Recent, it was published in Circulation that empagliflozin inhibits H₂O₂-induced cardiac late sodium current (late $I_{Na}$). Using computational modeling and point mutagenic approaches, Philippaert et al suggested a possible site of empagliflozin-binding within Na$_v$1.5 similar to that of local anesthetics, supportive of direct drug binding to Na$_v$1.5, although this remains to be determined conclusively and alternative mechanisms may exist. We have previously shown that CaMKII (Ca/calcium-dependent kinase II) binds to Na$_v$1.5, stimulates late $I_{Na}$, and affects its H$_2$O$_2$-dependent regulation. We also demonstrated that empagliflozin inhibits CaMKII in failing human and murine cardiomyocytes.

Here we show that inhibition of H$_2$O$_2$-induced late $I_{Na}$ by empagliflozin cannot solely be mediated by direct drug binding but depends on CaMKII-dependent phosphorylation of Na$_v$1.5 at serine 571. We demonstrate that empagliflozin inhibits late $I_{Na}$ in patients with aortic stenosis (AS) and phenotype features of heart failure (HF) with hypertrophy and preserved ejection fraction (59.4±1.7%). Murine models of CaMKIIδ knock-out (CaMKIIδ$^{-/-}$), inhibition of CaMKII-dependent Na$_v$1.5 phosphorylation at serine 571 (S571A), and with CaMKII phosphomimetic Na$_v$1.5 S571E mutation were tested for involvement of CaMKII-Na$_v$1.5 phosphorylation. Isolated ventricular myocytes were incubated (30 min) with empagliflozin (1 µmol/L) or control (dimethyl sulfoxide). Some cardiomyocytes were incubated with inhibitors of open-state Na channel inactivation (ATX-II or veratridine) or lidocaine (100 µmol/L, 30 min) for direct Na channel inhibition. H$_2$O$_2$ (100 µmol/L, 5 min) was used to induce reactive oxygen species, which stimulate late $I_{Na}$ in HF via CaMKII$^{3}$ (tested with CaMKII-inhibitor myristoylated-autocamtide-2-related inhibitory peptide (AiP); 2 µmol/L, 30 min). For some experiments, empagliflozin was washed in to ATX-II or H$_2$O$_2$ preincubated myocytes. Late $I_{Na}$ was measured as described previously.

Resting membrane potential was held at ~120 mV and $I_{Na}$ elicited by depolarizing to ~20 mV for 1000 ms, quantified by integrating from 100 to 500 ms of the start of depolarization (normalized to membrane capacitance). Western blots used human ventricular tissue exposed to empagliflozin/vehicle (30 min). Data were analyzed using mixed-effects analysis with Holm-Sidak, linear mixed model with random factor “individual” and Sidak correction, or paired t test (GraphPad Prism 9).

We demonstrate that late $I_{Na}$ in ventricular myocytes from patients with AS similar to CaMKII-inhibitor AiP (Figure [A]). ATX-II-dependent (Figure [B]) enhancement of late $I_{Na}$ in
Figure. Late \( I_{\text{Na}} \) inhibition by empagliflozin requires CaMKII.

A. Original recordings and mean data of empagliflozin- or AiP-mediated inhibition of late \( I_{\text{Na}} \) in human ventricular cardiomyocytes from patients with AS (n=patients). B. Original recordings and mean data of late sodium current (late \( I_{\text{Na}} \)) in murine cardiomyocytes from wild-type (WT) or CaMKII\( \delta \)-/- mice (n=cells per mice). The ATX-dependent enhancement of late \( I_{\text{Na}} \) could not be blocked by empagliflozin. C. In contrast, the \( \text{H}_2\text{O}_2 \)-dependent stimulation of late \( I_{\text{Na}} \) was blocked by CaMKII inhibition (AiP, CaMKII\( \delta \)-/-), by transgenic inhibition of CaMKII-dependent Na\( \text{V} \)1.5 phosphorylation (S571A), or in the presence of empagliflozin. D. In contrast with local anesthetic lidocaine, neither empagliflozin nor AiP could block enhanced late \( I_{\text{Na}} \) in mice with phosphomimetic substitution of glutamic acid for serine at 571 (S571E). E and F, Western blots of cardiomyocytes on empagliflozin show reduced CaMKII-autophosphorylation (T287) and reduced CaMKII-dependent Na\( \text{V} \)1.5 phosphorylation. For comparison of multiple groups, mixed-effects analysis plus Holm-Sidak (A) or linear mixed model plus Sidak were performed. For comparison of 2 groups, paired \( t \) test was done (F). A indicates ampere; AiP, autocamtide-2-related inhibitory peptide; AS, aortic stenosis; ATX II or ATX, Anemonia viridis toxin 2; CaMKII, Ca/calmodulin-dependent kinase II; CaMKII\( \delta \)-/-, CaMKII delta knock out \( \delta \); Empa; Empa, empagliflozin; F, farad; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; HF, heart failure; kDa= kilo Dalton; ms, miliseconds; p, phosphorylated; pA, picoampere; S571A and S571E: Nav1.5 with a phosphomimetic mutation at Ser571 (S571E), or Nav1.5 with the phosphorylation site ablated (S571A); V, vehicle; and WT, wildtype.
murine wild-type cardiomyocytes was not affected by empagliflozin (not even at 10 and 100 µmol/L), or after wash-in (at 1 µmol/L) to ATX-II preincubated myocytes, which would be expected if empagliflozin were a direct Na\textsubscript{\text{\text{\text{Na}}}} inhibitor. Moreover, wash-in of empagliflozin (up to 10 µmol/L) also did not inhibit late Na\textsubscript{\text{\text{\text{Na}}}} in myocytes preincubated with a moderate concentration of veratridine (16 nmol/L, experimentally determined as EC\textsubscript{50} by dose-response; data not shown). In sharp contrast, both veratridine and ATX-II-enhanced late Na\textsubscript{\text{\text{Na}}} were blocked by lidocaine (not shown). Empagliflozin robustly inhibited H\textsubscript{2}O\textsubscript{2}-induced late Na\textsubscript{\text{\text{Na}}} (Figure [C]), with maximal efficacy at 6 minutes but not at 2 minutes after onset of exposure (late Na\textsubscript{\text{\text{Na}}} integral during wash-in: 0 minutes, −50.8±4.3 A*F−1[ampere·farad]·ms; 2 minutes, −39.9±4.6 A*F−1·ms, \textit{P}=0.0934 versus 0 minutes; 4 minutes, −24.2±4.6 A*F−1·ms, \textit{P}=0.0007 versus 0 minutes; 6 minutes, −17.2±4.0 A*F−1·ms, \textit{P}<0.0001 versus 0 minutes, n=6). No additional effect of empagliflozin on late Na\textsubscript{\text{\text{Na}}} was observed with AIP (not shown) or in myocytes lacking either CaMKII\textsubscript{\text{\text{\text{δ}}}} (CaMKII\textsubscript{\text{\text{\text{δ}}}−/−}) or CaMKII-dependent Na\textsubscript{\text{\text{\text{Na}}}} phosphorylation at serine 571 (S571A, Figure [C]). Accordingly, the enhanced late Na\textsubscript{\text{\text{Na}}} in mice with CaMKII phosphomimetic Na\textsubscript{\text{\text{\text{Na}}}+/−}S571E was blocked by neither empagliflozin nor AIP (Figure [D]). In contrast, lidocaine inhibited late Na\textsubscript{\text{\text{Na}}} in S571E cells, underscoring that empagliflozin primarily acts by CaMKII-Na\textsubscript{\text{\text{\text{Na}}}} phosphorylation. Empagliflozin dose-response revealed an IC\textsubscript{50} for inhibition of H\textsubscript{2}O\textsubscript{2}-dependent late Na\textsubscript{\text{\text{Na}}} of 0.086 µmol/L in murine myocytes (not shown). Empagliflozin inhibited CaMKII autophosphorylation and CaMKII-dependent phosphorylation of Na\textsubscript{\text{\text{\text{Na}}}} in AS and HF (Figure [E and F]).

In conclusion, inhibition of late Na\textsubscript{\text{\text{Na}}} by empagliflozin is at least in part caused by inhibition of CaMKII-dependent regulation of Na\textsubscript{\text{\text{\text{Na}}}}.\textsuperscript{2,4} If cardiac Na channels were solely directly inhibited, empagliflozin, like local anesthetics, should have blocked ATX-II/veratridine–stimulated late Na\textsubscript{\text{\text{Na}}} but it did not. Nevertheless, the target of empagliflozin in the heart remains unclear,\textsuperscript{5} and further research is needed to better understand direct versus indirect effects on late Na\textsubscript{\text{\text{Na}}}.

We demonstrate that empagliflozin also inhibits late Na\textsubscript{\text{\text{Na}}} in patients with AS and features of HF with preserved ejection fraction, which may reduce the propensity for arrhythmias and contribute to the positive results of the EMPEROR-Preserved trial (Empagliflozin Outcome Trial in Patients With Chronic Heart Failure With Preserved Ejection Fraction).

**ARTICLE INFORMATION**

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**Disclosures**

None.

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