers require high concentrations to completely block CaCCs and are known to cause undesirable side effects and block other channels. For example, niflumic acid and 4,4’-disothiocyanatostilbene-2,2’-disulfonic acid (DIDS) which are widely used to block CaCC also block volume-regulated anion channel (VRAC) in some cell types [3,4]. Niflumic acid, flufenamic acid and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) are shown to have a blocking effect on K+ channel current [5,6]. Niflumic acid, flufenamic acid and NPPB also cause an increase in intracellular Ca2+ concentration ([Ca2+]i) in several cell types, which could elicit other cellular responses [7-11]. Therefore, due to these problems with low potency and selectivity, there is an eminent need for development of better blockers for CaCCs.

**Xenopus laevis** oocytes have been used widely in the field of electrophysiology to study the structure and the function of numerous ion channels and to screen for selective blockers. These oocytes express several native ion channels including CaCC in the plasma membrane [12], which are normally avoided when studying other ion channels by substituting Ca2+ with Ba2+ in the extracellular solution in attempt to prevent activation of endogenous CaCCs. These CaCCs have similar properties in many ways to those in cardiac muscle, smooth muscle, secretory epithelial cells and neurons [13]. There have been a numerous attempts to discover chemical compounds to block the endogenous CaCCs in *Xenopus laevis* oocytes using TEVC (two-electrode voltage clamp) technique. These studies reported half maximal inhibition concentration (IC50) of various compounds, including niflumic acid (17 μM of, IC50) [14], flufenamic acid (28 μM of IC50) [14], DIDS (48 μM of IC50) [13], diphenylamine-2-carboxylate (DPC, 111 μM of IC50) [13], 9-anthracene carboxylic acid (9-AC, 10.3 μM of IC50) [13] and NPPB (22–68 μM of IC50) [15]. But the potency of these blockers is relatively low. In other studies, CaCC current was evoked by direct intracellular injection of Ca2+ into an oocyte [12,16] or by depolarizing the membrane which allows Ca2+ entry through voltage gated Ca2+ channels [17-19]. However, these approaches can introduce complications of unstable baseline current due to irregular amplification of Ca2+ concentration upon Ca2+ entry, unpredictable concentration of Ca2+ release from intracellular stores and time dependent inactivation of CaCCs, making it difficult to perform large scale drug screening. Based on the observation that no reliable method of drug screening and no ideal blocker of CaCC in *Xenopus laevis* oocytes have yet been described, we sought to design an optimized experimental protocol ideal for large scale drug screening to find better blockers for CaCCs.

### Results

**Cl- current elicited by Ca2+ influx in oocytes permeabilized with ionomycin**

To find optimal conditions for drug screening using CaCCs in *Xenopus* oocytes, we first characterized the CaCC currents in *Xenopus* oocytes and compared various treatment conditions. To permeabilize the oocyte membrane to allow Ca2+ influx, oocytes were treated with 10 μM ionomycin for 30 min in Ca2+ free solution. In oocytes, Ca2+ entry and activation of an inward current were achieved by switching from Ca2+ free external solution to Ca2+ containing solution [20]. After membrane permeabilization, application of external Ca2+ evoked Cl- currents consisting of fast peak (Ifast) and slow steady state component (Islow, Figure 1C). The fast component was elicited by 5 s exposure to extracellular Ca2+ ([Ca2+]o) in a dose dependant manner (Figure 1A) and calculated EC50 for [Ca2+]o was 4.89 mM (n = 9) (Figure 1B). Therefore, 5 mM [Ca2+]o was used to induce Cl- current in all subsequent experiments. Ifast and Islow were not induced by substituting Ca2+ with Ba2+ in each condition (Figure 1E, F, I, J, N, O), indicating that both of these currents were activated by Ca2+ entry.

In response to repetitive 5 s applications of the same dose of [Ca2+]o, the amplitude of 2nd response was smaller compared to the initial response (I fast2nd/I fast1st = 0.89 ± 0.04, Figure 1M, white bar), most likely due to an activation of a Ca2+-dependent protein kinase C (PKC) [21]. To exclude the effect of PKC in CaCC current, PKC inhibitor chelerythrine was added to the intracellular solution. Inclusion of chelerythrine decreased the variability represented by the standard error of mean value (I fast2nd/I fast1st = 0.85 ± 0.013, Figure 1M, black bar), but still the 2nd response remained smaller than the 1st response (Figure 1D). Additional variability in peak amplitude can come from the Ca2+ induced Ca2+ release from intracellular stores. To eliminate the contribution of Ca2+ release from intracellular stores, Ca2+ ATPase inhibitor, thapsigargin was treated on ionomycin pretreated oocytes. Under the condition of ionomycin treatment followed by thapsigargin treatment and recording with chelerythrine added intracellular solution, the peak amplitudes of two consecutive responses to 5 mM external Ca2+ were almost the same with relatively low standard error of mean value (Figure 1H, I fast2nd/I fast1st = 1.01 ± 0.02 (n = 7), Figure 1M,
Figure 1
Endogeneous Ca$^{2+}$ activated Cl- channels in Xenopus laevis oocyte. (A) Currents induced by extracellular Ca$^{2+}$ in a dose dependent manner on ionomycin treated oocyte. (B) Dose response and EC$_{50}$ of Ca$^{2+}$ obtained from (A). (C–F) Currents recorded after treatment of ionomycin without thapsigargin treatment. (C, G) Fast peak and slow component during Ca$^{2+}$ applications. (G–J) Currents recorded after treatment of ionomycin followed by thapsigargin. (D, H) Second application of Ca$^{2+}$ induces slightly reduced fast peak amplitude compared to the first peak. (E, I) Ba$^{2+}$ does not induce the slow component. (F, J) Ba$^{2+}$ does not induce the fast peak. (K–O) Comparison of currents under each condition. CHE+ means that current was measured with chelerythrine added intracellular solution. TG+ indicates that thapsigargin was treated on ionomycin pretreated oocytes. (K) Fast peak amplitude. (L) Slow component amplitude. (M) Summary of the experiments shown in (D) and (H). Ratio of amplitude induced by the first and the second Ca$^{2+}$. (N) Summary of the experiments shown in (E) and (I). (O) Summary of the experiments shown in (F) and (J). n indicates number of oocytes. Error bars indicate SEMs. * indicates statistically significant difference by two-tailed t-test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
gray bar). Therefore, a reliable protocol for drug screening of CaCCs in Xenopus oocytes was established with treatment of 1 μM thapsigargin for 90 min to ionomycin treated oocytes followed by recording with microelectrodes filled with intracellular solution containing 1 μM chelerythrine.

**Effect of known blockers and commercially available chemical compounds on CaCC in Xenopus oocytes**

Using the optimized drug screening protocol, the effect of known blockers of CaCC was tested. Over the concentration range tested (1 μM – 300 μM), each of the typical CaCC blockers caused a concentration dependent block of CaCC currents (Figure 2A) and IC50s were obtained from the dose-response curves (Figure 2B). The name and structure for each chemical compound are listed in Figure 3, and each chemical compound was numbered as shown on top of each chemical structure. From the recordings, IC50s were found to be 10.7 μM for DIDS, 32.3 μM for NPPB, 94.3 μM for 9-AC, 37.3 μM for niflumic acid, and 35.4 μM for flufenamic acid (Figure 2C, Figure 3A, Table 1). Other blockers generally known for other Cl- channels were also tested under the same condition. IC50s were found to be 44.5 μM for mefenamic acid and 88.1 μM for N-Phenylanthranilic acid. Except for DIDS, most of known blockers displayed higher IC50 values compared to the previously reported values (Table 1).

We searched for other commercially available chemical compounds that have similar structure to known blockers. Since flufenamic acid, mefenamic acid and N-Phenylanthranilic acid commonly have anthranilic acid backbone, which is composed of two benzene rings, anthranilic acids containing a nitro group (-NO2) such as 5-Nitro-N-phenylanthranilic acid (a-8), N-(2-Nitrophenyl)anthranilic acid (b-1) and N-(3-Nitropheno)anthranilic acid (b-2) were tested (Figure 3A, B). IC50s were 42.5 μM for a-8 and 32.1 μM for b-2. b-1 showed low potency for CaCC block (Figure 3A, B, Table 1). Interestingly, even though a-8, b-1 and b-2 have a similar chemical composition, their blocking effect on CaCC was quite different. The only difference between three chemical compounds is the position of -NO2. Therefore, the position of substituent group on benzene ring of the anthranilic acid backbone apparently affected the blocking effect on CaCC.

**Positional effect of substituent group of benzene ring on block of CaCC current**

To examine the positional effect of -NO2 of benzene ring on block of CaCC current in more detail, we synthesized compounds with substitutions on the benzene ring (see additional file 1). We synthesized N-(4-nitropheno)anthranilic acid (b-3) that has -NO2 on its para position and tested for the block of CaCC current. This compound showed a significantly improved IC50 of 17.8 μM, compared to b-1 that has -NO2 on its ortho position (IC50 > 200 μM) or b-2 that has – NO2 on its meta position (IC50 > 32.1 μM Figure 4A). Based on the fact that blocking effects of -NO2 on the benzene ring of either side of

### Table 1: IC50s of known blockers and anthranilic acid derivatives.

| Compound number | Chemical compound | IC50 * | IC50 n |
|-----------------|-------------------|--------|--------|
| a-1             | DIDS              | 48     | 10.7   |
| a-2             | NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid) | 48–68  | 32.3   |
| a-3             | 9-AC (9-anthracene carboxylic acid) | 10.3   | 94.3   |
| a-4             | Niflumic acid     | 17     | 37.3   |
| a-5             | Flufenamic acid (N-(3-Trifluoromethylphenyl)anthranilic acid) | 28     | 35.4   |
| a-6             | Mefenamic acid    | 44.5   | 6      |
| a-7             | N-Phenylanthranilic acid | 88.1   | 6      |
| a-8             | 5-Nitro-N-phenylanthranilic acid | 42.5   | 8      |
| b-1             | N-(2-Nitrophenyl)anthranilic acid | LP     | 7      |
| b-2             | N-(3-Nitrophenyl)anthranilic acid | 32.1   | 7      |
| b-3             | N-(4-Nitrophenyl)anthranilic acid | 17.8   | 6      |
| b-4             | 5-Nitro-N-(4-nitrophenyl)anthranilic acid | 15.4   | 5      |
| b-5             | N-(2-Trifluoromethylphenyl)anthranilic acid | 29.5   | 6      |
| b-6             | N-(4-Trifluoromethylphenyl)anthranilic acid | 6.0    | 6      |
| b-7             | N-(4-Fluoro-3-trifluoromethylphenyl)anthranilic acid | 14.7   | 6      |
| c-1             | N-(4-Fluorophenyl)anthranilic acid | 63.1   | 6      |
| c-2             | N-(4-Chlorophenyl)anthranilic acid | 11.3   | 6      |
| c-3             | N-(4-Methylphenyl)anthranilic acid | 55.3   | 7      |
| c-4             | N-(4-Isopropylphenyl)anthranilic acid | 17.0   | 6      |
| c-5             | N-(4-tert-Butylphenyl)anthranilic acid | 22.9   | 7      |
| c-6             | N-(4-Decylphenyl)anthranilic acid | LP     | 6      |
| c-7             | N-(4-Methoxyphenyl)anthranilic acid | 102.3  | 5      |

IC50 * means IC50 previously studied. LP: Low Potency. IC50 > 200 μM n indicates number of oocytes.
anthranilic acid backbone (a-8, b-3) showed improved IC50 compared to the N-Phenylanthranilic acid itself (a-7). We synthesized 5-Nitro-N-(4-nitrophenyl)anthranilic acid (b-4) that has -NO2 on its para position on both benzene rings. Interestingly, IC50 of b-4 was further enhanced (IC50 = 15.4 μM) than a-8 and b-3, indicating that substituent group on para position should enhance the blocking effect.

Since flufenamic acid (N-(3-trifluoromethylphenyl)anthranilic acid, a-5) has trifluoromethyl group (-CF3) in meta position, we hypothesized that shifting -CF3 from meta position to ortho or para position would result in changes in IC50. We synthesized N-(2-trifluoromethylphenyl)anthranilic acid (b-5) that has -CF3 on its ortho position and N-(4-trifluoromethylphenyl)anthranilic acid (b-6) that has -CF3 on its para position. b-5 showed enhanced IC50 (IC50 = 29.5 μM) than a-5 (Figure 3B, 4B). In particular, b-6 dramatically enhanced the IC50 compared to any other compounds (IC50 = 6.0 μM, Figure 3B, 4B). These data indicate that changing the position of substituent group on the benzene ring of anthranilic acid backbone to para position leads to an increase in blocking activity.

Figure 2
Effect of known blockers on Ca2+ activated Cl- channel. (A) Trace of Ca2+ activated Cl- channel current before and during application of flufenamic acid (FA). (B) Dose response relation of flufenamic acid block of Ca2+ activated Cl- current. (C) Summary of IC50s of commercially available blockers for Ca2+-activated Cl- channel. n indicates number of oocytes. Error bars indicate SEMs.
Figure 3
Chemical structures and IC_{50s} of known blockers and anthranilic acid derivatives. (A) Known blockers. (B) Anthranilic acid derivatives; positional compounds. (C) Anthranilic acid derivatives that have variable substituent group on para position of benzene ring. LP: Low Potency. IC_{50} > 200 μM. n indicates number of oocytes.
Figure 4
Positional effect of substituent group on the phenyl ring of blocker that affects block of Ca\(^{2+}\)-activated Cl\(^-\) current. (A) Comparison of chemical structure, IC\(_{50}\) and dose response between N-(2-nitrophenyl)anthranilic acid, N-(3-nitrophenyl)anthranilic acid and N-(4-nitrophenyl)anthranilic acid in which the nitro (-NO\(_2\)) group on the benzene ring is positioned at ortho, meta and para position. (B) Comparison of chemical structure, IC\(_{50}\) and dose response between flufenamic acid and derivatives N-(2-trifluoromethylphenyl)anthranilic acid and N-(4-trifluoromethylphenyl)anthranilic acid in which the trifluoromethyl (-CF\(_3\)) group on the benzene ring is positioned at ortho, meta and para position. Shaded boxes indicate the substituent groups tested. n indicates number of oocytes. Error bars indicate SEMs.

Comparison of blocking effect about anthranilic acid derivatives on CaCC
Since para positioned anthranilic acid derivatives showed higher potency of block, we synthesized more anthranilic acid derivatives that have various substituents on para position of the benzene ring and tested on CaCC currents. However, blocking activity of these derivatives was not better than b-5 with -CF\(_3\) on para position as indicated by their IC\(_{50}\): c-2 (chloryl group, -Cl, IC\(_{50}\) = 11.3 \mu M), c-4 (isopropyl group, -C\(_3\)H\(_7\), IC\(_{50}\) = 17.0 \mu M) and c-5 (butyl
group, \(-C_4H_9\) IC50 = 22.9 \(\mu M\) (Figure 3C, Table 1). In addition, N-(4-Fluorophenyl)-anthranilic acid (c-1, IC50 = 63.1 \(\mu M\)), N-(4-Methylphenyl)-anthranilic acid (c-3, IC50 = 55.3 \(\mu M\)), N-(4-Decylphenyl)-anthranilic acid (c-6, IC50 > 200 \(\mu M\)) and N-(4-Methoxyphenyl)-anthranilic acid (c-7, IC50 = 102.3 \(\mu M\)) which have fluoro group (-F), methyl group (-CH3), decyl group (-CH2(CH2)2CH3) and methoxy group (-OCH3) showed relatively low potency for CaCC block, respectively (Figure 3C, Table 1). Therefore, the most potent derivative among the compounds tested was the N-(4-trifluoromethylphenyl)anthranilic acid that has -CF3 on its para position with IC50 of 6.0 \(\mu M\).

Discussion

Since the lack of useful tools for drug screening has impeded the development of better blocker for CaCCs, we designed an improved drug screening protocol utilizing endogenous CaCCs in Xenopus oocytes. Our newly designed protocol consists of Xenopus Leavis oocytes treated with ionomycin and thapsigargin followed by recording with intracellular solution containing chelerythrine. These manipulations provide a consistent induction of CaCC mediated currents with similar amplitude upon repeated application of 5 mM external Ca2+. Treatment of ionomycin allows constant entry of Ca2+ from extracellular solution, whereas thapsigargin prevents irregular concentration fluctuations caused by Ca2+ release amplification from internal stores. Elevation of intracellular Ca2+ due to Ca2+ influx through Ca2+ channels formed by ionomycin should cause inactivation of the CaCC conductance via activation of PKC [21]. Thus, inhibition of PKC should decrease the channel modulation by PKC phosphorylation, which was shown to modulate the CaCC current [22]. Thus, intracellular solution containing PKC inhibitor chelerythrine was used for recording of CaCC current to exclude the effect of PKC. Under these conditions, the intracellular Ca2+ concentration was kept constant during induction of CaCC current, and current rundown due to PKC inactivation was kept at minimal level. These manipulations made it possible to measure blocking effect of various compounds on CaCC-mediated current in Xenopus leavis oocytes more accurately.

In addition, some blockers such as niflumic acid change the intracellular Ca2+ buffering which causes an increase in intracellular Ca2+ concentration [7-11]. Since CaCC current is induced exclusively by external Ca2+ in our protocol, we were able to isolate the blocking effect of various compounds on channel activities from the effect of compounds on Ca2+ buffering capacity.

Utilizing this optimized protocol, we first compared the effect of known blockers of CaCC. We found that IC50s of DIDS, NPPB, 9-AC, niflumic acid and flufenamic acid on CaCC current in Xenopus oocytes were 10.7 \(\mu M\) (DIDS), 32.3 \(\mu M\) (NPPB), 94.3 \(\mu M\) (9-AC), 37.3 \(\mu M\) (niflumic acid) and 35.4 \(\mu M\) (flufenamic acid), whereas previously reported values are 48 \(\mu M\), 28-68 \(\mu M\), 10.3 \(\mu M\), 17 \(\mu M\) and 28 \(\mu M\), respectively (see Table 1). Blocking effect of each known blocker differed from previous reports. It is likely due to differences in our protocol. We suggest that our modified screening protocol provides more exact profile of CaCC blocker on CaCC in Xenopus Leavis oocytes due to the reliability and repeatability of our assay protocol. In addition, some blockers that were generally used for other Cl- channels such as mefenamic acid and N-Phe- nylantranilic acid showed comparable blocking effect on CaCC in Xenopus Leavis oocytes with IC50s of 44.5 \(\mu M\) and 88.1 \(\mu M\).

Based on the fact that several known blockers of CaCCs have structural similarity as anthranilic acid which is composed of two benzene rings, we searched for CaCC blocker candidates with the structural similarity among commercially available chemical compounds. Even though, the number of compounds were not enough to explain exact SAR (structure-activity relationship), we could figure out the correlation between biological activity and the kind and position of substituents. We found that 5-Nitro-N-phenylantranilic acid and N-(3-Nitrophenyl)anthranilic acid both showed similar level of blocking effect as niflumic acid or flufenamic acid. Interestingly, blocking potency was quite different between N-(3-Nitrophenyl)anthranilic and N-(2-Nitrophenyl)anthranilic acid even though their chemical composition is identical, except for the relative position of -NO2 in the benzene ring. This reflected that the position of -NO2 affected the blocking activity to CaCC. Synthesized N-(4-Nitrophenyl)anthranilic acid that has -NO2 on its para position showed improved blocking ability compared to N-(3-Nitrophenyl)anthranilic and N-(2-Nitrophenyl)anthranilic acid with -NO2 on meta and ortho position, respectively. Likewise, synthesized N-(4-trifluoromethylphenyl)anthranilic acid that has -CF3 on its para position blocked CaCC better than flufenamic acid (N-(3-trifluoromethylphenyl)anthranilic acid) and N-(2-trifluoromethylphenyl)anthranilic acid with -CF3 on meta and ortho position, respectively. These results suggested that the positioning of substituent group on para site contributes to a higher affinity of these compounds to CaCC. Therefore, we concluded that anthranilic acid derivatives containing para positioned substituent group have high potency of CaCC block.

Additionally synthesized chemical compounds are anthranilic acid derivatives that have various substituent groups such as various hydrocarbons introduced into the benzene ring at para position. IC50s of N-(4-Chlorophenyl)-anthranilic acid, N-(4-Isoproplyphenyl)-anthranilic acid and N-(4-tert-Butylphenyl)-anthranilic acid were...
similar in IC\textsubscript{50} of N-(4-Nitrophenyl)anthranilic acid. However, low blocking effect was shown in N-(4-Fluorophenyl)-anthranilic acid, N-(4-Methylphenyl)-anthranilic acid and N-(4-Decylphenyl)-anthranilic acid. These results suggested that there should be no strong correlation between blocking effect and size of substituent group in CaCC blockers. It has been reported that blocker size affects voltage dependence rather than potency of CaCC block because large blockers lodge at sites less deep in the channel [1]. Taken together, N-(4-trifluoromethylphenyl)anthranilic acid that has -CF\textsubscript{3} on its para position in one benzene ring is most potent CaCC blocker candidate so far.

Ion channels endogenously expressed in Xenopus Leavis oocytes have extensively used in biological and pharmacological research. CaCCs in Xenopus Leavis oocytes have similar properties in many ways to those in other tissue. It has been revealed that several human diseases are involved in CaCCs. However, its molecular identity is not clear yet. Recently, CLCA [23,24], bestrophin [25], tweety [26] and TMEM16A [27] have been proposed as the molecular candidates. It is important to note that blocker candidates confirmed in Xenopus Leavis oocytes should be also examined in these candidate molecules and CaCCs in other various tissues. Nevertheless, several common blockers for CaCCs have undesirable side effects: they can affect the cellular Ca\textsuperscript{2+} level [7-11], and block VRACs [3].

These results suggested that there should be no strong correlation between blocking effect and size of substituent group in CaCC blockers. It has been reported that blocker size affects voltage dependence rather than potency of CaCC block because large blockers lodge at sites less deep in the channel [1]. Taken together, N-(4-trifluoromethylphenyl)anthranilic acid that has -CF\textsubscript{3} on its para position in one benzene ring is most potent CaCC blocker candidate so far.

**Conclusion**

This study has shown the development of reliable screening method for CaCC blocker using endogeneous CaCCs in Xenopus laevis oocytes. We found that anthranilic acid derivatives containing para positioned substituent group have high potency of CaCC block and N-(4-trifluoromethylphenyl)anthranilic acid is most effective CaCC blocker among the synthesized chemical compounds.

**Methods**

**Preparation of oocytes**

Matured stage V–VI oocytes [29] harvested from adult Xenopus laevis females (Xenopus I, Michigan, USA) which were maintained in an automated maintenance system, Xenopus System (Aquatic Habitats, Florida, USA). The animals were anaesthetized by cooling with ice [30]. Ovarian follicles were surgically removed, treated with 2 mg/ml collagenase type IA at room temperature for 90 min in Ca\textsuperscript{2+} free Barth’s solution containing 89 mM NaCl, 1.0 mM KCl, 2.4 mM NaHCO\textsubscript{3}, 0.82 mM MgSO\textsubscript{4} and 10 mM HEPES (pH 7.4). The oocytes were extensively rinsed with normal Barth’s solution containing 88 mM NaCl, 1.0 mM KCl, 2.4 mM NaHCO\textsubscript{3}, 0.82 mM MgSO\textsubscript{4}, 0.33 mM Ca(NO\textsubscript{3})\textsubscript{2}, 1.41 mM CaCl\textsubscript{2} and 5 mM HEPES (pH 7.4), placed in culture Barth’s solution containing 88 mM NaCl, 1.0 mM KCl, 2.4 mM NaHCO\textsubscript{3}, 0.82 mM MgSO\textsubscript{4}, 0.33 mM Ca(NO\textsubscript{3})\textsubscript{2}, 0.91 mM CaCl\textsubscript{2}, 10 mM HEPES, 10 μg/ml streptomycin and 10 μg/ml penicillin (pH 7.4), and maintained at 18°C. Oocytes were used 1–4 days after isolation.

**Synthesis**

All commercially available chemicals were reagent grade and used as purchased unless stated otherwise. All reactions were performed under an inert atmosphere of dry argon or nitrogen using distilled dry solvents. Reactions were monitored by TLC analysis using Merck silica gel 60 F-254 thin layer plates. Flash column chromatography was carried out on Merck silica gel 60 (230–400 mesh) by preparative LC system. \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were recorded either on a spectrometer operating at Bruker 400 and 100 MHz, respectively. Preparations of chemicals are described in detail in Additional file 1.

**Electrophysiology**

To permeabilize the membrane to Ca\textsuperscript{2+}, oocytes were incubated in the oocyte recording solution containing 96 mM NaCl, 2 mM KCl, 2 mM MgCl\textsubscript{2}, 0.5 mM EGTA and 10 mM HEPES (pH 7.4), added with 10 μM ionomycin for 30 min. The ionomycin was then removed from the external solution by washing with oocyte recording solution. In case of thapsigargin treatment, ionomycin treated oocytes were subsequently incubated in the oocyte recording solution containing 1 μM thapsigargin for 90 min. Then thapsigargin was also washed with the oocyte recording solution. Two electrode voltage-clamp recordings were made using Warner model OC725B two-electrode voltage clamp amplifier (Warner Instruments, Inc., Hamden, CT) with 1 M KCl-filled microelectrodes (WPI; Sarasota, FL; 1B150F-4) pulled with a P-97 programmable pipette puller (Sutter Instruments Co.; Novato, CA). The pipettes had resistances of 1–3 MΩ. During the recording oocytes were continuously perfused with oocyte recording solution. All recordings were from a holding potential of -60 mV. Drugs were prepared in separate bottles and applied by gravity. Flow of solutions was approximately 1 ml/min.

**Chemicals**

Chemical compounds including the following reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA); Collagenase type 1A, ionomycin-Ca\textsuperscript{2+} salt, thapsi-
gargin, chelerythrine. HEPES (N-[2-hydroxyethyl]piperazine-N'-[ethanesulfonic acid]) was obtained from J.T Baker (Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA).

Analysis of data
Currents were digitally recorded with AxoScope software (Axon Instruments, Burlingame, CA, USA) and data analysis was done with SigmaPlot 10.0 (Systat Software, Inc., CA, USA). All the current responses during a blocker were normalized to the average of a Ca2+ induced Cl− current applied before blocker application. Normalized and averaged data were fitted to the SigmaPlot’s Logistic, 3 Parameter curve to determine dose-response relationship and IC50. All data are expressed as mean ± standard error of mean and statistical analysis was performed using a 2-tailed t-test.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
SJO carried out electrophysiological recordings, data analysis and manuscript preparation. JHP, IKL, and EJR designed and synthesized chemical compounds. SKH participated in electrophysiological recordings and synthesis of chemical compounds. CJL conceived the idea, coordinated the study, carried our data interpretation and drafted the manuscript. All authors have read and approved the manuscript.

Additional material

Additional file 1
Methods for synthesis of anthranilic acid derivatives.
Click here for file [http://www.biomedcentral.com/content/supplementary/1756-6606-1-14-S1.doc]

Acknowledgements
We thank to Dayoung Cho for her contribution to drug screening.

This work was supported by the Korea research foundation KRF-2005-070-C00096 (C.J.L), KIST Core Competency Program and KIST Institutional Program.

References
1. Hartzell C, Putzier I, Arreola J: Calcium-activated chloride channels. Annu Rev Physiol 2005, 67:719-758.
2. Frings S, Reuter D, Kleene SJ: Neuronal Ca2+-activated Cl− channels – homing in on an elusive channel species. Prog Neurobiol 2000, 60:247-289.
3. Xu WX, Kim SJ, So I, Kang TM, Rhee JC, Kim KW: Volume-sensitive chloride current activated by hypoxic swelling in antral gastric myocytes of the guinea-pig. Pflugers Arch 1997, 435(1):9-19.
4. Greenwood IA, Large WA: Properties of a Cl− current activated by cell swelling in rabbit portal vein vascular smooth muscle cells. Am J Physiol 1992, 265(5 Pt 2):H1524-H1532.
5. Wang HS, Dixon JE, McKinnon D: Unexpected and differential effects of Cl− channel blockers on the Kv4.3 and Kv4.2 K+ channels. Implications for the study of the I(to2) current. Circ Res 1997, 81:711-718.
6. Greenwood IA, Leblanc N: Overlapping pharmacology of Ca2+-activated Cl− and K+ channels. Trends Pharmacol Sci 2007, 28(1):1-5.
7. Poronnik P, Ward MC, Cook DI: Intracellular Ca2+ release by flufenamic acid and other blockers of the non-selective cation channel. FEBS Lett 1992, 296:245-248.
8. Reinsprecht M, Rohin MH, Spadenger I, Pecht I, Schindler H, Romanin C: Blockade of capacitative Ca2+ influx by Cl− channel blockers inhibits secretion from rat mucosal-type mast cells. Mol Pharmacol 1995, 47:1014-1020.
9. Lee J, Lee SJ, Park JD, Kim SJ: Action of diphenylamine carboxylate derivatives, a family of non-steroidal anti-inflammatory drugs, on [Ca2+]i and Ca2+-activated channels in neurons. Neurosci Lett 1995, 190:121-124.
10. Schultheiss G, Frings M, Hollingshaus G, Diener M: Multiple action sites of flufenamate on ion transport across the distal colon. Br J Pharmacol 2000, 130:875-885.
11. Partridge LD, Valenzuela CF: Block of hippocampal CAN channels by flufenamate. Brain Res 2000, 867:143-148.
12. Miledi R, Parker J: Chloride current induced by injection of calcium into Xenopus oocytes. J Physiol (Lond) 1984, 357:171-183.
13. Qu Z, Hartzell HC: Functional geometry of the permeation pathway of Ca2+-activated Cl− channels inferred from analysis of voltage-dependant block. J Biol Chem 2001, 276:18423-18429.
14. White MM, Aylin M: Niiflumic and flufenamic acids are potent reversible blockers of Ca2+-activated Cl− channels in Xenopus oocytes. Mol Pharmacol 1990, 37:720-724.
15. Wu G, Hamill OP: NPPB block of Ca2+-activated Cl− channels in Xenopus oocytes. Pflugers Arch 1992, 420:227-229.
16. Dascal N, Gillo B, Lass Y: Role of calcium mobilization in mediation of acetylcholine-evoked chloride currents in Xenopus laevis oocytes. J Physiol 1985, 366:299-313.
17. Miledi R: A calcium-dependent transient outward current in Xenopus laevis oocytes. Proc R Soc Lond B Biol Sci 1982, 215(1201):49-97.
18. Barish ME: A transient calcium-dependent chloride current in the immature Xenopus oocytes. J Physiol 1983, 342:309-325.
19. Dascal N, Snutch TP, Lubbert H, Davidson N, Lester HA: Expression and modulation of voltage-gated calcium channels after RNA injection in Xenopus oocytes. Science 1986, 231:147-50.
20. Boton R, Gillo B, Dascal N, Lass Y: Two calcium-activated chloride conductance in Xenopus laevis oocytes permeabilized with the ionophore A23187. J Physiol (Lond) 1989, 408:511-534.
21. Boton R, Singer D, Dascal N: Inactivation of calcium-activated chloride conductance in Xenopus oocytes: roles of calcium and protein kinase C. Pflugers Arch 1990, 416:1-6.
22. Hahnenkamp K, Durieux ME, van Aken H, Berning S, Heyse TJ, Hone mann CW, Linck B: Modulation of Xenopus laevis Ca2+-activated Cl− currents by protein kinase C and protein phosphatases: implications for studies of anesthetic mechanisms. Anesth Analg 2004, 99:416-422.
23. Gandhi R, Eble RC, Gruber AD, Ji HL, Copeland SM, Fuller CM, Pauli BU: Molecular and functional characterization of a calcium-sensitive chloride channel from mouse lung. J Biol Chem 1998, 273:22096-22101.
24. Gruber AD, Eble RC, Ji HL, Schreur KD, Fuller CM, Pauli BU: Genomic cloning, molecular characterization, and functional analysis of human CLCA1, the first human member of the family of Ca2+-activated Cl− channel proteins. Genomics 1998, 54:200-214.
25. Sun H, Tsunenari T, You KW, Nathans J: The vitelliform macular dystrophy protein defines a new family of chloride channels. Proc Natl Acad Sci USA 2002, 99(8):5008-13.
26. Suzuki M, Mizuno A: A novel human Cl(−) channel family related to Drosophila flightless locus. J Biol Chem 2004, 279(21):22461-8.
27. Yang YD, Cho H, Koo JY, Tak MH, Cho Y, Shim WS, Park SP, Lee J, Lee B, Kim BM, Raouf R, Shin YK, Oh U: TMEM16A confers
receptor-activated calcium-dependent chloride conductance. Nature 2008, 455(7217):1210-1215.
28. Ouellet M, Falgueyret JP, Percival MD: Detergents profoundly affect inhibitor potencies against both cyclo-oxygenase isoenzymes. Biochem J 2004, 377:675-684.
29. Dumont JN: Oogenesis in Xenopus laevis (Daudin). I. Stages of oocyte development in laboratory maintained animals. J Morphol 1972, 136(2):153-179.
30. Yoshida S, Plant S: Mechanism of release of Ca²⁺ from intracellular stores in response to ionomycin in oocytes of the frog Xenopus laevis. J physiol 1992, 458:307-318.