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A single point mutation in the TRPC3 lipid-recognition window generates supersensitivity to benzimidazole channel activators

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ABSTRACT

Mutation of a single residue within the recently identified lipid (diacylglycerol) recognition window of TRPC3 (G652A) was found to abolish channel activation via endogenous lipid mediators while retaining sensitivity to the non-lipid activator GSK1702934A (abb. GSK). The mechanism of this change in chemical sensing by TRPC3 was analysed by whole-cell and single channel electrophysiology as well as Ca^{2+} imaging. Currents initiated by GSK or the structural (benzimidazole) analog BI-2 were significantly larger in cells expressing the G652A mutant as compared to wild type (WT) channels. Whole cell patch-clamp experiments revealed that enhanced sensitivity to benzimidazoles was not due to augmented potency but reflected enhanced efficacy of benzimidazoles. Single channel analysis demonstrated that neither unitary conductance nor I-V characteristics were altered by the G652A mutation, precluding altered pore architecture as the basis of enhanced efficacy. These experiments uncovered a distinct gating pattern of BI-2-activated G652A mutant channels, featuring a unique, long-lived open state. Moreover, G652A mutant channels lacked PLC/diacylglycerol mediated cross-desensitization for GSK activation as typically observed for TRPC3. Lack of desensitization in G652A channels enabled large GSK/BI-2-induced Ca^{2+} signals in conditions that fully desensitized TRPC3 WT channels. We demonstrate that the lipid-recognition window of TRPC3 determines both sensitivity to lipid mediators and chemical gating by benzimidazoles. TRPC3 mutations within this lipid interaction site are suggested as a basis for chemogenetic targeting of TRPC3-signaling.

1. Introduction

Lipid sensing is common in TRP channels and represents part of a polymodal gating feature, which enables the channels to integrate multiple input stimuli. The lipid sensing ability appears crucial for the pathophysiological role of these important signaling molecules [1–4]. For TRPC channels, specifically the TRPC3/6/7 isoforms, interaction with the lipid mediator diacylglycerol (DAG) is considered as the primary gating stimulus [5], a mechanism that links these channels tightly to phospholipase C signalling pathways. Just recently, TRPC domains involved in DAG recognition have been resolved by high-resolution structural analysis of lipid-occupied channels and by refined functional approaches [5–7]. Importantly, these channels can be opened by synthetic small molecule agonists of which GSK1702934A (referred to as GSK further on for the sake of simplicity) was the first introduced and characterized [8,9]. The fact that a single point mutation (G652A) in TRPC3 is sufficient to drastically reduce PLC/lipid mediator regulation while GSK sensitivity of the channel is fully retained, indicates that GSK bypasses the lipid gating machinery of TRPC channels acting via a separate mechanism [11].

Here we set out to analyse the mechanism by which G652 interferes with pharmacological modulation of TRPC3. We found that the action of GSK-based benzimidazole activators was substantially enhanced in channels carrying the G652A mutation. Our whole-cell and single-channel analysis of TRPC3 function revealed that this mutation in the pore domain alters neither the potency of benzimidazole agonists nor the unitary conductance of the TRPC3 channel but profoundly stabilizes a benzimidazole-induced open state of the pore complex. Thereby, the G652A mutation generates Ca^{2+} entry via channels, which display unique chemosensitivity in that they are uncoupled from endogenous lipid regulation but displaying supersensitivity to the novel class of TRPC activators.

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2. Methods

2.1. Materials

If not mentioned otherwise, chemicals were purchased from Sigma Aldrich, TCI or ABCR. For synthesis of BI-2, a recently published, optimized microwave procedure was used [12].

2.2. Mutagenesis

Mutants were generated through site-directed mutagenesis using the QuickChange II Site Directed Mutagenesis Kit (Stratagene, USA). Human TRPC3 (Uniprot database ID: Q13507-3) cloned into pEYFP-C1 vector was used as template.

2.3. Cell-culture and transfection

HEK293 cells were cultured using DMEM medium (10% FBS). Transfection was performed with Fugene (Promega, USA) according to the manufacturer's protocol.

2.4. Electrophysiology

2.4.1. Single-channel in cell attached configuration

Characterization of unitary currents through TRPC3 channels was performed as published recently [11]. In brief, patch pipettes were pulled from borosilicate glass (Clark Electromedical Instruments, UK). Currents were recorded at RT using Axopatch 200 B amplifier (Molecular devices, USA). Single channel activity was recorded in cell-attached configuration. The bath solution contained (in mM) 145 potassium gluconate, 5.3 KCl, 3 $MgCl_2$ and 15 HEPES. The pipette solution contained 137 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$ and 10 HEPES. The pH of all solutions was adjusted to 7.4. Patch pipettes had resistances of 20–30 MΩ. Cells were stimulated with 100 µM carbachol (CCh) or 1 µM BI-2. Experiments were performed at room temperature. Single channel currents were digitized at a sampling rate of 50 kHz and filtered with the Axopatch-200B internal 4-pole low-pass Bessel filter (-3 dB cut-off at 2 kHz). The holding potential ranging from -100 to 100 mV and was controlled by the holding command function of the amplifier. Data acquisition, analysis and further filtering with a low-pass Gaussian filter (-3 dB cut-off at 1.5 kHz) was done using pClamp10 software (Axon Instruments, Foster City, CA). For open- and closed-time histograms in logarithmic scale, data were compiled in bins of variable width and fitted with multi-exponential functions as described in [13] using MATLAB 2016a software (MathWorks, Natick, MA).

2.4.2. Whole-cell configuration

Patch pipettes were pulled from thin-wall filament glass capillaries GC 150TF-7.5 (Havard Apparatus) to a resistance of 2–4 MΩ. An inverted microscope Axiovert 200 (Zeiss) was used to identify positively transfected cells by their green fluorescence when illuminated at 514 nm. Whole-cell experiments were performed at room temperature using an Axopatch 200B amplifier (Molecular Devices) and Digidata 1440 A/Digidata 1550B digitizer (Axon Instruments). Currents were filtered at 3 kHz by a 4-pole Bessel filter and digitized with 8 kHz. Application of linear voltage-ramp protocols ranging from −130 to + 80 mV (holding potential 0 mV) was controlled by Clampex 10.4/11 (Axon Instruments) software. Standard pipette solution contained (in mM) 120 cesium methanesulfonate, 20 CsCl, 15 HEPES, 5 $MgCl_2$, 3 EGTA adjusted to pH 7.3 with CsOH or NMDG. Patch clamp experiments were performed using extracellular solutions containing (ECS; in mM): 140 NaCl, 10 HEPES, 10 glucose, 2 $MgCl_2$, 2 $CaCl_2$ pH adjusted to 7.4 with NMDG.

2.5. Ca^{2+} imaging

Changes in $[Ca^{2+}]_i$ were monitored using the Fura-2 technique as previously described [13,14]. Briefly, cells on cover slips were loaded with 2 µM Fura-2 AM for 30 min in loading buffer (LB, containing in mM: 137 NaCl, 5.4 KCl, 10 HEPES, 10 glucose, 1 $MgCl_2$, 2 $CaCl_2$) at 37 °C in the dark. After the incubation period, cells were washed twice with LB, and left to equilibrate for at least 15 min in LB. The coverslip was then mounted in a perfusion chamber on an inverted microscope (Olympus IX71) and perfused with empty/ TRPC3 agonist containing LB buffer at room temperature. During the recordings using Live Acquisition 2.5 software (FEI, Germany), cells were excited alternately at 340 and 380 nm every second using an Oligochrome excitation system (FEI, Germany) and fluorescent images were captured at 510 nm with an ORCA-03 G digital CCD camera (Hamamatsu, Germany). The $[Ca^{2+}]_i$ imaging figures display the quantity: (Ratio F_{340}/F_{380}) = (F_{340} (cell) − F_{340} (background)) / (F_{380} (cell) − F_{380} (background)), where the "background" means fluorescence values were calculated from a region-of-interest (ROI) in each channel image (340 and 380 nm) drawn in an area without any cells.

2.6. Statistics

All two groups comparisons were carried out using student's *t*-test whenever normal distribution and equal variance of data set was observed otherwise the significance was evaluated using Mann-Whitney rank sum test. Comparison of multiple groups was performed using One Way ANOVA.

3. Results

By homology modelling, we have recently identified lateral fenestrations within the pore domain of the TRPC3 cation channel, which serve as crucial determinants of lipid recognition and gating. A single glycine residue G652 within this sensory region of the TRPC3 channel complex alters the channel's lipid recognition profile and renders the channel largely insensitive to physiological activation by receptor-PLC signaling [11]. Interestingly, mutant channels (G652A) retained sensitivity to modulation by the pharmacological tool GSK, a direct selective activator of lipid-sensitive TRPC3 and TRPC6 channels [10] (Fig. 1C). GSK is a benzimidazole derivative that reliably activates G652A mutant channels irresponsive to physiological regulation by muscarinic receptor agonists. Here we set out to characterize the pharmacological modulation of TRPC3 by benzimidazoles and the impact of impaired lipid-sensitivity due to G652A mutation.

3.1. $TRPC3_{G652A}$ channels display markedly enhanced responsiveness to benzimidazole activators

Whole-cell patch clamp recordings performed in HEK293 cells overexpressing WT TRPC3 channels revealed that peak current densities and current to voltage (I-V) relations induced by GSK (1 µM), its structural analog BI-2 (1 µM; Fig. 1D) or the muscarinic receptor agonist, carbachol (CCh, 100 µM), do not display any significant differences (Fig. 1). On the contrary, the sensitivity profile of the G652A mutant channels was profoundly altered. While CCh-induced barely any current responses as already reported [11], GSK and BI-2 administration resulted in activation of substantial current densities surmounting those mediated by WT channels (Fig. 1A-B). These results demonstrate that the G652A mutation, which has been described to blunt sensitivity of the channel to endogenous lipid mediators, strongly promote the currents activated by benzimidazoles. Consequently, we next investigated the mechanistic basis of this current facilitation.

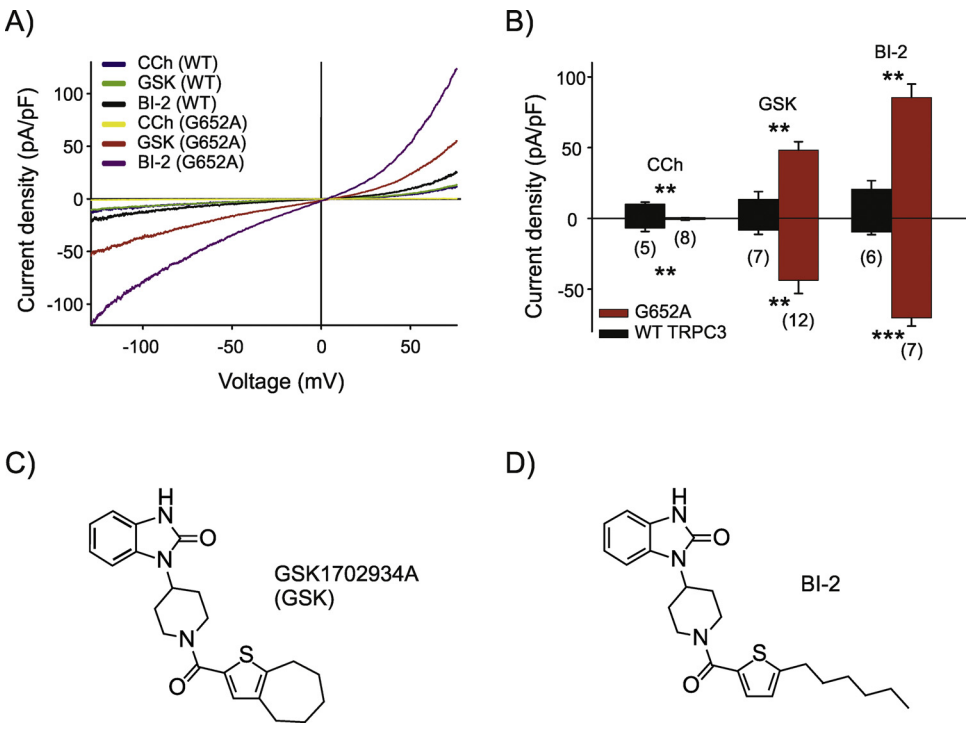

Fig. 1. Super-sensitivity of $TRPC3_{G652A}$ channels to benzimidazole activators in comparison with WT and their physiological activation.
A) Representative I-V curves obtained in whole-cell recordings of HEK293 cells over-expressing WT/G652 A TRPC3 channels activated with 100 μM CCh /1 μM GSK / 1 μM BI-2. B) Statistical evaluation of maximum current densities generated in the experimental setting of panel A) at -90/70 mV; mean ± SEM are shown; number of cells measured is indicated in parentheses; two tailed *t*-test (normally distributed values) or Mann–Whitney tests (non-normally distributed data sets) were applied, (**p < 0.01, ***p < 0.001). C) and D) molecular structures of the TRPC3 agonists, GSK and BI-2.

3.2. Facilitation of benzimidazole-induced currents in $TRPC3_{G652A}$ mutant channels is not caused by a gain in agonist potency

In view of the prominent impact of the G652A mutation on TRPC3 activation, we first tested the hypothesis that enhanced current densities generated by the mutant channels in response to a submaximum concentration of the benzimidazoles might be due to a gain in potency and affinity of activator. Consequently, we compared the concentration response relations for BI-2 activation of WT and mutant channels in whole-cell patch clamp recordings. As shown in Fig. 2A the EC_{50} values for generation of inward currents by BI-2 were indistinguishable between WT and G652A TRPC3 channels (203 and 282 nM respectively). These data demonstrate that the potency of BI-2, and therefore the binding affinity to its receptor is most likely unaffected by the G652A mutation. Enhanced current densities were observed at all BI-2 concentrations, suggesting a profound increase in efficacy of benzimidazole action.

3.3. Facilitation of benzimidazole-induced currents in $TRPC3_{G652A}$ mutant channels is not due to enhanced ion permeation through the open pore

In TRP channels a considerable plasticity of the pore domain, including gating processes within the selectivity filter and an allosteric coupling between the occluding S6 bundle crossing gate (BC) and the selectivity filter (SF), has been demonstrated [5,6,15,16]. To investigate the impact of G652A mutation, which localizes closely behind the SF, we analysed unitary conductance and gating pattern of the channels during activation by BI-2 compared to CCh in single-channel

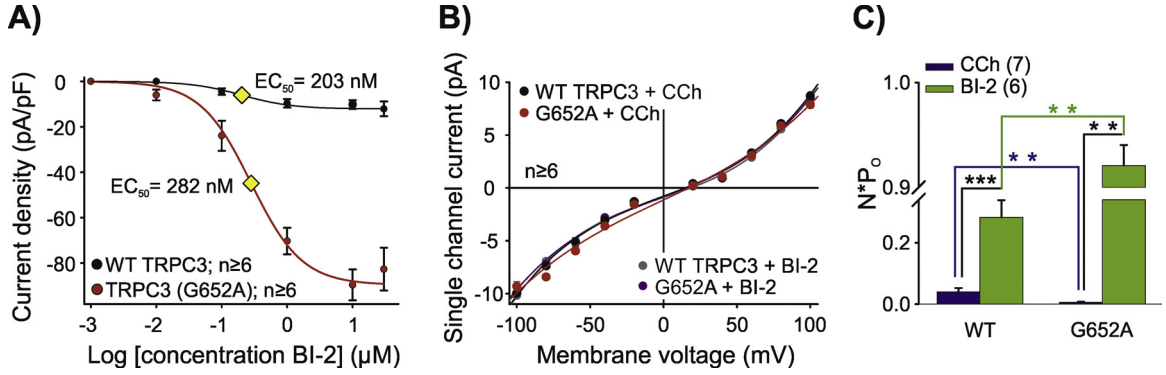

Fig. 2. Facilitation of benzimidazole-induced currents in $TRPC3_{G652A}$ mutant channels is neither caused by a gain in agonist potency nor by enhanced ion permeation through the open pore.
A) Concentration-response curve of BI-2 obtained from whole cell patch clamp recordings of HEK293 cells overexpressing either WT/G652A TRPC3 channels. EC_{50} reflecting BI-2's affinity to the channel is highlighted with yellow diamonds: 203 (WT) vs. 282 (G652A) nM respectively. B) Unitary current to voltage (I-V) relationships of WT TRPC3 and G652A channels activated by 100 μM CCh or 1 μM BI-2. Data points represent the mean ± SEM*, number n of cells/patches measured at each voltage as indicated, *note that symbol size exceeds error bars at most voltages. Curves represent the least-squares fit to the third-order polynomial relationship. Data were not corrected for liquid junction potentials. C) Mean values ± SEM of the open probability of TRPC3 WT vs. G652A mutant channels under CCh/BI-2 stimulated conditions. Values were derived from the single channel traces at a temporary steady state where only one channel was active. Number of cells is indicated in parenthesis. Two tailed t-test*t*-test or Mann-Whitney test were applied to assess the statistical significances between data sets, **p < 0.01, ***p < 0.001).

recordings.

Despite the use of high-resistance (20–30 MΩ) patch pipettes with essentially small openings (< 1μM), we observed mostly multiple channels per patch. For gating analysis, we selected patches with a minimum number of overlapping channel events during the initial transient peak of activation. The number of transiently available channels (N) in these patches was typically higher for G652 A mutant channels (N = 5–6) as compared to WT channels (N = 2). For both, WT and mutant channels, the availability per patch ceased rapidly (within 1–2 min) during activation resulting in most experiments in a prolonged phase displaying exclusively a single conductance level while modified gating was still evident. For gating analysis only recordings, which exhibited stable gating behaviour for about one minute and lacked multiple conductance levels, were used. Due to the limited time available for analysis, we were restricted to characterize gating only at relatively high agonist concentrations, which produce sufficient open probability and gating transitions for analysis. Fig. 2B displays the unitary current to voltage relations derived from cell-attached recordings in HEK293 cells overexpressing WT or G652 A TRPC3 channels activated by BI-2 or CCh. Unitary currents at any given potential were indistinguishable, demonstrating identical pore architecture of both WT and G652 A TRPC3 channels that were activated by either BI-2 or CCh. This conclusion was in line with results from whole-cell experiments showing that G652 A mutant channels retained sensitivity to the classical pore blocker ruthenium red as shown in Suppl. Fig. 4. Our findings demonstrate that the increased current densities obtained in whole-cell patch clamp recordings with BI-2 in the G652 A mutant are not a consequence of modified ion permeation and enhanced unitary conductance.

3.4. Enhanced benzimidazole-induced currents in $TRPC3_{G652A}$ channels are based on prolongation of open channel life times

Because of the transient nature of both BI-2 and CCh induced responses, we limited our analysis to the stable phase of gating behaviour, typically obtained after an initial, transient increase in channel activity initiated by agonist application. Our evaluation of this time period, in which channel activity was fairly stable over about 1 min, demonstrated a clear increase in open probability due to BI-2 administration of both WT and G652 A channels. As depicted in Fig. 2C the increase in open probability at this time period was substantially larger in G652 A channels as compared to WT (91% vs 24%).

Inspection of single channel traces at different membrane voltages revealed striking differences in the gating pattern of WT and G652 A channels when stimulated with BI-2 as illustrated in Fig. 3A. The mutant channel showed a high preference for a long-lived open state, which was not observed in WT channels. This is clearly evident from the dwell time histograms displaying a considerable shift towards long openings in BI-2 activated G652 A mutant channels (Fig. 3B).

Fig. 4 shows the statistical comparison of open state features observed with G652A mutant channels at basal conditions or activated by either CCh or BI-2 at a membrane potential of + 80 mV. When activated with BI-2, G652A mutant channels displayed a large proportion (about 58% in average) of exceptionally long-lasting (> 12 ms in average) openings. The population of short openings was in turn significantly reduced (Fig. 3B, Fig. 4). The observed long-lived open state of BI-2-activated mutant channels was absent in both, CCh-stimulated mutant and WT channels (Fig. 4) and hence, strictly associated with the G652A mutation.

Information about the closed time constants derived from these experiments is shown in the Suppl. Fig. 1. Our results demonstrate that BI-2 is able to activate both, WT and G652A TRPC3 channels in part by favouring short channel closures. The closed time constants of BI-2-activated channels were clearly reduced, demonstrating a general destabilization of closed states by benzimidazoles. The G652A mutation therefore appears to enhance efficacy of benzimidazoles by enabling a specific long-lived open state of the pore complex.

3.5. The G652A mutation eliminates cross-desensitization between physiological (lipid-mediated) and benzimidazole-induced TRPC3 activation

Based on the concept of benzimidazoles activating TRPC3 via a mechanism bypassing the PLC pathway and our findings of G652A facilitating this mechanism, we hypothesized additivity of PLC-mediated and benzimidazole-induced channel activation. We set out to test the assumption that physiological (PLC/lipid-mediated) and benzimidazole-induced activation may be independent and additive in nature. We first performed experiments in which the activating stimuli CCh and BI-2 were administrated consecutively to activate WT channels. As activation of TRPC3 by either physiological of pharmacological stimuli is typically transient, due to a yet unclear mechanism of desensitization or inactivation, we administered GSK at two different time points during the response of channels to CCh (100 μM). Fig. 5 shows that GSK fails to initiate currents subsequently to an activating stimulus conferred via the PLC pathway. This result was independent of the time point of administration and was observed also at lower CCh concentrations. Notably, even close to threshold stimulation of the channels with CCh resulted in slight inhibition of GSK responses as shown in Suppl. Fig. 5 Similarly, in experiments in which agonists were administrated in reversed order, no additive current responses were observed (Suppl. Fig. 2). These results suggest that PLC-mediated and benzimidazole-induced activation share some part of the activation machinery, and that PLC/lipid-mediated activation desensitizes the WT channel for the benzimidazole activation. Next, we tested for an interaction between the two activation pathways in G652A mutant channels. Due to the general impairment in sensitivity of the mutant channel to CCh we used in addition the lipid mediator, 1-oleoyl-2-acetyl-sn-glycerol (OAG), which retains the ability to generate currents in G652A mutant channels [11]. Interestingly, GSK was highly efficient in activation of G652A mutant channels in cells exposed to a desensitizing stimulus conferred by either CCh or the synthetic lipid-mediator OAG. Hence, the G652A mutation not only facilitated the action of benzimidazoles but also eliminated the channel´s desensitization towards benzimidazoles as a consequence of the lipid-mediated activation.

Preservation of TRPC3 channels from desensitization via the PLC pathway by the G652A mutation was further tested at the level of cellular Ca^{2+} signaling. Consistent with our electrophysiological data, cells expressing WT TRPC3 channels failed to respond with cellular Ca^{2+} elevation when GSK (1μM, Fig. 6A) or BI-2 (1 μM, Fig. 6B) were administered subsequently to CCh (100 μM). Surprisingly, we even observed a moderate reduction in cellular Ca^{2+} levels in the desensitized WT channels. By contrast, when $TRPC3_{G652A}$ mutant channels were expressed, cells responded with large Ca^{2+} elevations, demonstrating that benzimidazole-induced TRPC3 activation and Ca^{2+} signaling was indeed preserved. A statistical comparison of the peak amplitudes of benzimidazole-induced Ca^{2+} influx via WT/G652A TRPC3 channels in the absence and presence of CCh pre-stimulation is illustrated in Suppl. Fig. 3B.

In summary we have identified a single point mutation in close vicinity of TRPC3 channel pore that is not only crucial for endogenous lipid gating but also a determinant of pharmacological modulation via benzimidazole agonists. We suggest that modulation of TRPC3 channels by diacylglycerols and benzimidazoles involves different primary receptor sites but shares at least part of a common gating machinery, which harbors G652 and the adjacent lipid recognition domain as a pivotal element.

4. Discussion and conclusions

Here we report on the pharmacological role of G652, which is localized behind the selectivity filter in a lateral window of the TRPC3 channel complex. This residue was recently identified as an important

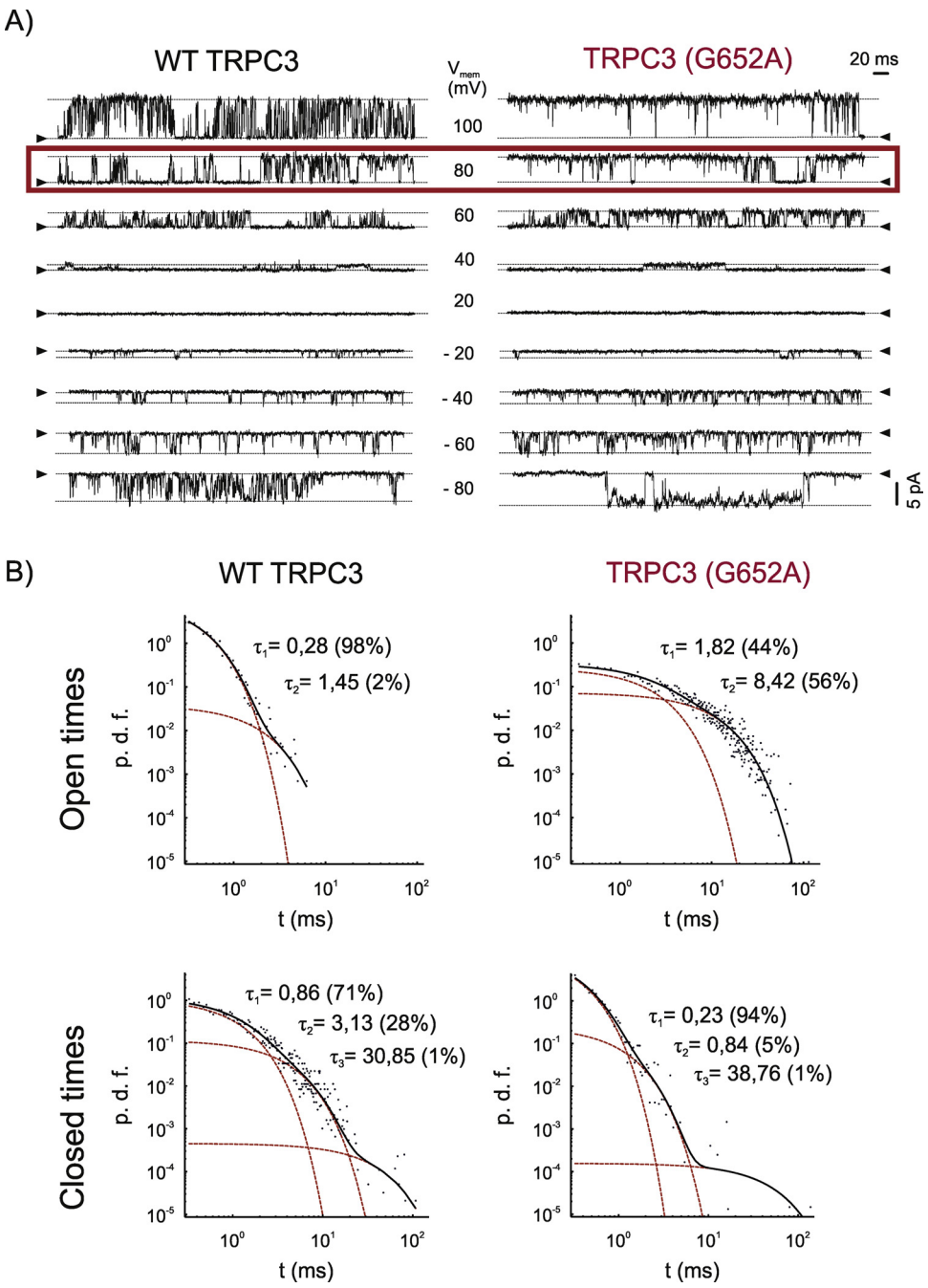

Fig. 3. Representative single channel traces and probability density function histograms of BI-2 activated channels.
A) Representative unitary current traces at various voltages (from -80 to 100 mV) recorded in HEK293 cells expressing either WT or G652A TRPC3 channels activated with 1 μM BI-2. Arrow head indicates the closed state. All traces displayed are filtered as described in the Method section. Red box indicates the membrane potential of + 80 mV at which further statistical analysis of open/closed dwell time constants and open probability was carried out. B) Representative probability density histograms (probability density function - p.d.f.) of open and closed time constants of BI-2 stimulated (1 μM) WT and G652A TRPC3 channels expressed in HEK293 cells. Data are derived from single-cell-attached patches at the membrane potential of + 80 mV. The sum of multiple exponential equations (black) and the individual components (red line) with time constants (τ_1–τ_2) are indicated. The data set size of the open/closed events is as follows: For WT channels 2044 / 1155 open events and 2405 / 1910 closures (detected/fulfilling the 0,3 ms delimiter criterion for further exponential function fitting) were collected. In G652A mutant channels 2657 / 2496 open events and 2665 / 1188 closures were collected.

determinant of lipid recognition and sensing [11]. The mutation G652A was found to largely eliminate physiological activation via Gq-coupled receptors, while channel opening by the benzimidazole compound GSK was retained. Characterization of two different benzimidazoles, GSK and its derivative BI-2 revealed a solid potentiation of benzimidazole action, and we consequently set out to delineate the mechanism by which the mutation within the lipid recognition domain affected the pharmacological modulation.

4.1. G652 is a critical determinant of both lipid and benzimidazole modulation

We demonstrated in whole-cell patch clamp experiments that benzimidazole-induced currents through G652A mutant channels are significantly larger in comparison to WT TRPC3 channels. Current facilitation was neither the result of an increased benzimidazole affinity of the mutant channels nor an increased unitary conductance. As the mutation did not affect benzimidazole potencies, we concluded that G652 is unlikely a part of the primary binding/recognition site for these activators. On the other hand, very recent high resolution structural information on TRPC3 supports the idea that G652 is an important flexible element within the inner lipid-binding pocket [6]. We have previously shown, that increasing the bulkiness of the side chain at position 652 determines the TRPC3 lipid recognition profile. Moreover, we observed that at a certain degree of rigidity in this position (G652L) introduces a substantial loss of function, indicating that flexibility and rearrangements within this region are indispensable for channel gating. Our current results support this view as they show that G652A mutation enhances currents initiated by benzimidazoles without a change in apparent affinity but by stabilizing a particularly long-lived open state of the channel. Hence, we hypothesize that G652 is an essential element of the TRPC3 channel gating machinery and determines both the

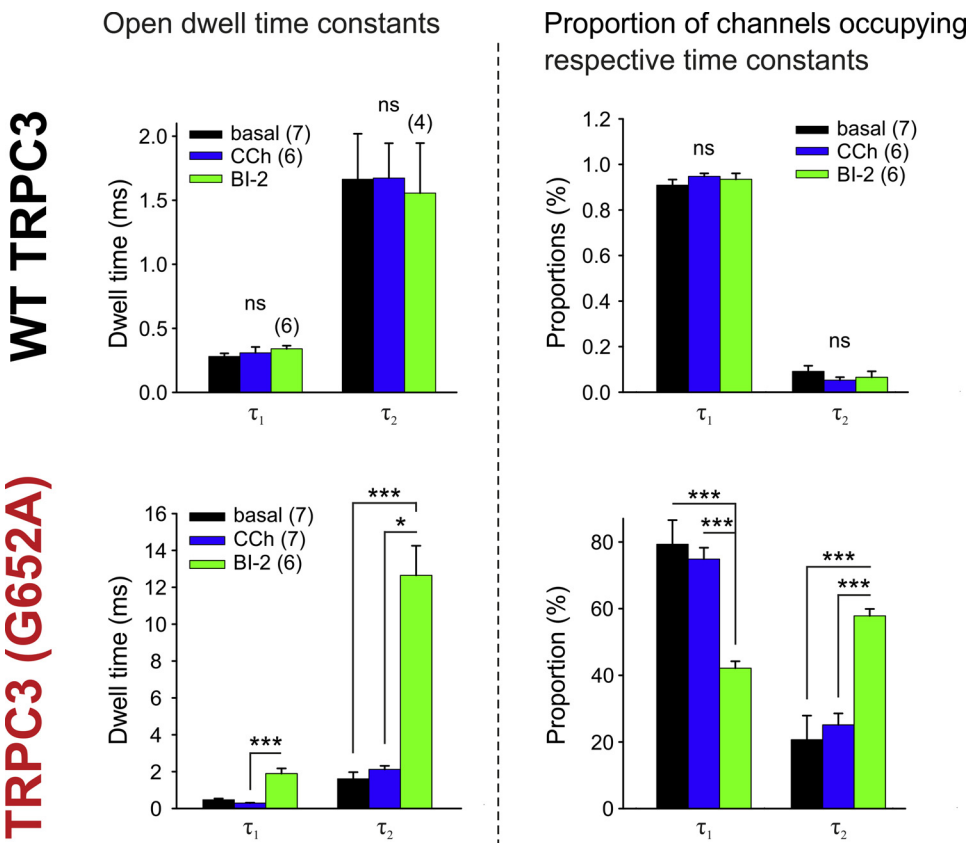

Fig. 4. BI-2 activated TRPC3$_{G652A}$ channels are characteristic by prolonged open channel life times.

Comparison of open dwell time constants and their occupancies derived from single channel recordings in cell-attached patches of WT (upper panel)/ G652A TRPC3 channels activated with 1 μM BI-2, 100 μM CCh or at basal conditions at V_{mem} = + 80 mV. Mean ± SEM, number of cells measured is indicated in parentheses. One Way ANOVA was employed to compare the triplets of data and two tailed *t*-test or Mann-Whitney test were used to evaluate the differences between two experimental groups (*p < 0.05, ***p < 0.001, ns = not significant).

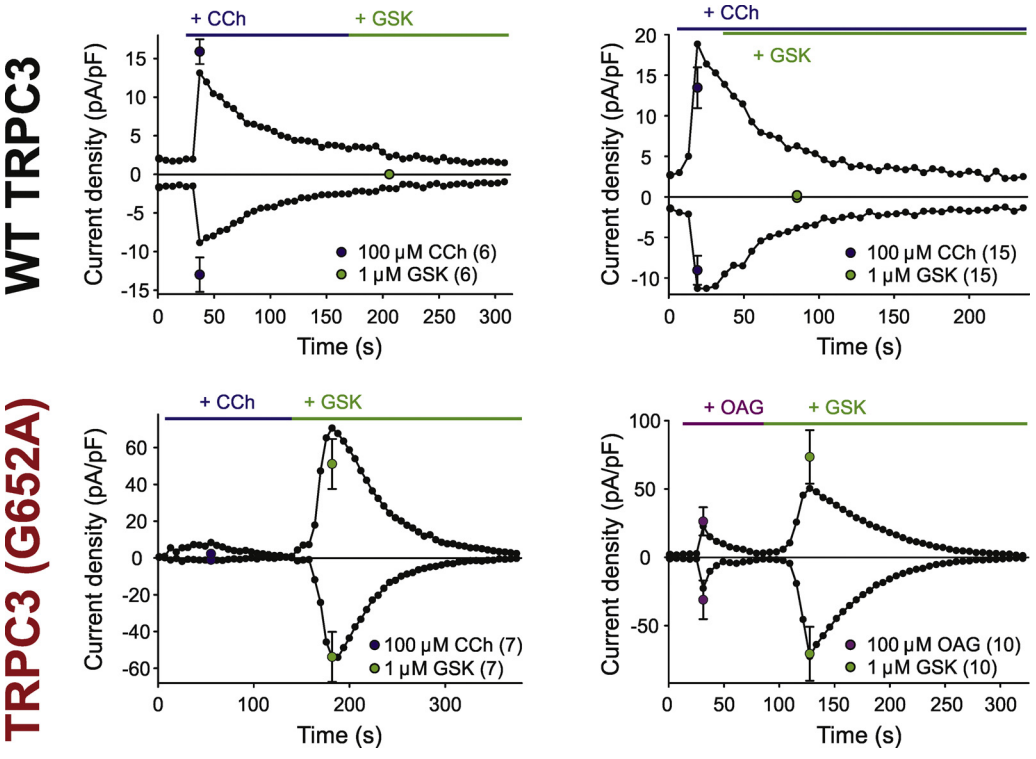

Fig. 5. The G652 A mutation blunts cross-desensitization between physiological and benzimidazole-induced TRPC3 activation as verified by electrophysiology experiments.

Representative time courses of current activation by 100 μM CCh followed by application of 1 μM GSK in WT (upper panel) and G652A (lower panel) TRPC3 channels derived from whole cell patch clamp recordings. In case of G652A mutant the current trace is also shown for modulation with 100 μM OAG followed by application of 1 μM GSK to compensate for lack of CCh response. Mean peak current densities ± SEM of the maximum responses due to activator addition are displayed as dots with error bars; number of cells measured is indicated in parentheses.

interaction and binding of lipids to the pore domain [6] as well as gating behaviour of the channel. Thereby, the recently identified lipid-gating fenestrations within the pore domain may serve as a pivotal hub connecting the modulation of TRPC3 by diacylglycerols and small molecule modulators. This concept is in accordance with the recently identified BTDM inhibitor binding pocket within TRPC6 channel structure [7], stretching diagonally from the middle of S6 over the S4-S5 linker of an adjacent subunit to its intracellular end of S1-4 voltage sensor. As the BTDM binding cavity can be found almost exactly in the middle of a hypothetical diagonal between the two proposed lipid

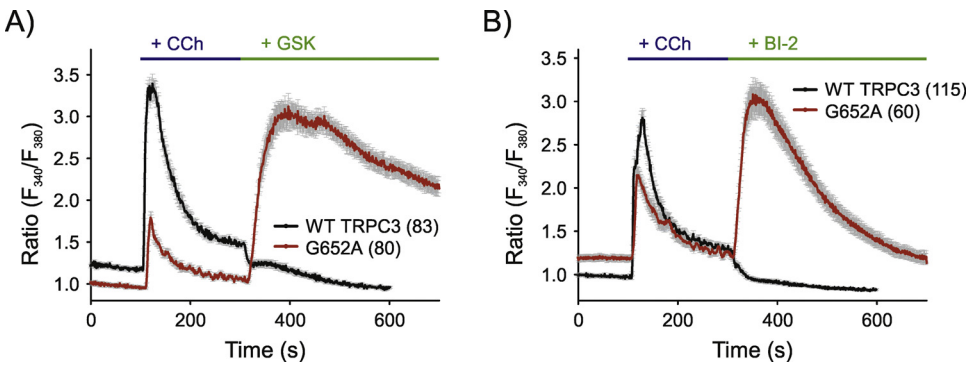

Fig. 6. Ca^{2+} imaging confirms $TRPC3_{G652A}$ channels do not cross-desensitize toward benzimidazole activation after physiological stimuli.
Time courses of single cell cytosolic Ca^{2+} $[Ca^{2+}]_i$ imaging experiments using FURA-2 dye in HEK293 cells overexpressing either WT or G652A TRPC3 channels stimulated with 100 μM CCh first and with A) 1 μM GSK/ B) 1 μM BI-2 s. Mean ± SEM are shown, number of cells measured is indicated in parentheses.

binding pockets of TRPC3 channel [6], its positioning makes it ideal to interfere with a potential crosstalk of the lipid gating machinery. Such a cross-talk and interdependence between benzimidazole and lipid actions on TRPC3 was substantiated by a test for additivity and cross-desensitization. Of note, benzimidazoles were unable to further increase currents maximally stimulated by CCh even at very early time points when inactivation/desensitization has just started. This indicates that the two activation pathways converge and may involve a common gating rearrangement within the channel. Of note, even at low, close to threshold levels of PLC-activating stimuli (CCh), moderate suppression of benzimidazol effects was observed (Suppl. Fig. 5). Although, we cannot entirely exclude a general, permissive role of basal DAG levels for channel activation by benzimidazoles, our results argue against promotion of channel-lipid interaction by benzimidazoles as a mechanism of activation.

Once WT channels were desensitized as a consequence of the PLC/lipid-mediated processes, benzimidazoles were unable to open those desensitized channels.

The inactivation/desensitization of TRPC3 is still incompletely understood but it has been proposed to involve current- and Ca^{2+}-dependent inactivation presumably via Ca^{2+}/calmodulin interactions and/or protein kinase C phosphorylation [17–21]. Here we add new information to this phenomenon in that we demonstrate rescue from desensitization by a mutation that affect primarily lipid interaction with the channel. Importantly, this rescue was observed even when desensitization was initiated by the synthetic lipid (OAG), which is expected to trigger substantial Ca^{2+}- and PKC-mediated inactivation processes [22,23]. It appears tempting to speculate that inactivation/desensitization of TRPC3 involves lipid interaction at the pore domain of the channel. Elucidation of the molecular details of this mechanism requires further investigation.

4.2. TRPC3 G652 mutations as a potential basis of chemogenetic targeting strategies

Collectively, our results demonstrate the potential value of mutations within the pore domain to generate TRPC channels that lack physiological, PLC/lipid-mediated activation but remain effectively controlled by small molecule activators. It appears reasonable to expect that for certain benzimidazoles, as shown for BI-2, efficacy in the mutant channels is high enough to enable selective activation of the recombinant mutant channels without significant interference with the endogenous TRPC channels. This scenario may allow for exceptionally selective manipulations to test and characterize TRPC functions. Such chemogenetic strategies combined with chemical desensitization of endogenous channels may turn out suitable for highly specific targeting of TRPC3 signaling.

Here we demonstrate that mutation of the TRPC3 lipid recognition domain enables pharmacological control over the channel complexes independent of PLC signaling and of PLC-mediated desensitization processes. Thereby our findings may pave the way towards the development of interventions to precisely manipulate TRPC3 signaling in complex native tissues such as the brain, heart or tumors.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ceca.2019.02.007.

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