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Cholesterol increases the open probability of cardiac K_{ACh} currents

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ABSTRACT

Cholesterol is one of the major lipid components of membranes in mammalian cells. In recent years, cholesterol has emerged as a major regulator of ion channel function. The most common effect of cholesterol on ion channels in general and on inwardly rectifying potassium (Kir) channels in particular is a decrease in activity. In contrast, we have recently shown that native G-protein gated Kir (GIRK or Kir3) channels that underlie atrial K_{ACh} currents are up-regulated by cholesterol. Here we unveil the biophysical basis of cholesterol-induced increase in K_{ACh} activity. Using planar lipid bilayers we show that cholesterol significantly enhances the channel open frequency of the Kir3.1/Kir3.4 channels, which underlie K_{ACh} currents. In contrast, our data indicate that cholesterol does not affect their unitary conductance. Furthermore, using fluorescent and TIRF microscopy as well as surface protein biotinylation, we also show that cholesterol enrichment in vitro has no effect on surface expression of GFP-tagged channels expressed in *Xenopus* oocytes or transfected into HEK293 cells. Together, these data demonstrate for the first time that cholesterol enhances Kir3-mediated current by increasing the channel open probability.

1. Introduction

Cholesterol is one of the major lipid components of the plasma membrane in mammalian cells constituting 10 to 45 mol% with respect to other lipids [1,2]. However, increases in blood and/or tissue cholesterol above the physiological level (up to 2–3-fold) are cytotoxic [1–3] and associated with the development of cardiovascular disease [4–6]. The major source for the cholesterol effects on cellular function is this sterol's ability to alter the function of multiple membrane proteins, including ion channels (see, for example, reviews [7–9]). Remarkably, the mechanisms of cholesterol regulation of ion channel function are only starting to emerge.

Here we focus on atrial G protein-gated inwardly rectifying potassium (GIRK or Kir3) channels. Kir3 channels are activated by the phosphoinositide phosphatidylinositol 4,5-bisphosphate $PI(4,5)P_2$ in the presence of an additional gating molecule, such as the G protein βγ subunits [10]. Recently, we have shown that both cholesterol enrichment in vitro and high cholesterol-dietary intake result in up to 3-fold increase in K_{ACh} currents in atrial myocytes freshly isolated from rabbit and rat [11]. These results were surprising: in most cases, including other types of inwardly rectifying K^+ channels, cholesterol enrichment suppresses channel function (e.g., reviews [7–9]). In particular, we have shown that Kir1.1, all members of the Kir2 subfamily, and Kir6.2Δ36 were significantly inhibited by cholesterol [12,13]. Furthermore, even within the Kir3 subfamily itself, Kir3.1* current was suppressed by cholesterol [11]. Kir6.2Δ36 refers to the C-terminal truncation mutant that renders Kir6.2 active as homomers in the absence of sulfonylurea receptor (SUR) subunits [14], and Kir3.1* refers to the homomerically active Kir3.1 pore mutant F137S [15]. Moreover, elevated levels of cholesterol and/or in vitro cholesterol enrichment also lead to decreased activity of Ca^{2+} sensitive K^+ channels [16,17], voltage-gated Na^+ channels [18], N-type Ca^{2+} channels [19], and volume-sensitive Cl^- channels [20]. In contrast, very few channels including specific transient receptor potential (TRP) channels [21] and Ca^{2+}-permeable, stretch-activated cation channels (SACs) [22] were shown to be inhibited by cholesterol depletion. In all these cases, it was proposed that cholesterol depletion disrupts the signaling platforms required for channel function. Also, while the effects of cholesterol depletion and high cholesterol-dietary intake on epithelial Na^+ channels (eNaC) provided indirect evidence that these channels may be enhanced by cholesterol enrichment [23–26], no other channels were demonstrated to be upregulated by cholesterol.

In the heart, atrial K_{ACh} channels are heterotetrameric proteins that consist of Kir3.1 and Kir3.4 subunits [27]. When Kir3.1 and Kir3.4 are

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co-transfected in mammalian cell lines or co-expressed in *Xenopus* oocytes, the resultant channel exhibits large whole-cell currents with single-channel conductance and open-time kinetics indistinguishable from those of native, atrial K_{ACh} channels [27,28]. Supporting conservation of cholesterol targets in native and heterologous membranes, our data from *Xenopus* oocytes demonstrated that the Kir3.1/Kir3.4 heterotetramer was up-regulated by cholesterol [11]. On the other hand, it has been previously shown that expression of either Kir3.1 or Kir3.4 homomers fails to display similar channel activity as the heteromeric complex. Specifically, homomeric expression of Kir3.1 in several mammalian cell lines does not yield detectable currents (e.g., [15,27,29]). Furthermore, homomeric expression of Kir3.4 gives several-fold smaller currents than the Kir3.1/Kir3.4 heteromers, with poorly resolved single-channel openings [15,30]. An S143T single point mutation at the pore helix of Kir3.4 channels, however, results in active homomeric channels (Kir3.4*) that have characteristics similar to those of the Kir3.1/Kir3.4 complex [15,31]. In particular, we have shown that similarly to atrial K_{ACh} currents, Kir3.4* channels are also up-regulated by cholesterol [11,12]. Yet, the mechanism that underlies cholesterol regulation of Kir3 channels has not been elucidated.

Our earlier data demonstrated that cholesterol did not affect Kir3.4* channels via $PI(4,5)P_2$ and that upregulation of Kir3.4* and Kir3.1/Kir3.4 channels by cholesterol was independent of G-protein βγ subunits [11], suggesting that cholesterol plays a critical role in modulating $I_{K,ACh}$ via a mechanism independent of the channel's major modulators. It remained unknown, however, whether the unique response of both the heterotetramer Kir3.1/Kir3.4 or the monomer Kir3.4* to cholesterol was a result of lipid action on unitary conductance, channel open probability, surface expression of the channel proteins, or a combination of these possibilities. Thus, here we focused on determining the biophysical characteristics of the modulation of these G-protein gated inwardly rectifying potassium channels by cholesterol using a combination of state-of-the-art experimental techniques including planar lipid bilayers, TIRF microscopy, and surface protein biotinylation.

2. Materials and methods

2.1. Expression of recombinant channels in Xenopus oocytes

cRNAs were transcribed in vitro using the "Message Machine" kit (Ambion, Austin, TX). Oocytes were isolated and microinjected as described previously [32,33]. Expression of channel proteins in *Xenopus* oocytes was accomplished by injection of the desired amount of cRNA. Oocytes were injected with 2 ng of cRNA of the channel per oocyte. All oocytes were maintained at 17 °C. Two-electrode voltage clamp recordings were performed 2 days following injection.

2.2. Cholesterol enrichment of Xenopus oocytes

Treatment of *Xenopus* oocytes with a mixture of cholesterol and lipids has been shown to increase the cholesterol/phospholipid molar ratio of the plasma membrane of oocytes [34]. Thus, in order to enrich the oocytes with cholesterol we used a 1:1:1 (w/w/w) mixture containing cholesterol, porcine brain L-α-phosphatidylethanolamine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (Avanti Polar Lipids, Birmingham, AL). The mixture was evaporated to dryness under a stream of nitrogen. The resultant pellet was suspended in a buffered solution consisting of 150 mM KCl and 10 mM Tris/HEPES at pH 7.4 and sonicated at 80 kHz in a bath sonicator (Laboratory Supplies, Hicksville, NY). *Xenopus* oocytes were treated with cholesterol for 1 h.

2.3. Two-electrode voltage clamp recording and analysis

Whole-cell currents were measured by conventional two microelectrode voltage clamp with a GeneClamp 500 amplifier (Axon Instruments, Union City, CA), as reported previously [32,33]. A high potassium solution was used to superfuse oocytes: 96 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$, 5 mM KOH/HEPES (pH 7.4). Basal currents represent the difference of inward currents obtained (at −80 mV) in the presence of 3 mM $BaCl_2$ in high potassium solution from those in the absence of Ba^{2+}.

2.4. Detection of surface expression of GFP-tagged channels in Xenopus oocytes

Detection of GFP-tagged channels in oocytes was performed as was previously described [35]. RNA from GFP-tagged channels was injected into oocytes and, 2–3 days after injection, *Xenopus* oocytes were fixed in 4% paraformaldehyde overnight at room temperature. Fixed oocytes were embedded in 2.5% agarose, and 50-μm sections were cut using a Vibratome. The cut sections were mounted on cover slips and imaged using a Zeiss Axiovert 100TV microscope (Zeiss). To compare fluorescence intensities among different oocyte sections, image acquisition parameters such as pinhole size, intensity, and offset were kept constant.

2.5. Cholesterol enrichment of HEK293 cells

HEK293 cells transfected with Kir2.1 were enriched with cholesterol by treatment with methyl-β-cyclodextrin (MβCD) saturated with cholesterol, a well-known cholesterol donor as described previously [36]. 5 mM MβCD solution in DMEM without serum mixed with saturated cholesterol was sonicated and shaken overnight in a 37 °C incubator. HEK293 cells were incubated with the MβCD solution with or without cholesterol for 1 h to enhance or reduce the cellular cholesterol level. Cellular cholesterol levels in HEK cells were measured using an Amplex Red cholesterol assay kit (Molecular Probes) according to the manufacturer's specifications.

2.6. TIRF microscopy

TIRF microscopy images were acquired using a motorized Zeiss Laser TIRF imaging system equipped with a high-speed EMCCD camera (Quantem 512SC; Photometrics) and a 63×/1.46 NA alpha Plan-Apochromat objective. Images from green and red channels (excitation 488 nm and 561 nm) were obtained by fast switching the excitation lasers by the AxioVision Software (Zeiss). For intensity comparisons the laser intensity, acquisition time and EMCCD gain were kept constant. Membrane intensities of the GFP tagged channels were measured by ImageJ (NIH).

2.7. Surface biotinylation of HEK cells

Surface biotinylation was performed as previously described [37]. In brief, HEK293 cells with 90–95% confluence were incubated in an EZ-Link Sulfo-NHS-SS-Biotin reagent in PBS for 30 min at 4 °C. After the quenching reaction, cells were lysed using a lysis buffer and sonication, followed by incubation on ice for 30 min. Isolation of the biotin-labeled proteins was performed by a NeutrAvidin Agarose attached column. The biotinylated surface proteins were eluted from the avidin beads by an SDS-PAGE sample buffer containing 50 mM DTT, as recommended by the manufacturer (Thermo Fisher Scientific). The purified surface protein fraction was analyzed by Western blotting.

2.8. Western Blotting of purified biotinylated surface proteins

Western blotting was performed as previously described [37]. In brief, the purified surface protein fraction from the biotinylation procedure (30 μg/lane) was separated on a 4–15% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% non-fat dry milk made in Tris-buffered saline containing 0.1% Tween 20 for 2 h. Membranes were then incubated with mouse monoclonal Anti-GFP antibody

(1:5000 dilution, AB184207, Abcam) overnight at 4 °C in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS-T) and 5% non-fat dry milk. Membranes were then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution, Millipore) for 1–2 h at room temperature. The proteins were visualized using a Super Signal West Pico Chemiluminescent Substrate kit (Thermo Fisher Scientific).

2.9. Densitometric analysis

Quantity One software v4.6.9 (Bio-Rad, Hercules, CA) was used to calculate the band density from the Western blotting experiments. Briefly, rectangular boxes of the same size were placed around the bands in each lane, and then the background-subtracted band density was calculated. The band density for a cholesterol enriched protein sample was compared to the band density of the corresponding control sample.

2.10. Bilayer experiments

Bilayer experiments were performed as described [38]. The experimental apparatus consisted of two 1.4 ml chambers separated by a Teflon film that contains a single hole (200 μm in diameter). The bilayer recording solution in both chambers consisted of 150 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris–Hepes (pH 7.4). Bilayer lipids were dissolved in chloroform, then dried under N_2 atmosphere and resuspended in n-decane as previously described [39]. A lipid bilayer was formed by "painting" the hole with a 1:1 mixture of L-α-phosphatidylethanolamine and 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-L-serine] sodium salt dissolved in n-decane to a final concentration of 5 mg/ml. For experiments with cholesterol, cholesterol powder was first dissolved in chloroform and then added to the lipid mixture to reach the desired concentration. The *cis* side was defined as the chamber connected to the voltage-holding electrode, and all voltages were referenced to the *trans* side (ground) of the chamber. The pellet

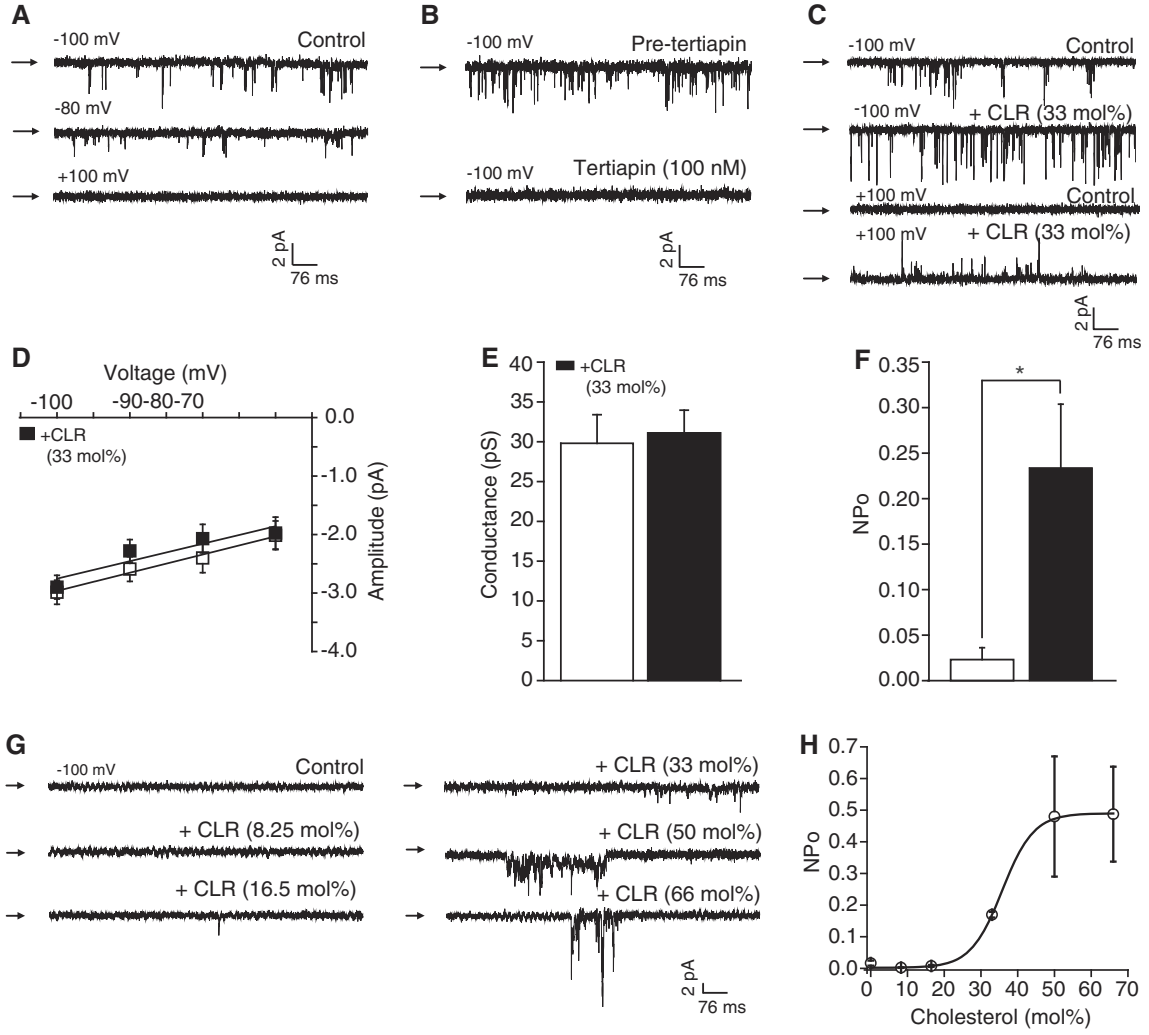

Fig. 1. Cholesterol leads to an increase in the open probability of Kir3.4*. Representative traces of Kir3.4* reconstituted in lipid bilayers with 12.5 μM diC8-PI(4,5)P_2 (A) at −100 mV, −80 mV and 100 mV and (B) blocked by tertiapin at −100 mV and at 100 mV. (C) Representative traces of Kir3.4* at −100 mV and at 100 mV reconstituted in lipid bilayers with 12.5 μM diC8-PI(4,5)P_2 and with 33 mol% cholesterol. (D) Current–voltage curves obtained from single channel recordings of Kir3.4* reconstituted in lipid bilayers with and without cholesterol. The conductance which was calculated from the slope of the linear fit of the currents recorded between −100 and −70 mV, is displayed in Figure E. (E) Summary data showing that cholesterol does not affect the conductance of Kir3.4* in the presence of 12.5 μM diC8-PI(4,5)P_2. (F) Summary data showing the effect of 33 mol% cholesterol on the open probability of Kir3.4* in the presence of 12.5 μM diC8-PI(4,5)P_2. (G) Representative traces of Kir3.4* reconstituted in lipid bilayers with 12.5 μM diC8-PI(4,5)P_2 at −100 mV and with varying concentrations of cholesterol ranging from 0 mol% (control) to 66 mol%. (F) Cholesterol concentration-curve obtained from recordings of Kir3.4* at a single channel resolution following reconstitution of the channel protein into the lipid bilayers with different cholesterol concentrations.

obtained from the membrane preparation of oocytes injected with the channel was re-suspended in 100 μl of bilayer recording solution. After bilayer formation (80–120 pF), 5 μl aliquot of resuspended membrane preparation was added to the *trans* side of the chamber and stirred for 5 min. The orientation of the channel insertion was defined by the inward rectification of ionic current in response to a set of voltages from −100 mV to +100 mV. Records were low-passed filtered at 1 kHz, and sampled at 5 kHz. When needed, the identity of the channel was confirmed by addition of the selective Kir3 blocker tertiapin (100 nM) [40] to the chamber corresponding to the extracellular side of the bilayer. Ion currents were obtained for up to 1 min of continuous recording using a Warner BC-525D amplifier, low-pass filtered at 1 kHz using the 4-pole Bessel filter built in the amplifier, and sampled at 5 kHz with Digidata 1322A/pCLAMP 9.2 (Molecular Devices). Cholesterol concentration-response data points were fitted with Boltzmann function, the fitting resulting in a sigmoidal curve. Fitting was performed using Origin 8.5.1 software (OriginLab).

3. Results

The biophysical basis of the cholesterol-induced increase in atrial Kir3 current may include an increase in the open probability of the channel, an increase in the channel's conductance and/or an increase in the surface expression of the channel protein.

3.1. Cholesterol enrichment leads to an increase in the open probability of Kir3.4 and Kir3.1/Kir3.4*

We first focused on determining the effect of an increase in cholesterol levels on the open probability and the conductance of Kir3 channels. Initially, we tried to use *Xenopus* oocytes for this purpose. However, following cholesterol enrichment, channel activity was increased to a level that prevented analysis at single channel-resolution, which is necessary to clearly distinguish between changes in conductance and modifications in open channel probability.

We thus reconstituted Kir3.4* protein in planar lipid bilayers that enable to control the amount of the enriching cholesterol. We first confirmed that the channel was incorporated in the bilayers by using the selective Kir3 channel blocker tertiapin [40] and by determining the conductance of the channel. Fig. 1A shows representative single channel traces of the reconstituted channel. As can be seen in Fig. 1B the resulting currents were sensitive to tertiapin (Fig. 1B). Moreover, the conductance of the channel was 30 ± 4 pS (Fig. 1D–E), further confirming the identity of the channel as Kir3.4* [31].

Next, we determined the effect of cholesterol on the conductance and open probability of the reconstituted channel in lipid bilayers with 33 mol% cholesterol. This molar fraction of cholesterol was chosen because it has been previously used for studying cholesterol effects on potassium channels [39,41], and is found in native membranes of mammalian cells [42]. Notably, a cholesterol concentration–response curve (Fig. 1G–H) shows that this physiological level of cholesterol is very close to the EC_{50} value of 35.8 ± 2.5 mol%. Representative single channel traces of the reconstituted Kir3.4* channel protein in control bilayers and in bilayers with cholesterol are depicted in Fig. 1C for both inward (−100 mV) and outward (+100 mV) currents. Analysis of the recordings showed that cholesterol had no effect on the conductance of the channel (see Fig. 2D–E). Single channel recording analysis showed that the conductance of Kir3.4* channels in the presence of 33 mol% cholesterol was 31 ± 3 pS, this value being similar to that in the absence of cholesterol (Fig. 1D–E). In contrast, the presence of cholesterol led to a 10.1 ± 5.1-fold increase in the NP_o, with records clearly demonstrating an increase in the overall frequency of channel transitions from closed to open state(s) (Fig. 1F).

In order to corroborate the results obtained for Kir3.4*, we tested the effect of cholesterol on the open probability of the heterotetramer Kir3.1/Kir3.4, which underlies the atrial K_{ACh} current. We first

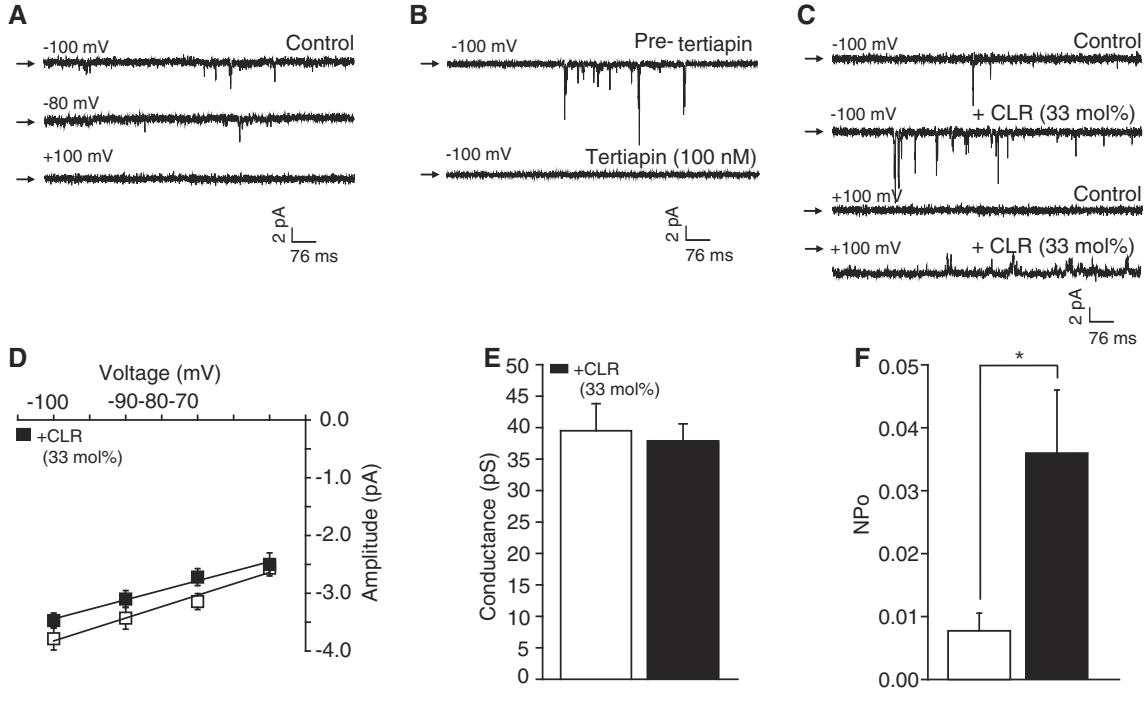

Fig. 2. Cholesterol leads to an increase in the open probability of Kir3.1/Kir3.4. Representative traces of Kir3.1/Kir3.4 reconstituted in lipid bilayers with 12.5 μM diC8-PI(4,5)P_2 (A) at −100 mV, −80 mV and 100 mV and (B) blocked by tertiapin at −100 mV and at 100 mV. (C) Representative traces of Kir3.1/Kir3.4 at −100 mV and at 100 mV reconstituted in lipid bilayers with 12.5 μM diC8-PI(4,5)P_2 and with 33 mol% cholesterol. (D) Current–voltage curves obtained from single channel recordings of Kir3.1/Kir3.4 reconstituted in lipid bilayers with and without cholesterol. The conductance which was calculated from the slope of the linear fit of the currents recorded between −100 and −70 mV, is displayed in Figure E. (E) Summary data showing that cholesterol does not affect the conductance of Kir3.1/Kir3.4 in the presence of 12.5 μM diC8-PI(4,5)P_2. (F) Summary data showing the effect of 33 mol% cholesterol on the open probability of Kir3.1/Kir3.4 in the presence of 12.5 μM diC8-PI(4,5)P_2.

confirmed the identity of the channel using the selective Kir3 channel blocker tertiapin [40] and by determining the channel unitary conductance. As seen in Fig. 2A–B, the channel was sensitive to tertiapin and had a unitary conductance of 40 ± 4 pS (see Fig. 2D–E), which is in agreement with previously published conductance values for this heteroteramethic channel [27,31].

We then investigated the effect of cholesterol on the conductance and open probability of the channel. As the representative traces in Fig. 2C show and the summary data in Fig. 2D–F indicate, similarly to the results obtained with Kir3.4*, cholesterol drastically increased Kir3.1/Kir3.4 NP_o, while having no effect on channel conductance. Specifically, the open probability of the channel was 4.7 ± 1.9-fold larger in lipid bilayers with 33 mol% cholesterol than in the absence of cholesterol. In contrast, there was lack of change in channel conductance: that was 40 ± 4 pS in the absence of cholesterol and 38 ± 3 pS in the presence of 33 mol% cholesterol.

3.2. Cholesterol enrichment of *Xenopus* oocytes and HEK293 cells does not affect surface expression of Kir3.4*

To test the possibility that cholesterol, in addition to an increase in channel open probability, increases in the surface expression of the channel protein, we used two different systems: *Xenopus* oocytes and Human Embryonic Kidney (HEK293) cells.

It has been shown that treatment of *Xenopus* oocytes with a mixture of cholesterol and lipids results in an increase in the cholesterol/phospholipid molar ratio of the plasma membrane of oocytes [34]. Furthermore, we have previously shown that Kir3.4* exhibits enhanced basal currents following cholesterol enrichment in *Xenopus* oocytes similarly to the native Kir3.1/Kir3.4 channels in atrial myocytes, and can thus be used as a model system for investigating the mechanism that underlies up-regulation of native atrial Kir3.1/Kir3.4 channels by cholesterol [11,12]. To test the effect of cholesterol on the surface expression of Kir3.4* channels expressed in *Xenopus* oocytes, we used GFP-tagged constructs of the channel. As can be seen in Fig. 3A–B., 1 h cholesterol enrichment treatment of *Xenopus* oocytes injected with GFP-tagged Kir3.4* led to a ~2.7-fold increase in Kir3.4* currents, which was similar to the effect of cholesterol on the untagged channel [12]. Yet, Fig. 3C–D demonstrate that cholesterol had no effect on membrane expression of the same oocytes whose functional data is depicted in Fig. 3A–B.

To confirm this observation, we turned to Human Embryonic Kidney (HEK293) cells that have been previously shown to express Kir3 channels following transfection [43]. In the heart, activation of K_{ACh} by acetylcholine (ACh) [44–46] via muscarinic M2 receptor and pertussis toxin-sensitive G proteins mediates the vagal negative chronotropic effect [47–50]. We thus co-transfected GFP-tagged Kir3.4* with hM2 in order to test the effect of cholesterol enrichment on GFP-tagged Kir3.4* expressed in HEK293 cells. As Fig. 4A–B show, exposing the cells for 1 h to methyl-β-cyclodextrin (MβCD) saturated with cholesterol, a well-known cholesterol donor [36], resulted in a ~2-fold increase in GFP-tagged Kir3.4* currents. As expected, exposing HEK293 cells to 1 h of cholesterol enrichment resulted in a ~1.5-fold increase in the cellular cholesterol levels (Fig. 4C).

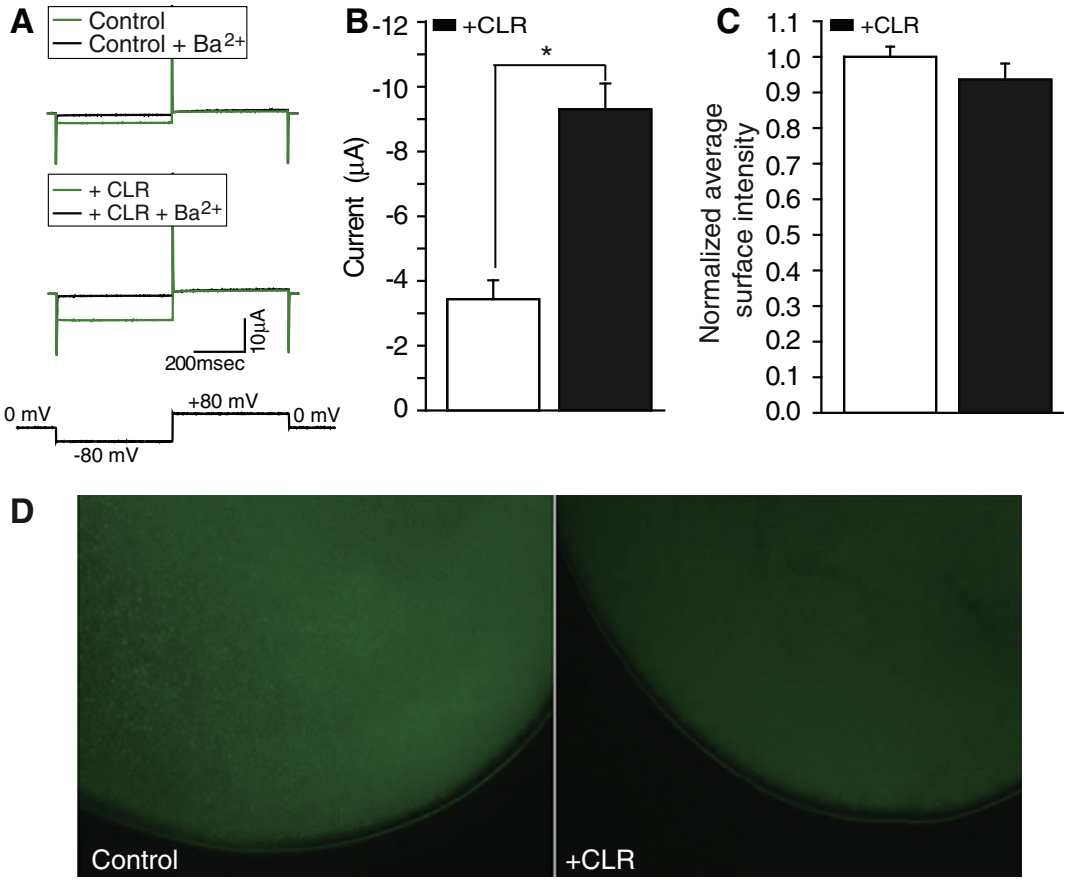

Fig. 3. Cholesterol does not affect the surface expression of Kir3.4* in *Xenopus* oocytes. (A) Representative traces of the whole-cell basal currents recorded at −80 mV/+80 mV from *Xenopus* oocytes expressing the GFP-tagged Kir3.4* subunits. The waveform is shown below the figure. (B) Summary data of whole-cell basal currents recorded in *Xenopus* oocytes at −80 mV showing the effect of cholesterol enrichment on GFP-tagged Kir3.4*. Significant difference is indicated by an asterisk (*, $p < 0.05$). (C) Summary data of the surface expression of GFP-tagged Kir3.4* channels in control and cholesterol enriched oocytes (1 h) ($n = 8–9$). (D) Representative images viewed using Zeiss Axio Vert 200M of oocyte sections expressing GFP-tagged Kir3.4* subunits in control (left) and cholesterol enriched (right) oocytes (1 h).

Having established that GFP-tagged Kir3.4* channels are up-regulated by cholesterol in HEK293 cells, we next tested the effect of cholesterol on the surface expression of the channel in these cells using total internal reflection fluorescence (TIRF) microscopy. The advantage of TIRF microscopy is that it is configured for the precise visualization of structures or processes near cell membranes (<200 nm), and is therefore able to distinguish between protein expression on the surface of the cell and expression in the intracellular membrane near the plasma membrane. As can be seen in Fig. 4D–E, our data demonstrate that cholesterol does not affect surface expression of Kir3.4* in HEK293 cells, corroborating the results obtained using *Xenopus* oocytes.

To further corroborate these results, we also determined the effect of cholesterol on the surface expression of the channel in HEK293 cells using surface protein biotinylation. As Fig. 4F–G show, our biotinylation data confirmed that cholesterol did not affect surface expression of the channel protein.

4. Discussion

An increase in cholesterol is commonly associated with suppression of channel function. Thus, the observation that atrial K_{ACh} currents are enhanced following a high cholesterol diet or acute enrichment of atrial myocytes with cholesterol was rather surprising [11].

We thus focused on determining the biophysical properties of Kir3 channels that are enhanced by cholesterol enrichment. It has been previously shown that while cholesterol suppressed whole cell currents

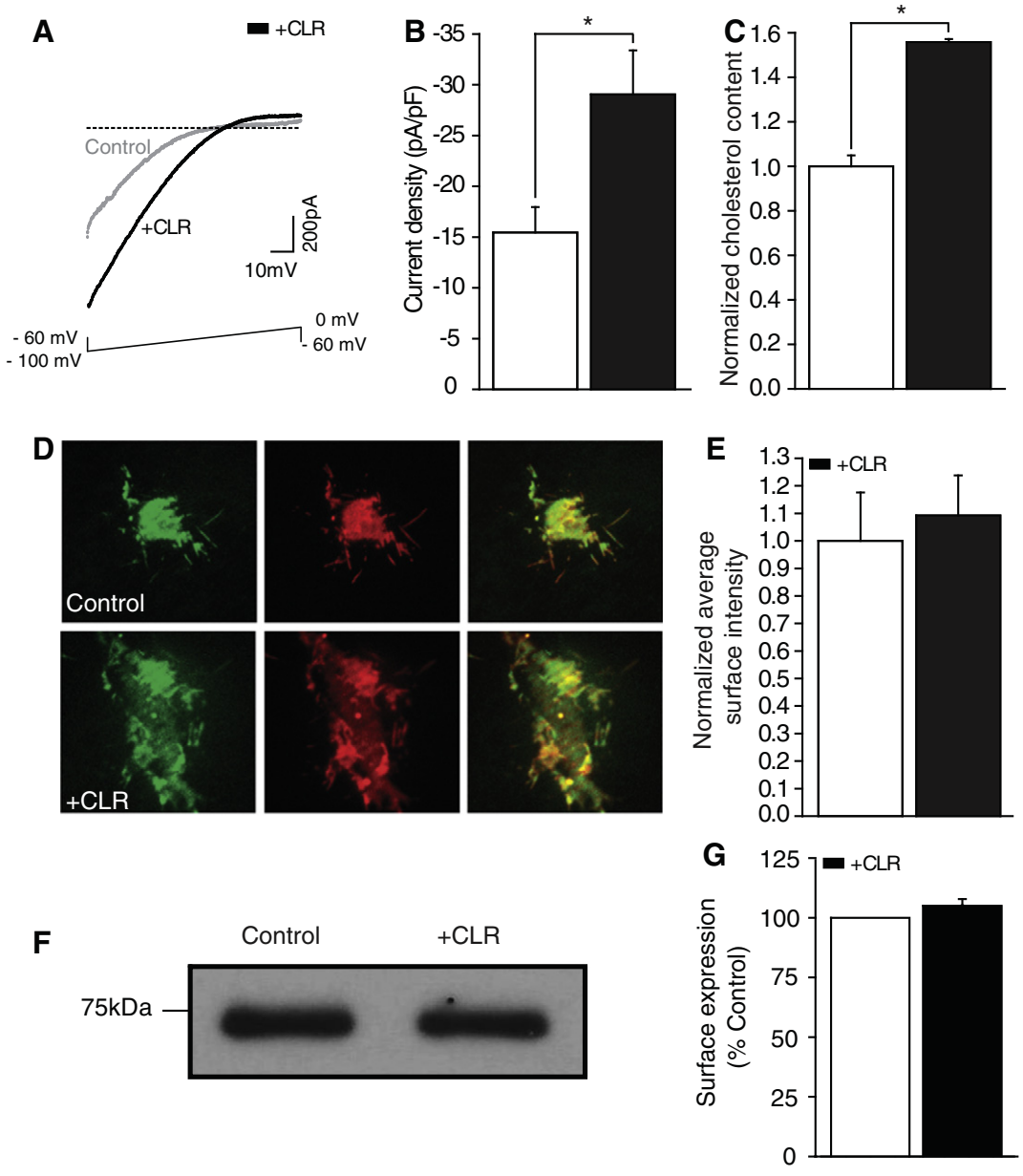

Fig. 4. Cholesterol does not affect the surface expression of Kir3.4* in HEK293 cells. (A) Representative traces and (B) ACh-activated mean peak current densities for control and cholesterol-enriched HEK293 cell population co-transfected with GFP-tagged Kir3.4* subunits, enhanced GFP and HM2 receptor. (C) Cholesterol content of HEK cell in control and cholesterol enriched cells. Significant differences are indicated by asterisks (*, $p < 0.05$). (D) TIRF images of HEK293 cells transfected with GFP-tagged Kir3.4* subunits in control (top) and cholesterol enriched (bottom) cells (1 h). On the left, in green are TIRF images showing the GFP-tagged channel expressed on the plasma membrane. In the center, in red are TIRF images showing the cell membrane that expressed CellLight Plasma Membrane-RFP, which is a modified insect virus (baculovirus) expressing a fusion construct of a plasma membrane marker and red fluorescent protein. On the right are merged figures. (E) Summary data (n = 24–29). (F) Representative Western blot reflecting the surface expression of Kir3.4* obtained by surface protein biotinylation of HEK293 cells expressing the GFP-tagged channel. (G) Summary data of the Western blots (n = 4).

of Kir2.1, it did not affect the surface expression, unitary conductance or open probability of the channel [13]. It was thus proposed that Kir channels exist in the plasma membrane in two modes: "active channel mode" in which the channels flicker between closed and open states leading to overall high P_o, and "silent channel mode" in which channels are stabilized in their closed state and thus, openings are not discernible in single channel recordings [13]. Further corroboration of the "silent channels" hypothesis was evident from more recent data showing that mutations leading to both loss of cholesterol sensitivity of Kir2.1 channels and decrease in the strength of channel-PI(4,5)P_2 interactions resulted in a decrease in open probability of the channels [51]. Since PI(4,5)P_2 is required for the activation of Kir channels (see, for example, reviews [9,52–55]), these observations suggested that only changes in channel-PI(4,5)P_2 interactions but not in the channel's sensitivity to cholesterol were accounted for [51].

We thus asked whether also in the case of Kir3.4* and Kir3.1/Kir3.4 channels, changes in the ratio between "silent" and "active" channels could underlie the effect of cholesterol in channel function. Although cholesterol has an opposite impact on whole cell currents of Kir3.4*, our earlier data has shown that mutations at equivalent positions in the cytosolic domains of the channels reduce or completely abrogate the sensitivity of both channels to cholesterol. Specifically, I229L of Kir3.4* and L222I of Kir2.1 abrogate the sensitivity of the channels to cholesterol [12,56]. Similarly, the D223N mutant of Kir3.4* and the N216D mutant of Kir2.1 lead to a similar effect on the sensitivity of the channels to cholesterol [11,56]. D223N abrogates the sensitivity of Kir3.4* to cholesterol and N216D significantly decreases the cholesterol sensitivity of Kir2.1. Accordingly, opposite effects of cholesterol on different ion channels may still be mediated by a common mechanism [12].

Our data indicate that, as found for Kir2.1, cholesterol does not affect the surface expression or conductance of Kir3.4*. However, cholesterol has a significant effect on the overall open probability of Kir3.4*. Our records demonstrate that this increase is the consequence of an increase in the frequency of channel openings (Figs. 1–2). This observation may originate from two sources: an increase in the frequency of openings of active channels and/or an increase in the number of functional channels in the plasma membrane. Because the frequency of openings of Kir3.4* channels is very low [57], determination of the contribution of each of these two sources from electrophysiological data may be inaccurate. Moreover, our earlier data indicated that an increase in channel activity by co-expressing the channel with the βγ subunits of the G-protein would dominate the effect of cholesterol [11], and therefore could not be used as a mean to enhance the accuracy of this analysis. Taking this limitation into account, we used single channel analysis to estimate the open probability, the P_o, of the active channels in the bilayers in the presence and absence of cholesterol by dividing the NP_o by the number of opening levels observed in each bilayer. This estimate suggested that in the presence of 33 mol% cholesterol the P_o was 7.7 ± 2.6 fold higher than the P_o of the channel in bilayers that lacked cholesterol. Since our data show that cholesterol leads to a 10.1 ± 5.1 fold increase in the overall open probability, the NP_o, of Kir3.4*, the difference between the increase in P_o and NP_o following an increase in cholesterol levels seems to be insignificant. This implies that there is no significant increase in the number of active channels (N) in the presence of cholesterol. Similarly, also for the heterotetramer Kir3.1/Kir3.4, there is no significant difference between the increase in the P_o and in the NP_o due to the presence of cholesterol. Specifically, our estimate suggests that in presence of cholesterol the P_o is 3.7 ± 1.0 fold higher than the P_o of the channel in bilayers that lacked cholesterol while NP_o is 4.7 ± 1.9 fold higher than the NP_o in the absence of cholesterol. These results suggest that, unlike Kir2.1 channels, cholesterol enhances Kir3.4* and Kir3.1/Kir3.4 currents predominantly by increasing the frequency of openings of the active channels, demonstrating that cholesterol enhances Kir channel function by increasing their open probability.

Two basic mechanisms have been proposed to account for cholesterol regulation of ion channels. Earlier studies have suggested that cholesterol regulates ion channel function by altering the physical properties of the lipid bilayer. These include membrane ordering, permeability to small molecules, lateral diffusion, and most importantly, changes in membrane rigidity. For the majority of ion channels, cholesterol suppresses activity (e.g., reviews [7–9]). Thus, it was proposed that cholesterol-induced channel inhibition occurred because cholesterol increased membrane rigidity, which in turn resulted in increased energetic cost for channel opening [58]. Because membrane rigidity is a monotonic function of cholesterol levels within the physiological range in membranes, such a mechanism cannot account for an increase in the open probability of ion channels that are enhanced by cholesterol. More recent evidence suggests that alternatively cholesterol may regulate ion channel function by binding to specific sites in the channel proteins. In particular, cholesterol binding sites have been proposed in several ion channels that are suppressed by cholesterol including Kir2.1 [59], TRPV1 [60], BK channels [36,61] and AChR [62]. Our single-channel data for Kir3.4* and Kir3.1/Kir3.4 showing that cholesterol increases the open probability of the channel reinforce the notion that cholesterol may directly bind to the channel to facilitate channel opening, i.e., destabilizing the closed state(s). Whether channels whose activity is potentiated by cholesterol share common structural motifs for cholesterol recognition remains to be explored.

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