Pharmacological inhibition of acetylcholine-regulated potassium current ($I_{K,ACh}$) prevents atrial arrhythmogenic changes in a rat model of repetitive obstructive respiratory events

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BACKGROUND In obstructive sleep apnea (OSA), intermittent hypoxemia and intrathoracic pressure fluctuations may increase atrial fibrillation (AF) susceptibility by cholinergic activation.

OBJECTIVE To investigate short-term atrial electrophysiological consequences of obstructive respiratory events, simulated by intermittent negative upper airway pressure (INAP), and the role of atrial acetylcholine-regulated potassium current ($I_{K,ACh}$) activated by the $M_2$ receptor.

METHODS In sedated (2% isoflurane), spontaneously breathing rats, INAP was applied noninvasively by a negative pressure device for 1 minute, followed by a resting period of 4 minutes. INAP was applied repeatedly throughout 70 minutes, followed by a 2-hour recovery period. Atrial effective refractory period (AERP) and AF inducibility were determined throughout the protocol. To study INAP-induced $I_{K,ACh}$ activation, protein levels of protein kinase C (PKC) were determined in membrane and cytosolic fractions of left atrial (LA) tissue by Western blotting. Moreover, an $I_{K,ACh}$ inhibitor (XAF-1407: 1 mg/kg) and a muscarinic receptor inhibitor (atropine: 1 µg/kg) were investigated.

RESULTS In vehicle-treated rats, repetitive INAP shortened AERP ($\Delta 37 \pm 6$ ms vs baseline $44 \pm 3$ ms; $P < .001$) and increased LA membrane PKC$\varepsilon$ content relative to cytosolic levels. Upon INAP recovery, ratio of PKC$\varepsilon$ membrane to cytosol content normalized and INAP-induced AERP shortening reversed. Both XAF-1407 and atropine increased baseline AERP (control vs XAF-1407: $61 \pm 4$ ms; $P > .001$ and control vs atropine: $58 \pm 3$ ms; $P = .011$) and abolished INAP-associated AERP shortening.

CONCLUSION Short-term simulated OSA is associated with a progressive, but transient, AERP shortening and a PKC$\varepsilon$ translocation to LA membrane. Pharmacological $I_{K,ACh}$ and muscarinic receptor inhibition prevented transient INAP-induced AERP shortening, suggesting an involvement of $I_{K,ACh}$ in the transient arrhythmogenic AF substrate in OSA.

KEYWORDS Sleep apnea; Atrial fibrillation; $I_{K,ACh}$; Cholinergic activation; Rats

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KEY FINDINGS

- In rats, short-term simulated obstructive respiratory events are associated with shortening of atrial refractoriness, which is reversible upon recovery.
- Throughout repetitive obstructive respiratory breathing events, a certain subtype of protein kinase C (PKC\(\varepsilon\)) involved in atrial \(I_{K,\text{ACh}}\) regulation, transiently translocated from cytosol to membrane in atrial tissue.
- Pharmacological \(I_{K,\text{ACh}}\) and muscarinic receptor inhibition prevented shortening of atrial refractoriness, suggesting an involvement of \(I_{K,\text{ACh}}\) in the transient arrhythmogenic AF substrate in OSA.

Introduction

Obstructive sleep apnea (OSA) is the most common sleep-related breathing disorder and is broadly associated with cardiovascular diseases.\(^1\) In patients with atrial fibrillation (AF), OSA is present in up to 70%\(^2,3\) and impairs catheter-based and pharmacological antiarrhythmic treatment.\(^4,6\) Some mechanisms, such as intrathoracic pressure changes, hypoxia, and fragmented sleep and intermittent arousals, have been suspected to contribute to a complex and dynamic AF substrate in the setting of OSA.\(^3\) However, mechanisms and cellular pathways involved in the arrhythmogenic changes contributing to the dynamic AF substrate are not fully understood.\(^7,8\)

Whereas long-term OSA is acknowledged to induce structural remodeling, acute OSA is suspected to be associated with a rather transient and reversible AF substrate that may depend on the severity of OSA during a specific night.\(^7,9,10\) Accordingly, the recent observational cohort study, VARIOUSA-AF, showed that the most severe sleep apnea night within each patient conferred a 1.7-fold increased risk of suffering at least 5 minutes from AF during the same night within each patient conferred a 1.7-fold increased risk of suffering at least 5 minutes from AF during the same night within each patient.\(^7,9,10\)

These clinical observations suggested that there might be a transient arrhythmogenic substrate prevailing throughout the day after a night of OSA. However, how acute OSA episodes transiently increase AF susceptibility remains unclear.

Cholinergic activity has been proposed as an important and potentially modifiable contributor to increased arrhythmogenic risk during acute OSA. In different animal models, pharmacological inhibition of cholinergic signaling, vagotomy, and ablation of the autonomic ganglionic plexi could blunt acute shortening in atrial refractoriness during simulated OSA.\(^5,12\) Shortening of atrial refractoriness during increased cholinergic signaling can be caused by an activation of the G protein–gated acetylcholine-activated inward rectifier channel (GIRK channel: GIRK\(_1\) and GIRK\(_4\))–mediated potassium current (\(I_{K,\text{ACh}}\)).\(^13,14\) The \(I_{K,\text{ACh}}\) represents an important downstream component of vagal motor activation in the atria and may play a crucial role in atrial arrhythmogenesis in OSA.\(^5,15\) The \(I_{K,\text{ACh}}\) is increased, among other means, by phosphorylation of the GIRK channels, which is regulated by the translocation of the stimulatory protein kinase (PKC\(\varepsilon\)) from cytosol to membrane, which can happen independently of muscarinic receptor stimulation in chronic AF.\(^16,17\) However, so far neither \(I_{K,\text{ACh}}\) nor its potential regulatory mechanisms have been systematically investigated as a pharmacological target in OSA.

In this study, we investigated short-term atrial electrophysiological consequences of obstructive respiratory events, simulated by intermittent negative upper airway pressure (INAP), and the role of atrial acetylcholine-regulated potassium current (\(I_{K,\text{ACh}}\)).

Methods

Herein we developed a rat model for OSA in which we investigated transient atrial electrophysiological effects of obstructive respiratory events simulated by INAP. Moreover, we investigated INAP-induced \(I_{K,\text{ACh}}\) activation, by pharmacological intervention with the \(I_{K,\text{ACh}}\) inhibitor (XAF-1407; 1 mg/kg) and the muscarinic receptor inhibitor (atropine; 1 \(\mu\)g/kg) (Figure 1A) and by analyzing PKC\(\varepsilon\) protein levels in the membrane and cytosolic fractions of left atrial (LA) tissue lysate.

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (eighth edition; revised 2011) and to the ARRIVE guidelines. Moreover, all animal experiments were executed in accordance with the Danish Animal Experiment Inspectorate (case number 2017-15-0201-01231).

Animals and anesthesia

Forty-four rats (male Wistar, 300–400 g body weight, aged 10–12 weeks) were purchased from Charles River (Denmark) and housed 4 per cage under standardized conditions (room temperature 24°C, relative humidity 55%, 12 hours dark/night cycle). Rats had free access to a standardized diet and tap drinking water ad libitum. During experiments, rats were anesthetized with 2% isoflurane and 98% oxygen.

Surgery and instrumentation

Throughout the experiments, vital parameters were monitored by a SpO\(_2\) probe, attached to the lower limb of the rat, and a 2-lead needle electrocardiogram (ECG). Body temperature was maintained at 37.0°C ± 0.5°C with the help of a heating lamp. For initial surgery, rats were anesthetized with 5% isoflurane, placed in supine position, and the right lateral neck area was dissected free. After exposure of the right external jugular vein, an octopolar electrode catheter (ERP-802; Millar, Houston, TX) was advanced through the jugular vein into the right atrium (RA). The position of the catheter in the RA was verified by intracardiac electrograms (EGMs) recorded by the catheter via Powerlab and LabChart 8 (ADInstruments, Dunedin, New Zealand). For a coordinated
intravenous drug perfusion, a Venflon cannula (G24; Henry Schein, Melville, NY) was placed in the tail vein.

Application of INAP
INAP, which might be seen as “inverse” CPAP (continuous positive airway pressure), was applied at a defined negative pressure of -50 mbar via a customized animal mask by a negative pressure device as a modification of the Mueller maneuver (forced inspiration against occluded airways), as described elsewhere.10 During INAP, respiration efforts (inspiration and expiration) and potential pressure leakages were documented via a pressure sensor and recorded with LabChart 8 (ADInstruments, Dunedin, New Zealand) (Figure 1B).

Intervention groups and INAP protocol
In order to investigate cumulative responses to repetitive INAP, rats underwent an INAP protocol, in which INAP was applied 14 times throughout 70 minutes (Figure 1B). Each INAP lasted for 1 minute with a 4-minute resting period afterwards.

Additionally, rats were randomized to receive either a buffer-based vehicle (controls, CTR; n = 11), $I_{K,ACH}$ inhibitor (XAF-1407; n = 11), or a muscarinic receptor inhibitor (atropine; n = 4) (Figure 1B, group 4). After the INAP protocol, cardiac tissue from CTR rats (n = 6) was harvested and frozen in liquid nitrogen (Figure 1B, group 3). Dedicated CTR rats were allowed to recover after the INAP protocol for either 1 hour (n = 6) or 2 hours (n = 3) under isoflurane anesthesia (Figure 1C, groups 1 and 2).

Detailed information about drug solution, administration, and dose-response experiments (n = 4) is shown in the Online Supplement (Supplemental Figure 1).

Electrophysiology
Atrial effective refractory periods (AERP) were determined using a programmed customized pacing protocol. Pacing trains of 6 stimuli (pulse width 0.5 ms, 2× threshold current) were applied with a fixed cycle length (S1 = 125 ms), followed by a shorter coupled stimulus 2 (S2), incrementing by 2 ms each train. AERP was defined as the last coupled stimulus S2, not resulting in an atrial activation. AERPs were determined before each INAP and shortly after. AF susceptibility was assessed by 100-Hz burst pacing (pulse width 2 ms, 4× threshold current) for 2 seconds before and during INAP in CTR (n = 7) and XAF-1407 (n = 6) from INAP 8 to

Figure 1  Potential pathways for increased atrial acetylcholine-regulated potassium current ($I_{K,ACH}$) and the rodent model. A: Conceptual overview of potential increase in $I_{K,ACH}$. In atrial myocardium, acetylcholine may activate muscarinic receptors, which might increase $I_{K,ACH}$. Alternatively, a translocation of protein kinase C from cytosol to membrane might contribute to an increase in $I_{K,ACH}$. Pharmacological interventions: atropine as a muscarinic receptor inhibitor or XAF-1407 as a direct $I_{K,ACH}$ inhibitor. G-protein gated acetylcholine-activated inward rectifier channel (GIRK channel)–mediated potassium current ($I_{K,ACH}$). Figure was created in BioRender.com. B: Representative traces of an atrial electrogram (EGM) and airway pressure during intermittent negative upper airway pressure application (INAP). C: Overview of the experimental protocol and visualization of different study groups. AERP = atrial effective refractory period.
14 (Figure 1C, group 4). AF was defined as more than 3 irregular R-R intervals and absence of the P waves in the ECG, and rapid and irregular activation in atrial EGMs. Inducible AF duration was measured from the end of burst pacing until the first regular 1:1 atrioventricular conduction detected by the surface ECG and endocardial bipolar electrograms. Atrial conduction velocity was evaluated by bipolar atrial EGM analysis of the octopolar catheter (ERP-802; Millar, Houston, TX). Conduction velocity was determined as the ratio between the distance and the time delay between the earliest and latest bipolar atrial EGM of the 2 poles of the catheter.

Preparation of cytosolic and membrane (particulate) fractions
After completion of the INAP protocol the LA tissue was immediately snap-frozen and stored at -80°C until further processing. For preparation of cytosolic and membrane fractions the tissue was first disrupted and homogenized in 1 mL ice-cold extraction buffer containing (in mM): HEPES, 20 (pH = 7.5); EDTA, 5; EGTA, 5; NaF, 20; Na3VO4, 0.2; β-glycerol phosphate, 20; benzamidine, 10; AEBSF, 0.5; leupeptin, 25 μg/mL; and DTT, 5. Homogenates were ultracentrifuged at 48,000 rpm for 30 minutes at 4°C and the supernatant corresponding to the cytosolic fraction was collected. The remaining pellet was then resuspended in the same buffer, to which was added 1% (v/v) Triton X-100, incubated for 30 minutes on ice, and subsequently again ultracentrifuged at 48,000 rpm for 30 minutes at 4°C. The supernatant containing the membrane (particulate) fraction was collected. All protein samples were stored at -80°C before further processing. Further in-depth explanation of Western blotting is provided in the Online Supplement.

Statistics
Data are expressed as mean ± standard error of the mean. An unpaired Student t test (2-tailed) was used for statistical analysis comparing 2 independent groups (CTR and XAF-1407; AF duration). A paired Student t test (2-tailed) was used for comparing changes induced by INAP within the same animal. A 2-way ANOVA with a Dunnett’s multiple comparisons test was used for examining the time effect of the INAP protocol between drug intervention groups. P values <.05 were regarded as statistically significant. Statistical analysis was carried out using GraphPad PRISM Version 8.0.1 (GraphPad Software, La Jolla, CA).

Results
Acute INAP application and effects on oxygen saturation
INAP was applied noninvasively via an animal mask for 60 seconds, which reduced upper airway pressure to around -20 mbar (representative traces in Figure 1B). During INAP application oxygen saturation dropped repeatedly
cytosolic protein concentration ratio balanced out again and demonstrated similar relations as seen in the time controls.

**I<sub>K,ACh</sub>** inhibition and arrhythmia susceptibility

In order to further assess I<sub>K,ACh</sub> involvement in INAP-induced AERP shortening, rats were randomized to receiving either a vehicle-based buffer (CTR, n = 13), an I<sub>K,ACh</sub> inhibitor (XAF-1407, n = 13), or a muscarinic receptor inhibitor (atropine, n = 4) prior to the INAP protocol (Figure 1A and 1C, group 4). Compared to vehicle-treated controls, XAF-1407 and atropine increased AERP at baseline (CTR: 45 ± 2 ms vs XAF-1407: 61 ± 4 ms; P > .001 and CTR vs atropine: 58 ± 3 ms; P = .011). With the initiation of the INAP protocol, controls decreased progressively in AERP throughout the first 7 INAP applications (Figure 4A, respective comparison to baseline: INAP4 “*” P = .01, INAP5 “##” P = .026, INAP 6 “§” P = .001, INAP7 “###” P = .001). In contrast to the controls, rats treated with either XAF-1407 or atropine did not shorten in AERP following INAP (Figure 4B and 4C).

Arrhythmogenic consequences of shortened AERP in this model were assessed by a burst pacing protocol throughout the second half of the INAP protocol (INAP 8–14) (Figure 5A). Overall, AF was induced in 5 out of 7 vehicle controls with an AF inducibility of 29% ± 12% and in 1 out of 6 XAF-1407-treated rats (3% ± 3%). Inducible AF durations were longer in control rats than in XAF-1407-treated rats.

**Discussion**

Repetitive obstructive respiratory events as depicted in this rat model were associated with progressive AERP shortening and a PKC<sub>E</sub> translocation from cytosol to membrane of the LA, both of which reversed upon INAP recovery. Pharmacological I<sub>K,ACh</sub> inhibition prevented INAP-associated AERP shortening and arrhythmogenic consequences.

**The transient arrhythmogenic substrate for AF**

Sleep apnea is recognized as an important contributor to atrial arrhythmia. Both clinical and basic science data underline that exposure to chronic sleep apnea is associated with atrial enlargement, local conduction disturbances, low-voltage areas, and connexin dysregulations. Additionally, individual acute obstructive respiratory events further contribute to arrhythmogenesis by impairing atrial refractoriness and increasing atrial premature counts.

Our study demonstrates that acute obstructive respiratory events can also be associated with transient arrhythmogenic changes, further dynamically contributing to the AF substrate. In our model, 70 minutes of repetitive simulation of obstructive respiratory events was associated with a progressive shortening in atrial refractoriness. The shortening in AERP reversed upon INAP recovery, indicating that despite a time delay the arrhythmogenic substrate is transient and reversible. Previously, we demonstrated that in the same model 4 hours of INAP decreased atrial antioxidative capacity, which was also reversible within 24 hours of INAP recovery. Taken together, a shortened AERP and a decreased antioxidative capacity may contribute to a temporal increase in AF risk, as shown in VARIOSA-AF.

**The I<sub>K,ACh</sub>-activating pathway**

Previous animal studies indicated that apnea-induced acute arrhythmogenic changes were mediated by cholinergic signaling. In order to scrutinize contribution of vagal motor activation to AF susceptibility in our model, we investigated one of the most downstream components of vagal activation on the atria, I<sub>K,ACh</sub>.

Generally, vagal motor activation may result in increased acetylcholine release, which in atrial myocardium can act on muscarinic receptors and activate GIRK channels, hence increasing I<sub>K,ACh</sub>. It has been postulated that a translocation of PKC<sub>E</sub> from the cytosol to the membrane and a subsequent phosphorylation increases opening probabilities of...
GIRK channels. In chronic AF patients this pathway was even found to contribute to a constitutively active $I_{K,ACh}$. We found the very same PKC isoform translocation pattern from cytosol to membrane, at a time point where atrial refractoriness was profoundly shortened by the INAP protocol. Even though PKC$_\varepsilon$ protein translocation was reversible, kinetics misaligned with the reversibility of atrial refractoriness, which reversed faster. This could indicate that PKC$_\varepsilon$ translocation due to our protocol is an independent pathway potentially contributing to increased $I_{K,ACh}$ compared to an

![Figure 4](image)

**Figure 4** Effects of pharmacological intervention. A: Progressive shortening of atrial effective refractory period (AERP) in control rats ($n = 11$) undergoing the intermittent upper airway pressure (INAP) protocol. Prevention of INAP-induced AERP shortening in rats pretreated with acetylcholine-regulated potassium current ($I_{K,ACh}$) inhibitor XAF-1407 (B, $n = 11$) and M2-receptor inhibitor atropine (C, $n = 4$). Data are expressed as mean ± SEM. For statistical analysis a 2-way ANOVA with a Dunnett’s multiple comparisons test was used. *: $P = .01$; **: $P = .026$; §§: $P = .001$; §§§: $P = .001$; demonstrated as respective comparisons to baseline.

![Figure 5](image)

**Figure 5** Atrial fibrillation (AF) susceptibility. AF inducibility (A) and inducible AF duration (B) by burst pacing in the second half of the intermittent upper airway pressure (INAP) protocol. Comparison between controls ($n = 7$) and XAF-1407 ($n = 6$). Data are expressed as mean ± SEM. No statistical analysis was performed owing to too few AF inductions in the XAF-1407-treated group.
activation due to potential cholinergic signaling. Other pathophysiological features of OSA that impact atrial electrophysiological integrity, such as negative thoracic pressure swings and subsequently cardiac stretch, could have been involved in a noncholinergic increase of $I_{K,ACH}$, as previously seen in chronic AF. Moreover, missing information about how much a translocation contributes to a direct effect on $I_{K,ACH}$ leaves the link between PKC translocation and increased $I_{K,ACH}$ putative. However, a direct in vivo pharmacological inhibition of $I_{K,ACH}$ by XAF-1407 increased baseline AERP dose-dependently and demonstrated effective protection against INAP-induced changes. Moreover, atropine depicted similar dynamics and protective effects against INAP, supporting a cholinergic-mediated activation of $I_{K,ACH}$ via the muscarinic receptors.

Clinical perspective
OSA is highly prevalent in AF patients and limits success rate of pharmacological and catheter-based treatment strategies. Although prevention of obstructive respiratory events by CPAP therapy seems to be the most logical approach for treating OSA patients, testing for sleep apnea and initiation of OSA treatment is not routinely accessible for the majority of AF patients owing to limited capacity of sleeping laboratories and missing reimbursement models for routine OSA screening. Moreover, compliance to CPAP therapy poses a problem to many OSA patients.

The transient INAP-associated arrhythmogenic changes presented in this study may represent an interesting target for a pharmacological $I_{K,ACH}$ inhibition in OSA patients with AF. Owing to its atria-specific profile, $I_{K,ACH}$ provides a tempting target for antiarrhythmic therapy without affecting ventricular repolarization. Impairing ventricular repolarization is often an undesirable side effect of antiarrhythmic therapy and might bear special implication in sleep-disordered breathing, since OSA itself has been linked to ventricular arrhythmia risk. Although pharmacological $I_{K,ACH}$ inhibition in several animal models, including ours, demonstrated an antiarrhythmic effect, clinical trials could not reproduce a reduction in AF so far. However, as AF as a consequence of acute OSA may be mediated by increased cholinergic signaling, AF patients with concomitant OSA may represent a subgroup of patients in whom $I_{K,ACH}$ inhibition is particularly effective in preventing AF. Whether a personalized and AF mechanism-guided selection of AF patients with concomitant OSA may translate in a better antiarrhythmic response and rhythm control upon pharmacological $I_{K,ACH}$ inhibition warrants further study.

Study limitations
In this animal model for repetitive obstructive respiratory events, we applied stable and reproducible INAP maneuvers at -50 mbar, which aligns with airway pressure measured in patients with OSA. This translated to airway pressures of about -20 to -25 mbar. Actual pressure in the thorax cavity, however, was not measured. To allow atrial electrophysiological studies throughout the experiment, we worked with spontaneously breathing animals in isoflurane anesthesia. Anesthesia induces a sleep-like state but cannot resemble all physiological features of sleep. Oxygen saturation was acquired by oximetry, which recorded intermittent signal losses during the end of obstructive respiratory events owing to potential peripheral vasoconstriction, which might have impacted the reliability of desaturation measurements. The described reversibility of INAP-induced changes of AERP and PKC, in particular in respect to the time dependency, might be rodent specific and attributable to increased metabolism faster than that of humans. Even though a translocation of PKC from cytosol to membrane, at a time point at which atrial refractoriness is shortened, suggests an interaction with GIRQ channel subunits, an actual interaction has not been proven and remains speculative. Pronounced increases in AERP owing to atropine and XAF-1407 could have masked INAP-related AERP shortening. Moreover, AERP measurements were acquired from the RA, whereas biochemical analysis was performed on the left atria. Atropine could have had effects on multiple muscarinic receptors and the arrhythmogenic contribution of different muscarinic receptors was not investigated in this study. AF inducibility was only determined in the second half of the protocol in order to evaluate AF susceptibility at a time point where atrial refractoriness was most impacted. A baseline evaluation and thus a clear paired comparison of AF inducibility prior to the INAP protocol is missing. This was not performed owing to lacking means of cardioversion in this presented set-up and potentially altered electrophysiology. Short periods of induced AF were measured, but clinical relevance of such short episodes remains limited and potentially insignificant. It has been reported, though, that AF durations do increase also in rodent models when considering and simulating AF comorbidities; this, however, was beyond the scope of this article. Electrophysiological properties and selectivity of XAF-1407 were analyzed in HEK293 cells, in which the compound selectively inhibited $K_{\alpha,3.1/3.4}$ and $K_{\alpha,3.4/3.4}$. However, this does not necessarily provide evidence for selectivity in native rodent or human cardiomyocytes.

Conclusion
Acute obstructive respiratory events create a transient arrhythmogenic substrate characterized by a shortening in AERP and increased AF susceptibility in rats. Selective pharmacological $I_{K,ACH}$ inhibition may represent a promising treatment strategy for AF patients with OSA. Additional preclinical studies in large animal models and clinical intervention studies are warranted to confirm the beneficial effects of $I_{K,ACH}$ inhibition seen in this rodent study on OSA-related atrial arrhythmogenesis.

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Authorship: All authors attest they meet the current ICMJE criteria for authorship.

Ethics Statement: This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (eighth edition; revised 2011) and to the ARRIVE guidelines. Moreover, all animal experiments were executed in accordance with the Danish Animal Experiment Inspectorate (case number 2017-15-0201-01231).

Data Availability Statement: The data underlying this article are available in the article and in its online supplementary material.

Appendix

Supplementary data
Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.hrroo.2021.11.013.

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