Ouabain stimulates slowly adapting pulmonary stretch receptors

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Abstract: Ouabain, a Na+/K+ -ATPase inhibitor, induces slowly adapting pulmonary stretch receptors (SARs) to discharge paradoxically. Paradoxical discharge is characterized by increased SAR activity during lung deflation coupled with silence during lung inflation. We hypothesized that over-excitation silences the SARs. Accordingly, if cyclic inflation pressure was reduced so as to lower SAR stimulation, paradoxical cycling would be prevented. In the present study, single-unit activity of SARs was recorded in anesthetized, open-chest and mechanically ventilated rabbits with positive-end-expiratory pressure (PEEP). After microinjection of ouabain into the receptive field, paradoxical discharge would be prevented. In the present study, single-unit activity of SARs was recorded in anesthetized, open-chest and mechanically ventilated rabbits with positive-end-expiratory pressure (PEEP). After microinjection of ouabain into the receptive field, SAR activity initially increased and then gradually became paradoxical. During paradoxical cycling, SAR activity started and stopped abruptly, oscillating between high frequency discharge during lung deflation and silence during peak inflation. Removing PEEP reduced basal cyclic stimulation and returned the discharge pattern to normal, that is, SAR activity was highest at peak inflation pressure but silent during deflation. It is speculated that stretching SARs causes Na+ influx, producing generator potential (GP). Normally, GP recovers by Na+ extrusion via Na+ /K+ -ATPase. Ouabain inhibits the ATPase, which limits Na+ extrusion, and thus sustains the GP. Therefore, after ouabain microinjection, lung inflation will further increase GP, causing over-excitation to silence the SARs.

Key words: vagus nerve; lung; reflex; mechanoreceptor; afferent nerve

The contribution of the sensory vagal nerves to the control of breathing has been investigated for almost half a century. While slowly adapting pulmonary stretch receptors (SARs) have not escaped scrutiny[1], the cellular mechanisms that prompt their activation remain to be defined. Using anesthetized rabbits, Matsumoto et al.[10] observed that some...
SARs discharge in a paradoxical manner after Na⁺/K⁺-ATPase blockade with intravenously injected ouabain. SAR activity increased during deflation and ceased during inflation. This reversal in SAR activity was attributed to a reduction of transmembrane Na⁺ gradient due to intracellular Na⁺ accumulation. In other words, ouabain suppresses SAR responsiveness by an inhibitory process, rendering the SAR less excitable. Yet if inhibition causes the paradoxical discharge, SAR activity should also be repressed during deflation, but SAR activity increases instead. Note that the paradoxical discharge of the SARs occurs on the order of minutes, whereas running down the Na⁺ gradient usually takes much longer, so that it is unlikely that the increased SAR activity during deflation is caused by dissipation of the Na⁺ gradient. Additionally, ouabain is known to lower activation thresholds and stimulate stretch receptors, such as renal pressure mechanoreceptors\(^{20}\), atrial stretch receptors\(^{20}\), and baroreceptors\(^{21}\). In light of these known properties, it seems that a mechanism other than inhibition is operating. We hypothesized that paradoxical SAR discharge induced by ouabain is not due to an inhibitory process, but rather to receptor over-excitation. Accordingly, a microinjection technique was used to study transduction properties of airway receptors in intact animals\(^{17}\). Microinjection permits the focused use of chemicals in higher concentrations than achievable by intravenous injection or aerosol inhalation. This method is similar to the topical application of chemicals used to study sensory properties in other visceral organs. This technique has been used in other studies to apply high concentrations of hydrogen peroxide and bradykinin directly to receptive fields to investigate lung reflexes\(^{21}\). The present study examines SAR responses to lung mechanical changes before and after ouabain microinjection to block Na⁺/K⁺-ATPase at the receptive field. Our data support the hypothesis that the cessation of SAR activity during inflation is due to receptor over-excitation.

1 MATERIALS AND METHODS

1.1 General

Experiments were carried out in 19 male New Zealand white rabbits anesthetized with 20% urethane (1 g/kg, i.v.). Study procedures were in accordance with ethics codes set by the NIH and approved by the IACUC of the University of Louisville. A midline incision was made to expose the trachea and vagus nerve. The trachea was cannulated low in the neck and the lungs were mechanically ventilated with a Harvard ventilator (Model 683, South Natick, MA). Positive-end-expiratory pressure (PEEP) was maintained by placing the expiratory outlet under 3–4 cm H₂O. Airway pressure was monitored at the tracheal tube with a pressure transducer (Statham P23). The chest was opened widely in the midline to allow locating the receptive field for single unit recording (see below). Receptor responses to cyclic changes in airway pressure, PEEP removal, and lung inflation were examined before and after microinjection of 1 µmol/L ouabain (Sigma Chemical). The agent was injected into the receptive field with a needle (30 GD) in a volume of 10 to 20 µl. Airway pressure and afferent activities were recorded by a thermorecorder (Astro-Med Dash IV).

1.2 Recording of afferent activity

Single-unit activities from the vagal afferent were recorded according to conventional methods\(^{19}\). The vagus nerve (either right or left) was separated from the carotid sheath, placed on a dissecting platform, and covered with mineral oil. A small afferent bundle was cut from the vagus nerve, leaving the main trunk intact. This bundle was dissected into thin filaments with two pairs of forceps. The filaments were further divided and placed on recording electrodes to measure action potentials. The electrodes were connected to a High Impedance Probe (Grass Model HIP 511), from which the output was fed into an amplifier (Grass P 511). After suitable amplification, action potentials from a single-unit of the vagal sensory receptors were displayed on an oscilloscope and monitored by a loudspeaker. In addition, a voltage analogue of impulse frequency was produced by a rate meter (Frederick Haer, Brunswick, ME) at a band width of 0.1 s. Adaptation rate was determined by an adaptation index\(^{17,14}\). The receptive field was initially located by gently exploring the external surface of the lungs with a cotton tip and then more precisely with a glass rod having a 0.5 mm round tip. Specifically, the receptor field is located by identifying the most sensitive point on the lung surface. At the center of this point, touching elicited a high discharge frequency, which can be easily identified by listening to the monitor and hearing a high pitch burst of action potentials. This response subsided, as the distance away from the center increased. Among nineteen rabbits, a total of 124 SARs were located. Of these, 38 were high-threshold in type\(^{15}\) and their receptive fields could be easily identified in the lung periphery for ouabain injection. These SARs characteristically discharged during lung inflation and then became silent during deflation. Activity decreased during PEEP removal with an adaptation index of less than 20% at 20 cm H₂O during constant pressure inflation.
2 RESULTS

2.1 General
Thirty-eight typical, high-threshold SARs located in the peripheral airways were examined. Twenty-four were immediately stimulated by microinjection of ouabain (1 µmol/L, 20 µl), 8 were stimulated after a long latency period (1 to 3 min), and 6 were not stimulated. The 8 SARs having the long latency period were excluded from data analysis, based on the belief that their receptive fields had not been precisely identified. The 6 non-responding SARs were re-examined after a second injection of ouabain, and all 6 responded in a manner consistent with the 24 SARs that responded to the first ouabain injection. Data from these 6 responders were combined with the 24, giving a total of 30 SARs. After microinjection of ouabain, high-threshold SARs increased their activity and altered their discharge pattern. The latency of stimulation was short [(8.4 ± 1.1) s, n=30], ranging from 1 to 20 s. Once influenced by ouabain, SARs progressed through three identifiable phases: tonic, paradoxical, and irregular.

2.2 Tonic phase
In the tonic phase, the cyclic discharge pattern was dampened, and the SARs discharged continually at high frequencies during lung inflation and deflation (Fig. 1). The receptor response to mechanical stimulation was greatly reduced. The SARs discharged continuously during PEEP removal. This tonic phase lasted for (580 ± 85) s, with a range of 64 to 1370 s. Seventeen of 30 SARs entered the second, paradoxical phase.

2.3 Paradoxical phase
The paradoxical phase is characterized by a high discharge frequency during lung deflation followed by an absence of firing during inflation. The oscillating discharge had a one-to-one relationship to the ventilator cycle. Discharge of the receptors switched ‘on’ and ‘off’ abruptly. In 9 of 17 SARs that expressed a paradoxical phase, we tested the response to PEEP removal. Activity of all 9 receptors shifted from a paradoxical pattern to a normal pattern during PEEP.

Fig. 1. Afferent activity of a slowly adapting receptor (SAR) recorded from an anesthetized, open-chest and artificially ventilated rabbit. The traces are: IMP/s, impulse activity per second, counted every 0.1 s; IMP, impulses (afferent activity); Paw, airway pressure. A: Control. The arrows above A denote gentle touching of the receptive field. B: Injection of ouabain into the receptive field, stimulated the SAR immediately and initiated tonic discharge. The arrow denotes the time of ouabain injection. C: Ninety seconds after injection, the SAR displayed tonic discharge. No substantial change in activity was observed during either inflation or deflation. D: Ten minutes after injection, upon lung inflation the SAR responded with a short surge discharge followed by silence. After pressure release, the SAR discharge became paradoxical, i.e., the receptor discharged during deflation and ceased activity during inflation. Note that receptor activity stops and starts abruptly, suggesting the sensory endings oscillate between extreme excitation and cessation.
removal; i.e., receptor activity increased during inflation and decreased during deflation (Fig. 2). Comparing the peak discharges of the SARs during PEEP and during PEEP removal, the discharge frequency was lower during PEEP removal [(55±10) imp/s] than during PEEP [(62±9) imp/s, n=9, P=0.0114], except in one case (Fig. 3). It is worth

Fig. 2. Comparison of SAR response to changes in lung mechanics before and after microinjection of ouabain. A, B: Controls. C, D, E: Records during the paradoxical phase. C was 15 min after ouabain injection. D was a chronological continuance of C. E was 5 min after D. Note that in the paradoxical phase, the SAR discharged during deflation and ceased discharge during inflation (D). During positive-end-expiratory pressure (PEEP) removal (C), which is indicated by the black bracket under the figure, the SAR reverted to a normal pattern. It discharged continuously and became more active during inflation. Constant pressure inflation ceased discharge completely (D and E). This SAR became silent after lung inflation in E. Note that the peak activity at lung inflation during the period of PEEP removal is less than the peak activity with PEEP. This observation supports the contention that the normal discharge pattern exists during PEEP removal because the generator potential does not reach the inactivation level.

Fig. 3. Illustration of a SAR that discharged only during inflation after PEEP removal at the paradoxical phase. A, B: Controls. C, D: Records after injection of ouabain. This is the only SAR whose peak activity during PEEP removal seems higher than that during PEEP ventilation (C). However, the peak frequency at the SAR inactivation could be higher (D). Please note that it still holds true that the threshold for the SAR to discharge is lowered after ouabain injection (comparing A and C during PEEP removal). Again, this indicates SAR stimulation instead of inhibition.
noting that general activity of the receptors declined with

time (Fig.2). The paradoxical phase was short-lived, last-
ing for (124±22) s, followed by an irregular phase. Thir-
eteen of the 30 SARs progressed directly into the irregular

phase, bypassing the paradoxical phase.

2.4 Irregular phase

After entering the irregular phase, some SARs were

inactivated, while others exhibited an irregular discharge

pattern. During the irregular phase, 73% of the SARs (22

out of 30) ceased activity, whereas the remaining 8 SARs

had oscillation patterns unrelated to the ventilator cycle.

The irregular discharge pattern was characterized by high

frequency discharge alternating with absence of discharge.

This irregular discharge pattern is similar to the paradoxi-
cal discharge pattern, in which the receptor discharge is

abruptly either ‘on’ or ‘off’. Figure 4 illustrates the irregu-
lar pattern. The alternating firing was unrelated to the ven-
tilator cycle. The SARs did not respond to lung inflation or
deflation. In two cases, however, removing PEEP during
silence (the irregular phase) resulted in a reversion to the
paradoxical discharge pattern (Fig.5).

2.5 Controls

In order to eliminate the possibility that the alterations in
discharge patterns from the SARs were due to physical
stimulation or volume distention after injecting ouabain,
control experiments were carried out in five SARs. In
these receptors, 0.9% NaCl was injected into the receptive
field instead of ouabain. Normal saline did not cause any
change in receptor behavior in these control SARs. Figure
6 is an illustration of the control SARs.

![Fig. 4. Illustration of the irregular phase of SAR in response to ouabain injection. A, B: Traces from two SARs recorded 22 and 21 min after
injecting ouabain, respectively. Please note that the SAR discharge bursts had varied duration and occurred during all ventilatory phases. C, D,
E: Records from a different SAR 46 min after ouabain injection. In contrast to traces A and B, this SAR had a uniform discharge period and
duration (C). However, like the other two SARs in A and B, the SAR bursts were not affected by lung inflation (D) or PEEP removal (E).]

![Fig. 5. Illustration of SAR activity 4 min after ouabain injection. This trace shows a transition in SAR activity from a tonic phase through a brief
paradoxical phase and into a silent phase. When PEEP was removed at this early silent stage (indicated by the bracket), SAR activity resumed,
although in a paradoxical pattern. Restoration of PEEP inactivated the SAR.]

3 DISCUSSION

These findings show that the paradoxical activity of SARs
induced by ouabain (silent during lung inflation but active
during deflation) can revert to the normal pattern after re-
ducing SAR stimulation by removing PEEP. Therefore, the
paradoxical discharge is most likely caused by over-excitatio,
as opposed to inhibition. In addition, this study contributes to the assessment of generator potential (GP) by indirect means. The response of SARs to microinjection of ouabain (1 µmol/L, 20 µl) into the receptive field can be divided into three phases: (1) immediate excitation manifested by tonic activity, (2) paradoxical discharge, and (3) irregular or cessation of discharge. Although the underlying cause of the alteration in discharge patterns is unknown, it is reasonable to infer that they arise out of the progression from a low excitation state to a high excitation state.

SARs are mechanosensors innervated by the vagus nerves; however, the exact mechanisms of their activation and sensory transduction are unknown[1]. SAR activation may find support in some mechanosensors, including the baroreceptors of the arterial wall[3]. In baroreceptors, degenerin/epithelial sodium channels (ENaC) are responsible for mechanical signal transduction. These receptors are sensitive to amiloride, an inhibitor of passive carrier facilitated Na+ transport. Similarly, mechanoreceptor activation in the spider is amiloride sensitive[6]. While GP can be measured from the stretch receptor in crayfish abdominal muscle[6], it is difficult to record in mammalian visceral organs. In mammals, GP has been extensively investigated in the pacinian corpuscles, the hair cells responsible for hearing and balance, and the muscle spindle[4,12]. Assuming SARs operate in a similar manner, detailed mechanisms for activation of the mechanosensory unit have been proposed[18]. The following is a brief account.

SARs may contain stretch-sensitive ion channels in the sensory terminal membrane. These channels are activated by a deformation force, causing cation influx. Cation entry into the cell induces local depolarization. Summation of these local potentials forms the GP. When the GP reaches threshold, voltage-dependent sodium channels open, causing action potentials. The voltage-dependent sodium channel is responsible for the increase in sodium permeability during the rapid rise of the action potential in SARs, which can be effectively blocked by tetrodotoxin (TTX) locally applied to the receptive field (personal observation). The efflux of K+ ions through the K+ channel, which is sensitive to 4-aminopyridine (4-AP)[9,11], may be responsible for repolarization. Discharge frequency in the sensory axon is proportional to the GP magnitude, i.e., greater stretch induces a larger GP and higher discharge frequency. Thus, the graded response of the receptor (analog signal) is converted into discharge frequency of action potentials (digital signal). Like most excitable cells, SARs must maintain high extracellular sodium and intracellular potassium concentrations by Na+/K+-ATPase or the sodium pump[14]. Local depolarization disturbs the ion concentration across the sensory membrane. The disturbance is restored by the electrogenic Na+/K+-ATPase. The Na+/K+-ATPase pumps 3 Na+ out of the cell in exchange for 2 K+ [14]. Inhibiting Na+/K+-ATPase may prevent or attenuate restoration of GP, that is, it will increase GP and discharge frequency of action potentials, causing repetitive firing[10].

In whole cell patch clamp studies, the relationship be-
between sodium current and membrane potential is U-shaped\[2\]. When the membrane depolarizes, sodium permeability increases along with the sodium current. The sodium current continues to increase with further depolarization. After reaching a peak current, further depolarization decreases the sodium current. Eventually, a point of membrane potential will be reached where the sodium channels close and are unresponsive to further efforts at depolarization. That is, at very positive membrane potentials, the sodium channels are completely inactivated.

If the GP is kept above the firing threshold, the SAR will discharge repetitively. As the lung stretches during inspiration, stretch-sensitive channels open, allowing more Na\(^+\) or other cations to influx, and GP further increases. If the GP is in the range to permit sodium conductance, the receptor fires action potentials. However, when the GP exceeds that range, the receptor will cease firing. This explains activity cessation during inspiration and the sudden increase in discharge frequency immediately before the cessation after ouabain injection. Firing cessation is related to over-excitiation and not an inhibitory process. Over-excitiation also explains the abrupt oscillation of SAR activity. Figure 5 illustrates that reducing stimulation to the SAR during the silent phase may re-activate it. This observation supports a contention that the silent phase is a more excited phase than the paradoxical phase.

While the SAR silence may be due to over-excitiation, the gradual decline in unit discharge after ouabain is not easily explained. During normal activity, SARs may discharge at 200 imp/s\[18\]. In the present study, the peak discharge frequency was approximately 66 imp/s after ouabain, which is lower than the maximal achievable discharge. This indicates that some other mechanism(s) are involved. Our current technique does not allow us to further define the underlying mechanisms. Nevertheless, the silence of the SARs during inspiration is most likely to be caused by stimulation and not by inhibition.

In summary, SAR activity was assessed by altering lung mechanics to provide indirect insight into the cellular mechanisms involved. The results indicate that the paradoxical discharge produced by ouabain is due to over-excitiation, possibly because of an excessive increase in GP. Further attempts to link SAR discharge pattern with GP will facilitate our understanding of receptor activity.

REFERENCES
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