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Characterization of the Pharmacology of YM-198313 on Volume-Regulated Anion Channels

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Activation of the volume-regulated anion channels (VRAC) is considered to be involved in arrhythmia, but it has not yet been fully elucidated because of the lack of its high affinitive and selective compounds. A newly synthesized compound, YM-198313 (sodium 4-({[2-(methylthio)benzyl]amino}-5-[(1-phenylethyl)thio]isothiazol-3-olate), strongly inhibited VRAC in HeLa cells with an IC_{50} of 3.03±0.05 μM. However, YM-198313 weakly affected both the Ca^{2+}-activated Cl^- channels in HTC cells and the cAMP-activated Cl^- channels in T84 cells, demonstrating that this compound is selective for VRAC among Cl^- channels. At 10 μM, YM-198313 almost completely (100±7.8%) inhibited the VRAC current in guinea pig atrial myocytes. However, at the same concentration, YM-198313 showed little inhibitory effect on the cardiac cation currents in ventricular myocytes. We believe that YM-198313 is a potent and selective VRAC inhibitor, therefore, it should be use to clarify the role VRAC plays in arrhythmia.

Key words YM-198313; volume-regulated anion channel; chloride channel; cardiac myocyte; hypotonic solution

Volume-regulated anion channels (VRAC), a class of Cl-channel, are activated by cell swelling. This type of the channel is ubiquitously expressed in mammalian cells and plays an important role in the control of cell volume homeostasis.[2] The VRAC is characterized by an outwardly-rectifying current, and may be permeated by both inorganic and organic anions.[3] Various proteins have been proposed as molecular candidates for the composition of VRAC: Mdr (multidrug resistance protein) or P-glycoprotein, pICln (protein associated with a nucleotide-sensitive chloride current), phospholemman, ClC-2, and ClC-3.[4,5] However, the molecular identification of the protein the VRAC consist of remains unclear.

In mammalian cardiac myocytes, VRAC activation has been reported in some experimental pathophysiological conditions, such as cardiac ischemia,[6—8] endotoxic shock,[9] and tachycardia-induced congestive heart failure.[10] Accordingly, the activation of VRAC due to the swelling of cardiac myocytes under the pathophysiological conditions may contribute to heart diseases such as arrhythmia and/or heart failure. However, validation of this notion suffers from the lack of selective high-affinity inhibitors. A number of small organic molecules with VRAC-blocking properties have been described, but they are neither very potent nor selective. New molecules with a more selective inhibitory effect on VRAC should therefore be a useful tool for further investigation of the pathophysiological role of VRAC in cardiac myocytes.

In this study, we investigated the inhibitory effects of a newly-synthesized molecule, YM-198313, on VRAC in HeLa cells and guinea pig cardiac myocytes. In order to demonstrate the selectivity of YM-198313 among Cl^- channels, we also evaluated the effects of this compound on Ca^{2+}-activated Cl^- channels (CaCC) and cAMP-activated Cl^- channels (to be referred to from this point as "CFTR"; cystic fibrosis transmembrane conductance regulator, because it was shown to be identical to CFTR[11]), both of which exist in cardiac myocytes. Furthermore, in order to examine the effect of YM-198313 on cardiac cation channels, the compound was tested as an inward rectifier K^+ current (I_{Kir}), Na^+ current (I_{Na}), L-type Ca^{2+} current (I_{Ca-L}), and voltage-activated K^+ current (I_{Kv}) using the signature current measurement method.

MATERIALS AND METHODS

Materials [^{14}C]Taurine (108.5 Ci/mol) was purchased from NEN Life Science Products (Boston, MA, U.S.A.). Pentobarbital sodium was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). NPPB (5-nitro-2-(3′-phenylpropyl-amino)-benzoic acid) was obtained from Nacalai Tesque (Kyoto, Japan). DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) was from Wako Pure Chemicals Industries (Osaka, Japan). YM-198313 (sodium 4-({[2-(methylthio)benzyl]amino}-5-[(1-phenylethyl)thio]isothiazol-3-olate) and mibefradil were synthesized at Yamanouchi Pharmaceutical (Ibaraki, Japan). The chemical structure of YM-198313 is shown in Fig. 1. All other chemicals were from Sigma Chemical (St. Louis, MO, U.S.A.). All test compounds were dissolved in dimethyl sulfoxide at 100×concentrated stock solutions and diluted each experimental solutions. Cell culture solutions and reagents were obtained from Gibco (Grand Island, NY, U.S.A.).

Animals Male Hartley guinea pigs (300—400 g) were obtained from Japan SLC (Shizuoka, Japan). Pelleted food

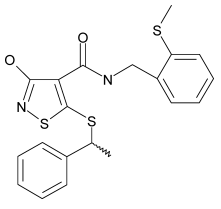

Fig. 1. Chemical Structure of YM-198313

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and municipal tap water were made freely available to them. Animals were maintained in accordance with the Institutional Guides for the Care and Use of Laboratory Animals, and the study was approved by the Animal Ethical Committee of Yamanouchi Pharmaceutical.

Cell Culture Human cervix epitheloid carcinoma (HeLa) cells (ATCC, VA, U.S.A.) and rat hepatoma (HTC) cells (ECACC, Wiltshire, U.K.) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 100 U/ml penicillin, and 100 mg/l streptomycin. Human colon carcinoma (T84) cells (Dainippon Pharmaceutical, Osaka, Japan) were maintained in a mixture of Ham's F12 medium and DMEM with 2.5 mM L-glutamine, 5% FBS, 100 U/ml penicillin, and 100 mg/l streptomycin. These cells were cultured in 5% CO_2 with 100% humidity at 37 °C, and maintained in the exponential growth phase by passaging them twice weekly.

Taurine Efflux Measurement The taurine efflux from HeLa cells was measured at room temperature as described previously.[12,13] In brief, HeLa Cells grown to 80% confluence in 24-well culture plates were loaded with [^{14}C]taurine by incubation for 6 h in growth medium containing [^{14}C]taurine (0.1 μCi/ml). Immediately before beginning the efflux experiment, the loading solution was removed and the cells were washed twice with isotonic solution which containing (in mM): KCl 150, $MgCl_2$ 0.5, $CaCl_2$ 1.3, and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) 10. To measure the amount of [^{14}C]taurine remaining inside the cells under basal conditions and after hypotonic-stimulation, the cells were incubated for 30 min with an appropriate isotonic solution, or a hypotonic solution with the KCl concentration reduced to 95 mM. After incubation, the cells were washed twice with isotonic solution, and at the end of the experiment, 0.5 ml of 0.1 N NaOH was added to each well to lyse the cells. The lysate was transferred to a vial containing scintillation liquid, and the [^{14}C]taurine was counted using a liquid scintillation counter (LS6000LL, Beckman Coulter. Fullerton, CA, U.S.A.). The amount of [^{14}C]taurine efflux from hypotonic cells was calculated by subtracting the amount of [^{14}C]taurine remaining inside the cells in hypotonic solution from that from cells in isotonic solution. This difference was taken to represent the control taurine efflux. In inhibitor experiments, the cells were incubated for 30 min with hypotonic solution. The inhibitory effect due to the test compounds was estimated as the decrease in the amount of [^{14}C]taurine efflux from the cells as compared to that in the control experiments. The inhibitory effect of the test compounds on the [^{14}C]taurine efflux was expressed as a percent of the amount of control taurine efflux.

I^- Efflux Measurement HTC cells or T84 cells grown to 80% confluence in 6-well culture plates were preloaded with I^- by incubation for 1—2 h at 37 °C in a loading solution containing (in mM) NaI 140, KI 5, $Ca(NO_3)_2$ 1.3, $Mg(NO_3)_2$ 0.5, HEPES 20, and glucose 5. The loading solution was removed and the cells washed five times in solution that contained (in mM): $NaNO_3$ 150, KNO_3 5, $Ca(NO_3)_2$ 1.3, $Mg(NO_3)_2$ 0.5, HEPES 20, and glucose 5, at room temperature (*ca.* 20 °C). Upon removal of the final NO_3^- wash solution, 1 ml of activating solution was added. The final wash solutions were reserved in 24-well culture plates for the measurement of I^-. The activation of CaCC was achieved by using NO_3^- solution containing 100 μM ATP. For CFTR activation, the NO_3^- solution contained 10 μM forskolin (FK). After 2 min, aliquots were removed to 24-well culture plates and I^- concentrations were measured using an I^--sensitive electrode (Model 9653, Orion Research, Inc., Beverly, MA, U.S.A.). The I^- efflux stimulated by ATP or FK was calculated by subtracting the amount of I^- in the final wash solutions from that in the stimulating solutions. In inhibitor experiments, the inhibitors were included in the final wash solutions as well as in the stimulating solution. Inhibition was expressed as the percentage reduction of the amount of control I^- efflux.

Isolation of Cardiac Myocytes Atrial and ventricular myocytes were isolated from the hearts of guinea pigs for electrophysiological experiments. Single cardiac myocytes were obtained using standard enzymatic techniques similar to those described previously.[14] Briefly, animals were bled to death, their hearts were rapidly removed and retrogradely Langendorff-perfused at 37 °C with oxygenated modified Tyrode's solution contained (in mM) NaCl 145, KCl 4, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, glucose 10. The heart was then perfused with Ca^{2+}-free modified Tyrode's solution for approximately 10 min, and subsequently with the same solution containing collagenase (0.04% w/v Type 1) for 20 min. Cells were obtained by gentle mechanical agitation in Modified Tyrode's solution containing Ca^{2+} (0.2 mM). Cells from the atrial and the ventricles were used for VRAC and signature currents, respectively.

Electrophysiological Recording Membrane currents were acquired using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, U.S.A.). Signals were digitized at 10 kHz and stored on digital audiotape (DTC 1000; Sony, Tokyo, Japan). Digitized membrane currents were acquired by computer-driven software (pCLAMP v.6.0.3, Axon Instruments). Recordings were low-pass filtered at 1 kHz. During whole-cell VRAC current recordings, junction potential changes were minimized by using a continuous agar bridge (4% agar in 3 M KCl) where the reference Ag/AgCl electrode was immersed in a 3 M KCl solution. During signature current recordings the Ag/AgCl electrode was immersed directly into the perfusate in the recording bath. Non-heparinized haematocrit glass tubes were pulled using a micropipette puller (P-97/IVF, Sutter Instruments, Novato, CA, U.S.A.). The tubes had a tip resistance of 2 to 5 MΩ when filled with the various pipette solutions.

Isolated cardiac myocytes were placed in a Perspex chamber mounted on an inverted microscope (TE300; Nikon, Tokyo, Japan). The cells were allowed to settle, and then superfused at 20—25 °C[15—17] with a Modified Tyrode's solution until the whole-cell recording configuration had been obtained, after which the cell interior was equilibrated with the pipette solutions at the relevant holding potential. The pipette solution used when measuring the VRAC current contained (in mM) $MgCl_2$ 2, HEPES 5, $CsCl_2$ 30, Tetraethylammonium chloride (TEA-Cl) 20, Tris–ATP 5, Li_2-GTP 0.1, and EGTA 5. The pipette solution used when measuring the signature current contained (in mM) K-glutamate 75, piperazine-*N*,*N*′-bis[2-ehtaneslphonic acid] neutralized to pH 7.1 with KOH 30, KCl 20, $MgCl_2$ 0.5, K_2EGTA 0.05, Mg-ATP 10, Tris phosphocreatine 5, Tris GTP 0.1, and pyruvic acid 5.0.

Voltage-ramp recordings from guinea pig atrial myocytes were used for measuring the VRAC current. K^+-free isotonic solution contained (in mM): $MgCl_2$ 2, HEPES 10, glucose 5.5, NaCl 100, $BaCl_2$ 2, Na-aspartate 40, and nicardipine 0.001. To activate the VRAC current, this solution was replaced by hypotonic solution of the same composition, except for the Na-aspartate. Applying the hypotonic solution swelled the cell and if cell size and current amplitude remained relatively constant for 5 min in the absence of drugs, the current–voltage (*I–V*) relationship of the VRAC current was determined by a linear membrane potential ramp from +70 to −90 mV, followed by a voltage-ramp step to +70 mV from a holding potential of −50 mV, at a rate at 0.32 V/s. This step-ramp voltage protocol for VRAC current was repeated at a frequency of 0.1 Hz.

For measuring signature currents, voltage-ramp recordings from rat ventricular myocytes were made as described previously.[18] Signature currents were evoked by a linear membrane potential ramp from −90 to +70 mV, followed by a voltage-ramp step to −90 mV from a holding potential of −50 mV, at a rate at 0.32 V/s. A basic stimulation frequency of 0.33 Hz was used.

RESULTS

Effect of YM-198313 on Hypotonic-Stimulated Taurine Efflux (VRAC) in HeLa Cells The efflux of organic osmolytes, such as taurine, accompanies cell volume increases in HeLa cells.[19] Therefore, to evaluate the effects of YM-198313 and other Cl^- channel blockers (DIDS, NPPB, and glybenclamide) on VRAC, [^{14}C]taurine efflux, stimulated by hypotonic conditions, was measured (Fig. 2). YM-198313 inhibited the taurine efflux stimulated by hypotonic solutions in a concentration-dependent manner. The IC_{50} of YM-198313 was 3.03±0.05 μM (n=3). DIDS, NPPB, and glybenclamide also inhibited the taurine efflux in a concentration-dependent manner. The IC_{50} values of these Cl^- channel inhibitors were 251±23 μM (n=3), 53.8±1.74 μM (n=3), and 218±34 μM (n=3), respectively. Based on these IC_{50} values, we determined that YM-198313 inhibited VRAC stimulated by hypotonic conditions at least 18-fold more potently than other Cl^- channel inhibitors.

Effect of YM-198313 on I^- Efflux The rationale behind the use of I^- efflux to monitor Cl^- channel activity lies in the greater relative permeability of Cl^- channels to this ion compared with most other anion exchangers and transporters.

1) ATP-induced I^- Efflux (CaCC): The inhibitory effects of YM-198313 and other Cl^- channel inhibitors, like NPPB and mibefradil,[20,21] on CaCC were evaluated using an ATP-induced I^- efflux assay in HTC cells.[22] YM-198313 and the other Cl^- channel inhibitors concentration dependently inhibited ATP-induced I^- efflux through the CaCC in HTC cells (Fig. 4). The IC_{50} values of YM-198313, NPPB and mibefradil were 15.4±0.69 μM (n=3), 13.2±6.8 μM (n=3), and 21.2±3.15 μM (n=3), respectively.

2) FK-induced I^- Efflux (CFTR): We also used FK-induced I^- efflux in T84 cells[23] to compare the inhibitory effect of YM-198313 and glybenclamide against CFTR.[24] Fig. 4 shows the effects of YM-198313 and glybenclamide on the FK-induced I^- efflux mediated by CFTR in these cells. Glybenclamide inhibited the I^- efflux in T84 cells with an IC_{50} value of 319±21 μM (n=3). At 100 μM, the maximum soluble concentration, YM-198313 inhibited I^- efflux by 40.8±5.1% (n=3).

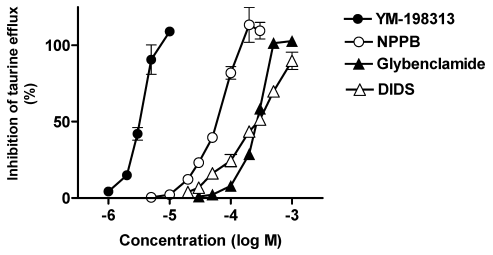

Fig. 2. Concentration–Response Curves of YM-198313 and Other Cl^- Channel Inhibitors, NPPB, Glybenclamide, and DIDS *versus* Taurine Efflux from HeLa Cells Stimulated by Hypotonic Solution

Data are presented as the percentage of the control efflux, with the mean and S.E.M. of 3 experiments, each performed in triplicate.

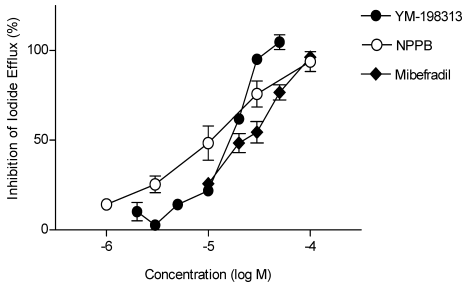

Fig. 3. Concentration–Response Curves of YM-198313 and CaCC Inhibitor, NPPB and Mibefradil, for I^- Efflux from HTC Cells Stimulated by ATP

Data are presented as the percentage of the control efflux and the mean±S.E.M. of 3 experiments.

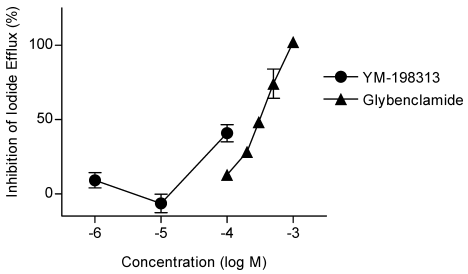

Fig. 4. Concentration–Response Data for YM-198313 (●) and the CFTR Inhibitor, Glybenclamide (▲), *versus* I^- Efflux from T84 Cells, Stimulated by FK

Data are presented as the percentage of the control efflux and the mean±S.E.M. of 3—4 experiments.

Effect of YM-198313 on VRAC Current in Guinea Pig Atrial Myocytes The effects of YM-198313 on VRAC current induced by hypotonic solutions in an isolated guinea pig atrial myocyte is shown in Fig. 5. Changes in the whole-cell current were monitored by applying a triangular voltage ramp. During the hypotonic condition, the whole cell current increased from 180±39 pA to 828±194 pA (n=5) at +60 mV indicating that the VRAC current was activated. This hypotonically-induced VRAC current was rapidly and almost completely suppressed to a level of 134±18 pA (n=5) at +60 mV by the subsequent addition of 10 μM of YM-198313.

Influence of YM-198313 on Signature Currents in Guinea Pig Ventricular Myocytes Figure 6 shows the influence of YM-198313 on signature currents in isolated

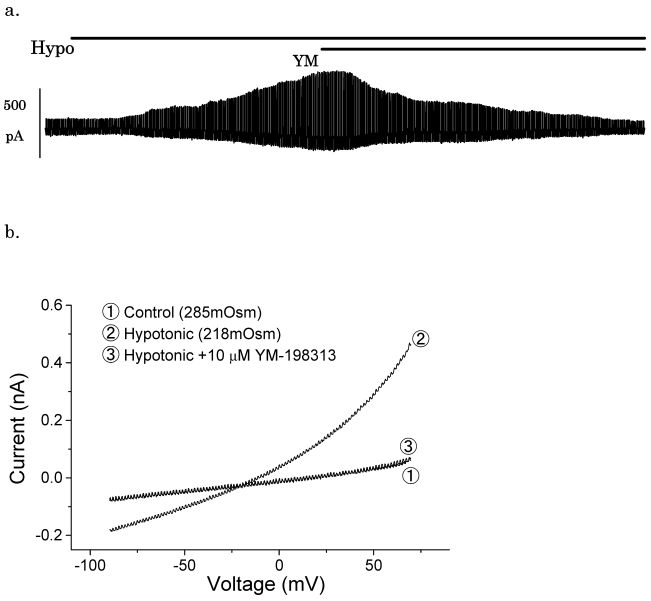

Fig. 5. Effects of YM-198313 on VRAC Current in Guinea Pig Atrial Myocytes

(a) Chart record of whole-cell current responses to voltage ramps applied every 3 s. The bars over the chart represent the periods of exposure to the hypotonic solution and hypotonic solution with 10 μM YM-198313. (b) *I–V* relationships of the VRAC current obtained during voltage ramps in isotonic solution (isotonic), after cell swelling in hypotonic solution (hypotonic), and after treatment of swollen cells with YM-198313 at 10 μM (hypotonic+YM-198313).

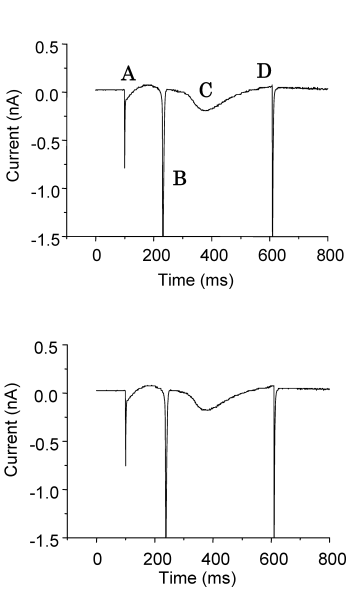

Fig. 6. YM-198313 Fails to Inhibit Signature Currents in Guinea Pig Ventricular Myocytes

Signature currents were recorded before (a) and 5 min after (b) exposure to 10 μM of YM-198313. The alphabets indicate each component in the following order of activation: I_{Kir} (A), I_{Na} (B), I_{Ca} (C), and I_{Kv} (D).

guinea pig ventricular myocytes. In the response to voltage ramp from −90 to +70 mV, a complex current profile was observed. Three of the four main current components, consistently recognized as I_{Kir}, I_{Na}, and I_{Ca-L}, produced inward currents which peaked at −87 mV, −54 mV, and −4.7 mV, respectively (Spencer *et al.*, 2000). The outward current, at very depolarized voltage-ramp potentials, was most likely carried by I_{Kv}, a number of which exist in cardiac muscle. As shown in Fig. 6, YM-198313 at 10 μM had almost no effects on the conductances underlying signature currents (n=3).

DISCUSSION

In cardiac myocytes, recent electrophysiological studies have provided evidence for three main classes of Cl^- channels: CaCC, which is activated by elevation of the intracellular Ca^{2+} concentration[25]; CFTR, which is activated by elevation of the intracellular cAMP concentration[26,27]; and VRAC, which is activated by cell swelling following a hypotonic stimulus.[28,29]

Some evidence suggests that VRAC contributes to cardiovascular diseases in which cell swelling occurs. During ischemia, the cytoplasm of cardiac myocytes becomes hypertonic due to the accumulation of the metabolic by-products of anaerobic metabolism, which creates an osmotic load.[30] Intracellular hypertonicity causes an increase in cell volume, follow by activation of VRAC.

In cardiac myocytes, at positive membrane potentials, VRAC is outwardly rectifying and contributes to the enhancement of the plateau phase and the rapid repolarization phase of the action potential duration[31,32] (APD). On the other hand, at negative membrane potentials, VRAC is inwardly rectifying and causes membrane depolarization when activated.[33] Therefore, it can be concluded that VRAC activation in cardiac myocytes causes shortening of APD and membrane depolarization. Such APD shortening and membrane depolarization caused by the activation of VRAC in swollen cells may possibly promote reentrant arrhythmia and accelerate the development of spontaneous activity in otherwise quiescent cells, respectively. Indeed, DIDS and DCPIB (4-(2-butyl-6,7-dichlor-2-cyclopentyl-indan-1-on-5-nyl), which are described as VRAC inhibitors, have been reported to attenuate the shortening of APD induced by swelling in guinea-pig myocytes.[31,32] Under hypoxic conditions, APD shortening has been observed in rabbit hearts. This shortening has been prevented by DIDS and SITS (4-acetamido-4′-isothyocyanatostilbene-2,2′-disulfonic acid).[34,35] DIDS was also reported to inhibit membrane depolarization.[31] DIDS and SITS were reported to inhibit the ischemia-induced delayed after depolarization in guinea-pig papillary muscles.[34] In addition, it has been demonstrated that DIDS inhibits ischemia/reperfusion arrhythmias.[36,37] These evaluation suggest that the activation of VRAC could accelerate and/or cause the development of the arrhythmia.

However, this hypothesis is based on evidence using standard Cl^- channel inhibitors. From pharmacological point of view, these Cl^- inhibitors have multiple effects on ion transport systems. For instance, DIDS has been reported to modulate K^+ channels.[25,38,39] Thereby, the contribution of VRAC to the cardiac pathophysiological condition is still unclear. Accordingly, hi-affinity and selective VRAC inhibitors are needed to be developed to clarify the role of VRAC in cardiac pathophysiology.

In this study, we found that YM-198313 is a potent and selective VRAC inhibitor. To investigate the inhibitory effect of YM-198313 on VRAC, we firstly measured [^{14}C]taurine efflux stimulated by hypotonic solutions in HeLa cells. Cells which undergo volume expansion in hypotonic or hypoxic conditions must control their volume through a process

known as regulatory volume decrease. In regulatory volume decrease, ion and organic osmolytes such as taurine leave the cells through VRAC, followed by water. YM-198313 and the Cl^- channel inhibitors blocked the [^{14}C]taurine efflux stimulated by the hypotonic solution. Based on the IC_{50} value, it is evident that YM-198313 inhibited [^{14}C]taurine efflux at least 18-fold more potently than the others. Furthermore, in the electrophysiological evaluation in cardiac myocytes, YM-198313 at 10 μM completely inhibited VRAC current. These data demonstrate that YM-198313 potently inhibits the VRAC current.

In order to assess whether or not YM-198313 has an inhibitory effect on two other Cl^- channels, CaCC and CFTR, we conducted I^- efflux assays. YM-198313 inhibited CaCC, with an inhibitory effect that was 5-fold weaker than that against VRAC. At 100 μM, YM-198313 inhibited only 41% of CFTR, which is at least 33-fold weaker than the effect on VRAC. Therefore, YM-198313 appears to be selective for VRAC among the Cl^- channels.

We performed patch-clamp experiments to observe ionic currents evoked by membrane potential ramps to evaluate the effect of YM-198313 on cardiac cation currents. The considerable utility of ascending voltage ramps, over the ranges of potentials encountered in the action potentials, has been shown to determine qualitatively which ionic currents are modified by experimental compounds.[18] These signature currents were therefore continuously recorded before and during superfusion with 10 μM YM-198313. As shown in Fig. 6, YM-198313 had almost no effect on signature currents, suggesting that YM-198313 has no significant inhibitory effect on the major cation channels in cardiac myocytes. However, some compounds such as Ca^{2+} channel blockers have been reported to be voltage-dependent and/or state-dependent,[40,41] thus further studies of YM-198313 on voltage- and state-dependency against each channel are needed.

Since YM-198313 seems not to have any effects on several cardiac currents that essentially generate and shape the action potential, we regard this compound as a useful tool to analyze the participation of VRAC in abnormal electrical activities during pathophysiological conditions in which cell swelling occurs. If the participation of VRAC in abnormal electrical activity in cardiac myocytes is clarified, VRAC inhibitors, such as YM-198313, could be use as anti-arrhythmic agents.

In conclusion, the newly developed compound, YM-198313, has a potent inhibitory effect on VRAC, but a lesser effect on major cardiac cation channels. Therefore, YM-198313 might be able to be used for clarification of the molecular identification of VRAC and its involvement in ischemic arrhythmia.

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