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Acute exposure of methylglyoxal leads to activation of KATP channels expressed in HEK293 cells

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Abstract

Diabetes is characterized by hyperglycemia and excessive production of metabolites. One of these metabolites is the highly reactive carbonyl, methylglyoxal (MGO). MGO can readily react with biomolecules leading to cellular dysfunction. Here we showed that acute MGO application led to a dose-dependent activation of K_{ATP} channels, a major vascular tone regulator and a critical pharmacological target for treating diabetes. Both Kir6.1 and Kir6.2 containing K_{ATP} channels were targeted by MGO in a SUR subunit independent manner. Single channel analysis showed that MGO mediated K_{ATP} channel activation via enhancement of channel open probability, leaving the channel conductance unaltered. This modulation appeared to be due to the direct interaction of MGO with the K_{ATP} channel, without the need for additional cell signaling pathways. Moreover, MGO mediated K_{ATP} channel activity was completely reversed with bath solution washout. Taken together, these data suggest that acute exposure to MGO activates K_{ATP} channels through direct, non-covalent and reversible interactions with the Kir6.x subunit, suggest a potential target for pharmacological intervention towards vascular complications of diabetes.

Keywords

potassium channel; ATP-sensitive potassium channel; diabetes; reactive carbonyl species; RCS; diabetic vascular complications

Introduction

Diabetes affects ~25 million people in the United States [1]. Hyperglycemia, a major hallmark of diabetes, serves as a trigger for the development and progression of many diabetes-associated complications on a systemic level. One of the key consequences of hyperglycemia is the alternation of the metabolic pathways leading to the production and accumulation of metabolic byproducts. Among these metabolites is the highly reactive carbonyl methylglyoxal (MGO), a key player mediating oxidative stress and carbonyl stress [2]. MGO is mainly produced by the transformation of triosephosphate intermediates during

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glycolysis and in limited quantities via protein catabolism and fatty acid oxidation mechanisms [2]. Patients with type-I diabetes have been reported to have a 5-6 fold increase in plasma levels of MGO while in type-II patients a 2-3 fold increase was observed [3]. Excess MGO when present, as a result of either impaired carbonyl clearance mechanisms, increased availability of precursor molecules or both, are seen in many pathological conditions including diabetes. Increased levels of MGO can lead to structural/functional changes in the cell, by attacking the molecules like transporters, ion channels, transcription factors, signal transduction components at both mRNA and protein levels [4, 5] [6, 7].

Diabetic vascular complication is a severe complication associated with many diabetic patients, and hyperglycemia is suggested to be a major contributor. Despite numerous studies hinting towards the importance of MGO in the regulation of the vascular functions in carbonyl stress [8, 9], the response pattern of key molecules in vasculature to the presence of reactive carbonyl species is yet to be fully understood. K_{ATP} channel is a major regulator of vascular tone and a critical pharmacological target for treating diabetes [10-13]. It couples the cellular metabolism to membrane excitability [14]. The activation of K_{ATP} channel in smooth muscle cells produces hyperpolarization of smooth muscle cells, which lowers the activity of voltage-dependent calcium channels (VDCC) leading to vasorelaxation. *Kcnj8* (encodes Kir6.1, vascular K_{ATP} channel pore) null mice show vasospasm in coronary arteries and sudden death [15, 16]. In diabetic patients, the response of K_{ATP} channel to stimuli is impaired, resulting in defective vasodilation of the vascular rings [17, 18]. As carbonyl stress has been suggested to play a critical role in the development of diabetes associated vascular complications [19], we tested if MGO, a major reactive carbonyl species, may regulate K_{ATP} channel. Interestingly, we found that prolonged exposure of vascular smooth muscle cells to MGO leads to the mRNA instability of K_{ATP} channel, likely contributing to the dysfunction of the vasculature [5]. However, it is not known yet if acute MGO exposure may also exert an effect on K_{ATP} channel function. Therefore, in this current study, we investigated the effect of acute exposure of MGO on the K_{ATP} channel activity. We found that acute MGO treatment causes the activation of vascular K_{ATP} channel in a receptor-independent mechanism, mediated through non-covalent interactions with the pore-forming Kir subunit of the K_{ATP} channel.

Materials and Methods

Chemicals and reagents

All reagents and chemicals used in this study were purchased from Sigma-Aldrich unless stated otherwise. Reagents were freshly made and prepared in high-concentration stocks in double-distilled water or dimethyl sulfoxide (DMSO). The final concentration of DMSO in the solution for experiments was less than 0.1%, which did not have any detectable effect on the channel activity.

Cell culture and heterologous expression of K_{ATP} channels

The HEK293 cells were maintained in Dulbecco's modified Eagle medium (DMEM/F12 with 10% fetal bovine serum and Penicillin/Streptomycin) at 37°C in a humidified 5% CO_2 atmosphere.

K_{ATP} channels were expressed in HEK293 cells as previously described [20-22]. Rat Kir6.1 (GenBank No. D42145), mouse Kir6.2 (GenBenk No. D50581) and SUR2B (GenBank No. D86038, mRNA isoform NM_011511) coding sequences were cloned into pcDNA3.1 (a eukaryotic expression vector). HEK293 cells cultured in 35 mm petri dish were transfected with 1 μg Kir6.1 (or Kir6.2) and 3 μg SUR2B using Lipofectamine^{2000} (Invitrogen Inc., Carlsbad, CA). Kir6.2Δ36 (3 μg) was transfected to HEK293 cells without SUR2B. Green fluorescent protein (GFP) cDNA (0.4 μg, pEGFP-N2, Clontech, Palo Alto, CA) was included in the cDNA transfection mixture to facilitate the identification of positively transfected cells. One day after transfection, cells were disassociated with 0.25% trypsin, split and transferred to cover slips. Electrophysiology experiments were performed on the cells grown on cover slips for two continuous days.

Electrophysiology

Patch clamp experiments were performed as described previously at room temperature [23] [24] [25]. In brief, 1 2 mm borosilicate glass capillaries were fire-polished to make patch pipettes of 2-5 MΩ resistance. Whole-cell currents were recorded in single-cell voltage clamp with a holding potential 0 mV and stepped to -80 mV every 3 s. The bath solution contained (in mM): KCl 10, potassium gluconate 135, EGTA 5, glucose 5, and HEPES 10 (pH=7.4). The pipette was filled with a solution containing: KCl 10, potassium gluconate 133, EGTA 5, glucose 5, K_2ATP 1, NaADP 0.5, $MgCl_2$ 1, and HEPES 10 (pH=7.4). All solutions containing ATP and/or ADP were freshly made and used within 4 h to avoid nucleotide degradation. The recordings were obtained from the Axopatch 200B amplifier (Axon Instruments Inc., Foster City, CA), low-pass filtered (2 kHz, Bessel 4-pole filter, -3 dB), and digitized (10 kHz, 16-bit resolution) with Clampex 9 (Axon Instruments Inc.). Macroscopic currents were recorded from giant inside-out patches and single-channel currents were recorded from normal inside-out patches with a constant single voltage of − 80 or − 60 mV. Symmetric high K^+ (145 mM in total) was used in both bath and pipette solution to make the reverse potential of K^+ close to 0 mV. K_2ATP 1 mM and NaADP 0.5 mM was also included in the bath solution for the maintaining the channel activity. For inside-out patch experiment, higher sampling rate (20 kHz) was used to digitize the recorded currents. Data was analyzed using Clampfit 9 (Axon Instruments Inc.).

Data analysis

Data are presented as mean±S.E. Differences in the means were evaluated with Student's *t*-test and was accepted to be statistical significant when $P < 0.05$.

Results

1 Acute MGO treatment leads to activation of Kir6.1/SUR2B isoform of K_{ATP} channel

Kir6.1/SUR2B is the major isoform of vascular K_{ATP} channel, though the presence of Kir6.2 subunit has also been detected. To test if acute MGO treatment can affect the activity of K_{ATP} channels, we expressed the Kir6.1/SUR2B channel in HEK293 cells and used whole cell voltage-clamp configuration to test the channel activity. Equal concentrations of K^+ (145 mM) were applied to both sides of the patch membranes. Membrane potential was held at 0 mV and a -80 mV command potential was given to the cells every 3s. The activity

of the Kir6.1/SUR2B channel was low at the basal level (Fig. 1A); however it could be fully activated by K_{ATP} channel specific opener Pinacidil (Pin, 10 μM) and inhibited by channel specific blocker Glibenclamide (Glib, 10 μM) (Fig. 1A). The Kir6.1/SUR2B channel was steadily activated in response to increasing dosage of MGO: a 300 μM MGO treatment increased the channel activity by 22.0±10.6% (n=4) while exposure to 3 mM MGO caused a 57.4±14.3% (n=4) increase in the channel activity (Fig. 1A). The relationship between MGO dosage and K_{ATP} channel activities was described using the Hill equation with EC_{50} ~1.7 mM (Fig. 1D). In the presence of Glib, MGO concentration as high as 10 mM was not able to induce the channel activity, suggesting that Glib and MGO target the same molecule (K_{ATP} channel) (Fig. 1B, C). Interestingly, MGO mediated channel activation was reversible: the application of control (bath solution) caused a significant decrease (from 57.4±14.3% to 29.3±10.7%, $P<0.05$) in MGO induced channel activity (Fig. 1B), thereby suggesting an involvement of non-covalent interactions between MGO and K_{ATP} channels.

2 MGO activates Kir6.1 and Kir6.2 subunit containing vascular K_{ATP} channel, independent of the SUR subunit

To determine whether the effect of MGO on K_{ATP} channel activity was isoform-specific, additionally we tested the effect of MGO on Kir6.2/SUR2B isoform of the K_{ATP} channel, another isoform found in vascular smooth muscle cells. In whole cell patch configuration, we found that the application of MGO (3 mM) caused an increase in the Kir6.2/SUR2B activity (65.4±7.9%, n=5, Fig. 2A) similar to what we saw from that of Kir6.1/SUR2B recording (57.4±14.3%, n=4). These data thus indicate that both Kir6.1 and Kir6.2 containing channels can be targeted by MGO.

Naturally occurring K_{ATP} channels need both Kir and SUR subunits to be functional. However, previous studies have suggested that the truncation of several dozen amino acids in the C-terminus of Kir6.2 subunit (Kir6.2Δ36) results in detectable currents, thereby eliminating the need for the SUR subunit for the K_{ATP} channel to be functional. (This SUR-independent expression of Kir is isoform specific, as truncated Kir6.1 isoform cannot express itself without SUR.) To further investigate if the Kir subunit alone was sufficient for MGO to exert its activating effect, we took advantage of Kir6.2Δ36 channel. Because of the absence of the SUR subunit, the Kir6.2Δ36 channel was not sensitive to regulation by either Pin or Glib, but could still be blocked effectively by Ba^{++}. In this condition, we quantified the effect of MGO against the Ba^{++} mediated channel blocking and expressed them as fold changes. In the absence of ATP and ADP in the pipette solution, the Kir6.2Δ36 channels readily opened and the application of MGO led to a 3.87±1.81 fold increase in the channel currents (n=4, $P<0.01$, Fig. 2B, D). In the presence of 1.0 mM ATP and 0.5 mM ADP, the basal currents of Kir6.2Δ36 remained small and the application of MGO (3 mM) was able to augment the channel activity by 3.35±0.6 fold (n=5, $P<0.01$, Fig. 2C, D). Additionally, in both of the above experimental conditions, Ba^{++} treatment was able to almost completely block MGO induced channel activity (Fig. 2B, C). These data thus suggest that MGO is targeting Kir rather than SUR subunit.

3 Biophysical properties of MGO mediated channel activation

MGO mediated activation of the K_{ATP} channel might be due to the direct modulation of the channel protein or involving the activation of other signaling pathways that regulate K_{ATP} channel activity. To investigate these two possibilities, we studied the channel activity in giant inside-out patch configuration, in which the cytosolic components of the cell were excluded. In this condition, a 3 mM MGO treatment led to a 52.7±12.1% (n=6) increase in the channel activity (Fig. 3A, B), which was comparable to the results found in our whole-cell study.

Moreover, we performed single-channel patch experiments to study the effect of MGO on channel open probability (Po) and channel conductance. In single channel study, a few channel openings were observed and the Po was averaged to be 0.183±0.069 at the basal level (n=4, Fig. 3C). With a 3 mM MGO treatment, the Po of Kir6.1/SUR2B channel increased to 0.444±0.084 (n=7, Fig. 3D). Pin was able to further augment the channel Po to 0.675±0.159 (n=6). Besides, we tested the channel conductance using a ramp protocol with voltage ranging from -100 mV to 100 mV. Three active channels were observed with both MGO and Pin treatments (Fig. 3E, F). The straight lines represented a slope conductance of 36 pS for all three channels, indicating that the channel conductance remains unchanged upon MGO treatment (Fig. 3E, F). Taken together, these data indicate that the modulation of vascular K_{ATP} channels by MGO is mediated via increase in the Po of channel without changing the channel conductance or recruitment of other cellular signaling machinery.

Discussion

In this current study, we found that acute MGO exposure led to the activation of K_{ATP} channels through direct targeting of the Kir6.x subunit. Furthermore, we found that this effect was independent of the SUR subunit and MGO did not cause covalent modifications on the K_{ATP} channel. Single channel analysis revealed that MGO augmented the K_{ATP} channel open probability without altering the channel conductance. In addition, MGO mediated activation of K_{ATP} channel did not require signal transduction components including receptors, secondary messenger molecules or cytosolic effectors.

In several instances, the concentration of MGO has been determined to be sub-millimolar range: in cultured mammalian cells, a ∼300 μM MGO concentration was detected [26] while ∼400 μM MGO was shown in diabetic patients with poorly controlled hyperglycemia [27, 28]. In other studies, the concentration of MGO seems to be much lower [29] [30] [31]. It is possible that the reported concentrations of MGO represent the average concentration on a systemic level instead of the local concentration at a specific site. The levels of MGO in the body over a period of time could be varying depending on the availability of the precursors and the efficiency of the carbonyl clearance system. In particular, a recent study reported that 3 mM MGO treatment for 90s resulted in similar intracellular MGO concentration compared with 1 μM MGO exposure for about 12 hours [6]. During carbonyl stress, MGO levels in the system might follow a sudden burst pattern followed by a recession in the MGO spikes due to the “catching up” by the carbonyl detoxification system with the extent of carbonyl stress present. In our experimental condition, we found the EC_{50} of MGO on Kir6.x activation appears to be 1.7 mM. Our findings could thus be interpreted

as an experimental condition that may represent the initial response of the vasculature to a rapid increase in the levels of MGO in localized environment as seen in pathological conditions.

Oxidative stress, characterized by the presence of excess ROS as a result of overproduction of ROS and impairment of cellular anti-oxidant machinery, have been long thought to be a major contributing factor for diabetes associated vascular complications [32], with several previous studies suggesting that important vascular tone regulators including the vascular K_{ATP} channel are targeted by ROS [33-35]. In agreement, we found that ROS modulates vascular K_{ATP} channel by covalent post-translational modification mechanism S-glutathionylation [21, 36]. In addition to oxidative stress, recently carbonyl stress has been proposed to be an underappreciated stress that may play a more dominate role in progression of diabetes associated vascular complications via the action of reactive carbonyl species (RCS). Among a variety of RCS, MGO is highly reactive. Excess MGO thus readily reacts with nucleophilic groups on proteins and nucleic acids, leading to cellular dysfunction and propagation of carbonyl stress [37, 38]. Indeed, in our recent study, we found that a prolonged MGO treatment leads to disruption of K_{ATP} channel activity via mRNA instability, which is likely to be a contributing factor for impairment of arterial function in carbonyl stress [5]. Interestingly, in the current study, we found that acute MGO treatment can activate the K_{ATP} channel. This differential response of the K_{ATP} channel to MGO treatment is dependent on the exposure time via different mechanisms of action [5, 21].

MGO is well known to interact with positively charged lysine residues on proteins to form advanced glycation adducts [39]. This kind of modulation involves covalent interactions, which is not likely to be broken by a washout alone. Interestingly, in our present study, we found that MGO mediated K_{ATP} channel activation can be rapidly reversed with washout, suggesting an activation mechanism not involving a covalent bond formation. We suspect that MGO can interact with the residues near the gating area to affect the channel gating directly or disrupt the sensitivity of the channel to inhibition by ATP. It is also possible that MGO can target the phospholipid bilayer of the membrane associated with the K_{ATP} channel to affect the channel gating. Future studies addressing these questions will provide valuable information regarding carbonyl stress mediated K_{ATP} channel activation.

It has been shown that MGO contributes to the development of vascular complications of diabetes [40], mainly through the formation of advanced glycation end products (AGEs) that interacts with receptor for advanced glycation end products (RAGEs) [41]. Interestingly, in this current study, this “classical” AGE/RAGE interaction does not seems to play a role in MGO mediated K_{ATP} channel activation, as our single channel preparation, which lacks all the signaling components, still yields similar results as our whole cell patch experiments.

In summary, our study demonstrated that acute MGO treatment led to reversible K_{ATP} channel activation in a signaling cascade-independent manner via interacting with the Kir6.x subunit, augmenting channel open probability. This finding may help us to better understand the response pattern of the vasculature to RCS and provide information to design effective pharmacological treatments against carbonyl stress mediated vascular dysfunction.

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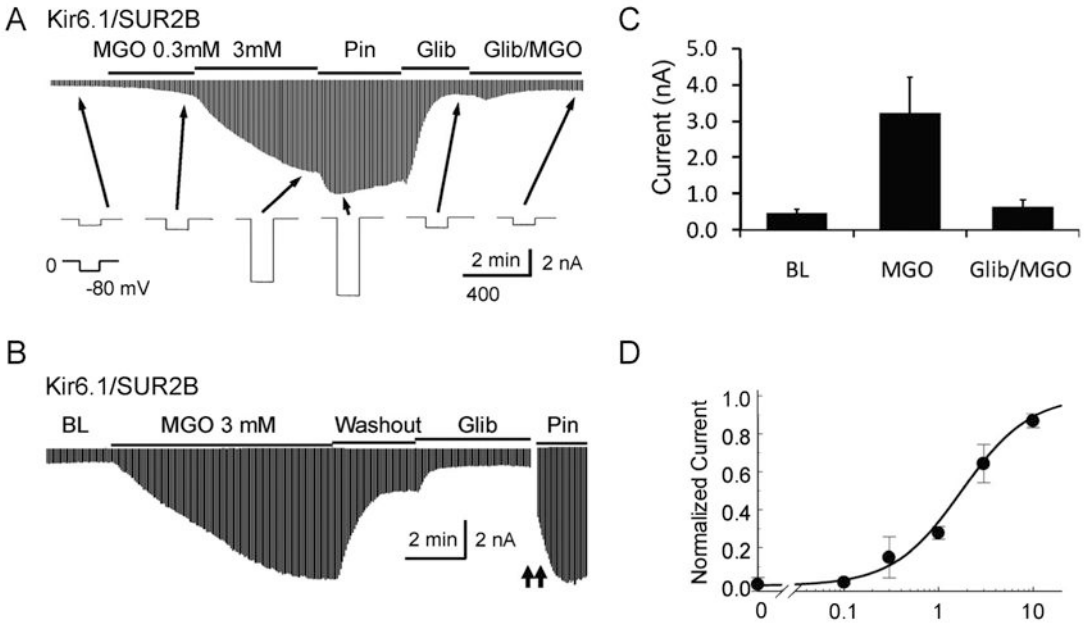

Figure 1. Acute MGO treatment led to activation of Kir6.1/SUR2B channel

A, Kir6.1/SUR2B channel was expressed in HEK293 cells. Whole-cell currents were recorded from cells two days after transfection in voltage-clamp configuration. MGO (0.3 mM – 3 mM) led to the activation of Kir6.1/SUR2B currents in a concentration-dependent manner. Pinacidil (Pin, 10 μM), a K_{ATP} channel specific opener, could further open the channel. The application of K_{ATP} channel specific inhibitor glibenclamide (Glib, 10 μM) dramatically reduced the current. In the presence of Glib (10 μM), the effect of higher concentration of MGO (10 mM) on channel activation was completely blocked. **B**, The MGO-mediated channel activation was reversible. Following channel activation, MGO washout with the bath solution returned the K_{ATP} channel currents to almost baseline level. Glib caused a further decrease in the K_{ATP} channel currents. **C**, Summary of the effect of MGO on the K_{ATP} channel currents in the presence and absence of Glib. **D**, Dose-response relationship between the concentration of MGO and the normalized current. Data was described by the Hill-equation with EC_{50} of 1.7 mM.

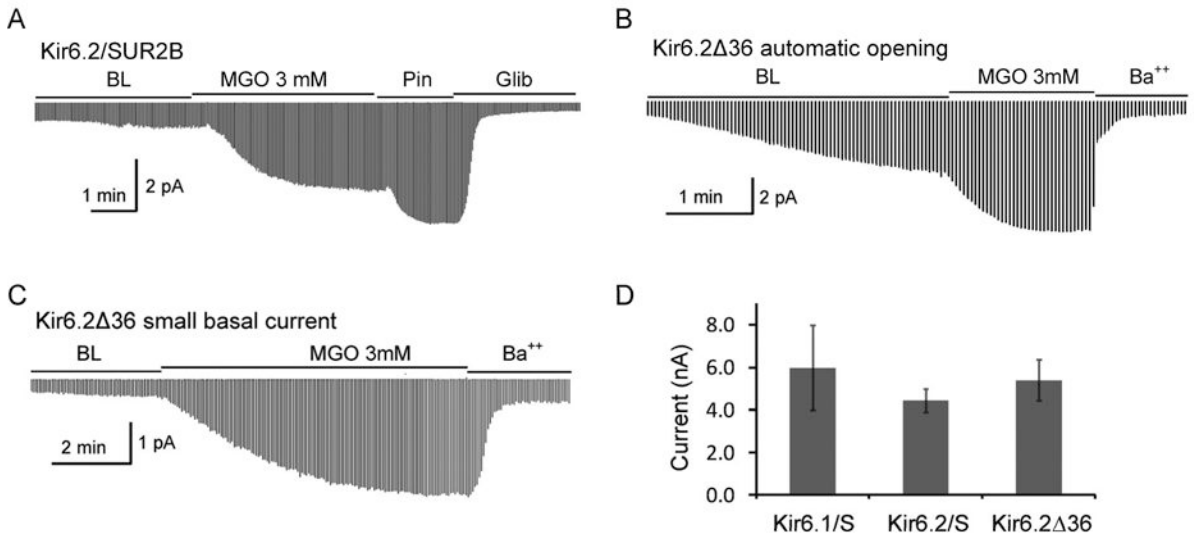

Figure 2. MGO mediated activation was dependent on Kir but not SUR subunit of the K_{ATP} channel

A, MGO (3 mM) activated Kir6.2/SUR2B isoform of the vascular K_{ATP} channel to a similar extent as seen in Kir6.1 isoform. **B**, A truncated form of Kir6.2 subunit (Kir6.2Δ36) channel, capable of expressing itself without the need for SUR subunit, opened automatically, and the channel activity was further enhanced in the presence of MGO. **C**, In the presence of 1.0 mM ATP and 0.5 mM ADP, the basal currents of Kir6.2Δ36 channel remained small and the application of MGO (3 mM) was able to augment the channel activity significantly. **D**, Summary of the effect of MGO on Kir6.1/SUR2B (Kir6.1/S), Kir6.2/SUR2B (Kir6.2/S) and Kir6.2Δ36 channels (Note that unlike Kir6.2, truncated Kir6.1 channel was not able to express by itself).

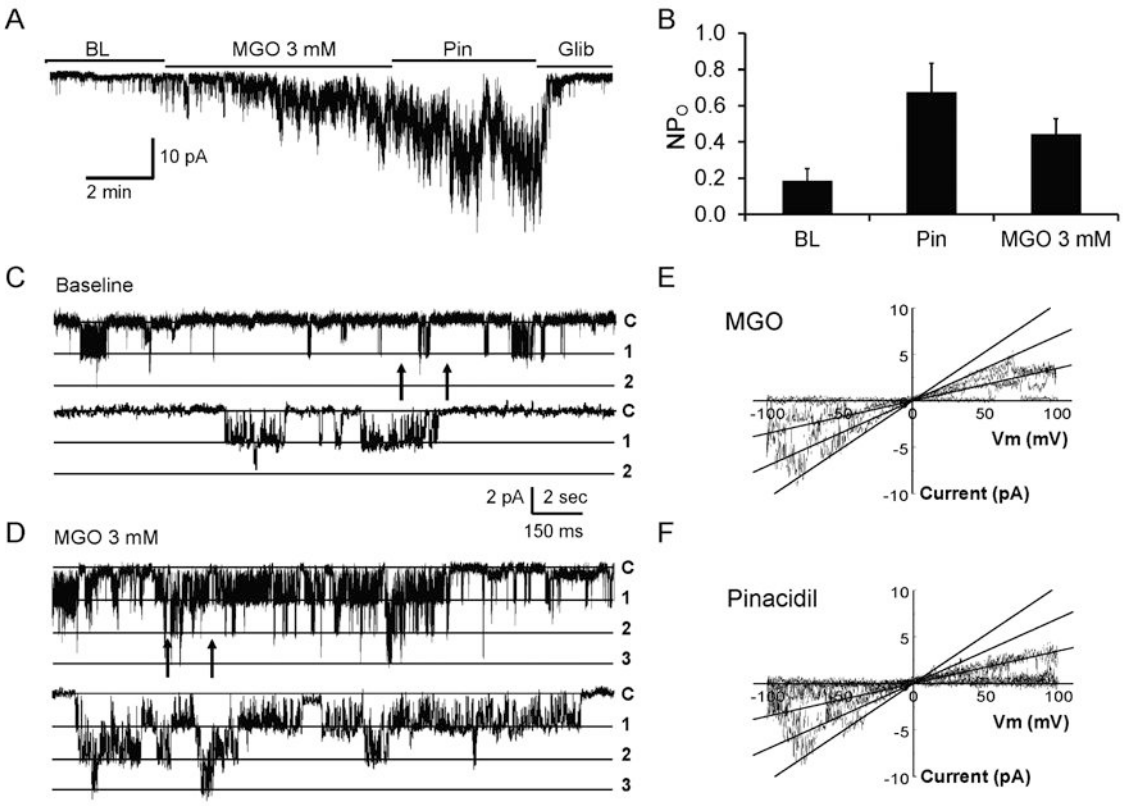

Figure 3. MGO affected single-channel properties of K_{ATP} channel

A, In giant inside-out patch configuration, 3 mM MGO activated the Kir6.1/SUR2B currents in a manner similar to that seen in the whole cell configuration. This current could be further activated and inhibited by Pin and Glib, respectively. **B**, Summary of MGO treatment in inside-out patch study. **C**, Single-channel level currents were recorded in inside-out patches with a holding potential of -80 mV. The lower trace was an expansion from the upper trace between the arrows. The presence of two active channels was evident at baseline level. The NPo averaged 0.183 (n=4) in the baseline. **D,** With an exposure of the inside-out patch to 3 mM MGO, the single-channel activity was augmented, as a result of the appearance of one more active channel. The NPo averaged 0.444 with MGO treatment. **E**, **F**, Single-channel conductance was measured with a ramp protocol with voltage ranging from -100 mV to 100 mV. Three active channels were observed with both MGO alone (**E**) and Pin alone (**F**) treatments. The straight lines represented a slope conductance of 36 pS for all three channels.