Effective Accentuation of Voltage-Gated Sodium Current Caused by Apocynin (4′-Hydroxy-3′-methoxyacetophenone), a Known NADPH-Oxidase Inhibitor

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Abstract: Apocynin (aPO, 4′-Hydroxy-3′-methoxyacetophenone) is a cell-permeable, anti-inflammatory phenolic compound that acts as an inhibitor of NADPH-dependent oxidase (NOX). However, the mechanisms through which aPO can interact directly with plasmalemmal ionic channels to perturb the amplitude or gating of ionic currents in excitable cells remain incompletely understood. Herein, we aimed to investigate any modifications of aPO on ionic currents in pituitary GH3 cells or murine HL-1 cardiomyocytes. In whole-cell current recordings, GH3-cell exposure to aPO effectively stimulated the peak and late components of voltage-gated Na+ current (I\textsubscript{Na}) with different potencies. The EC\textsubscript{50} value of aPO required for its differential increase in peak or late I\textsubscript{Na} in GH3 cells was estimated to be 13.2 or 2.8 \mu M, respectively, whereas the K\textsubscript{D} value required for its retardation in the slow component of current inactivation was 3.4 \mu M. The current–voltage relation of I\textsubscript{Na} was shifted slightly to more negative potential during cell exposure to aPO (10 \mu M); however, the steady-state inactivation curve of the current was shifted in a rightward direction in its presence. Recovery of peak I\textsubscript{Na} inactivation was increased in the presence of 10 \mu M aPO. In continued presence of aPO, further application of rufinamide or ranolazine attenuated aPO-stimulated I\textsubscript{Na}. In methylglyoxal- or superoxide dismutase-treated cells, the stimulatory effect of aPO on peak I\textsubscript{Na} remained effective. By using upright isosceles-triangular ramp pulse of varying duration, the amplitude of persistent aPO-stimulated I\textsubscript{Na} measured at low or high threshold was enhanced by the aPO presence, along with increased hysteretic strength appearing at low or high threshold. The addition of aPO (10 \mu M) mildly inhibited the amplitude of erg-mediated K+ current. Likewise, in HL-1 murine cardiomyocytes, the aPO presence increased the peak amplitude of I\textsubscript{Na} as well as decreased the inactivation or deactivation rate of the current, and further addition of ranolazine or esaxerenone attenuated aPO-accentuated I\textsubscript{Na}. Altogether, this study provides a distinctive yet unidentified finding that, despite its effectiveness in suppressing NOX activity, aPO may directly and concertedly perturb the amplitude, gating and voltage-dependent hysteresis of I\textsubscript{Na} in electrically excitable cells. The interaction of aPO with ionic currents may, at least in part, contribute to the underlying mechanisms through which it affects neuroendocrine, endocrine or cardiac function.

Keywords: apocynin (4′-Hydroxy-3′-methoxyacetophenone); NADPH-dependent oxidase (NOX); voltage-gated Na+ current; persistent Na+ current; erg-mediated K+ current; current kinetics; voltage-dependent hysteresis; electrically excitable cell

1. Introduction

Apocynin (aPO, 4′-Hydroxy-3′-methoxyacetophenone), a polyphenolic compound, is a naturally occurring ortho-methoxy-substituted catechol isolated from a variety of plant sources, including Apocynum cannabinum, Pierorhiza kurroa, and so on [1]. Of note, this compound has been widely used as a selective inhibitor of NADPH-dependent oxidase (NOX) [2–5]. Alternatively, it has been recognized to be one of the most promising drugs in
a variety of pathophysiological disorders, such as inflammatory and neurodegenerative
diseases, glioma, and cardiac failure [1,3,5–11]

*aPO* has been recently shown to ameliorate cardiac function (e.g., structural remodeling) in heart failure [6,7,11–13]. Pituitary cells were previously demonstrated to be expressed in the activity of NOX [14,15]. *aPO* has been reported to blunt the progression of neuroendocrine alterations induced by social isolation, which were thought to be mainly through its inhibition of NOX activity [16]. However, whether *aPO* exercises any modifications on ionic currents remains largely unknown.

The voltage-gated Na⁺ (Naᵥ) channels, nine subtypes of which are denoted Naᵥ1.1 through Naᵥ1.9, belong to the larger protein superfamily of voltage-dependent ion channels and their activity plays an essential role in the generation and propagation of action potentials (APs) in electrically excitable cells. The Naᵥ channels contain four homologous domains (DI-DIV), each of which consists of a six α-helical transmembrane domain (S1–S6) and a reentry P loop between S5 and S6. Naᵥ1.5 channels primarily underlie AP initiation and propagation in the heart, these channels have also been shown to be critical determinants of AP duration, particularly in the setting of certain arrhythmias (e.g., LQT-3 syndrome) [17,18]. Previous studies have demonstrated the ability of *aPO* to attenuate angiotensin II-induced activation of epithelial Na⁺ channels in human umbilical vein endothelial cells as well to blunt activation of these channels caused by epidermal growth factor, insulin growth factor-1 or insulin [19,20]. However, the issue of how *aPO* or other related compounds could perturb the amplitude or kinetic gating of transmembrane ionic currents (e.g., voltage-gated Na⁺ current [I₉Na]) still remains unmet.

Therefore, in the present study, the electrophysiological effects of *aPO* and other related compounds in pituitary GH₃ cells and in HL-1 atrial cardiomyocytes were investigated. We sought to (1) evaluate whether the *aPO* presence has any effect on the amplitude, gating and voltage-dependent hysteresis (Vhys) of I₉Na residing in GH₃ cells; (2) compare the effect of other related compounds on the peak amplitude of I₉Na; (3) study the effect of *aPO* on erg-mediated K⁺ current in GH₃ cells; and (4) investigate the effect of *aPO* on I₉Na in HL-1 cardiomyocytes. Findings from this study, for the first time, provide distinctive evidence to show that, in addition to its effectiveness in suppressing NOX activity, the differential stimulation by *aPO* of peak and late I₉Na may be engaged in varying ionic mechanisms underlying its perturbations on the functional activities of electrically excitable cells (e.g., GH₃ or HL-1 cells).

2. Materials and Methods

2.1. Chemicals, Drugs and Solutions Used in the Present Work

Apocynin (*aPO*, NSC 2146, NSC 209524, acetovanillone, acetoguaiacone, 4′-Hydroxy-3′-methoxyacetophenone, 1-(4-Hydroxy-3-methoxyphenyl)ethanone, C₉H₁₀O₃, CAS number: 498-02-2, https://pubchem.ncbi.nlm.nih.gov/compound/Acetovanillone (accessed on 16 September 2004)), methylglyoxal (MeG, acetylformaldehyde, pyruvaldehyde, pyruvic aldehyde), norepinephrine, superoxide dismutase (SOD), tefluthrin (Tef), tetrothylammonium chloride (TEA), and tetrodotoxin (TTX) were acquired from Sigma-Aldrich (Merck, Taipei, Taiwan), rufinamide (RFM, 1-[(2,6-difluorophenyl]-1H-1,2,3-triazole-4-carboxamide), E-4031 and ranolazine (Ran) were from Tocris (Union Biomed, Taipei, Taiwan), and esaxerenone (ESAX) was from MedChemExpress (Gene-chain, Kaohsiung, Taiwan). Unless noted otherwise, culture media (e.g., F-12 medium), horse serum, fetal bovine or calf serum, L-glutamine, and trypsin/EDTA were purchased from HyCloneTM (Thermo Fisher Scientific, Tainan, Taiwan), while all other chemicals were of laboratory grade and taken from standard sources.

The HEPES-buffered normal Tyrode’s solution used in this work had an ionic composition, comprising (in mM): NaCl 136.5, KCl 5.4, CaCl₂ 1.8, MgCl₂ 0.53, glucose 5.5, and HEPES 5.5, and the pH was adjusted with NaOH to 7.4. For measurements of I₉Na or I₉Na(P), we kept GH₃ or HL-1 cells immersed in Ca²⁺-free, Tyrode’s solution in attempts to avoid the contamination of Ca²⁺-activated K⁺ currents and voltage-gated currents. To record K⁺
currents, we filled up the recording pipette with a solution containing (in mM): K-aspartate 130, KCl 20, KH$_2$PO$_4$ 1, MgCl$_2$ 0.5, Na$_2$ATP 3, Na$_2$GTP 0.1, EGTA 0.1, HEPES 5, and the pH was titrated to 7.2 by adding KOH, while to measure $I_{Na}$ or $I_{Na(P)}$, we substituted K$^+$ ions in internal pipette solution for equimolar Cs$^+$ ions and the pH in the solution was adjusted to 7.2 by adding CsOH. All solutions used in this study were prepared using demineralized water from Milli-Q purification system (Merck). On the day of experiments, we filtered the bathing or filling solution and culture medium by using Acrodisc® syringe filter with a 0.2-µm pore size (Bio-Check, Tainan, Taiwan).

2.2. Cell Preparations

These are provided in the Supplemental Materials mentioned in previous studies [21,22].

2.3. Electrophysiological Measurements

Shortly before experiments, we dispersed cells with 1% trypsin/EDTA solution and an aliquot of cell suspension was quickly placed in a custom-built chamber affixed to the stage of a CKX-41 inverted microscope (Olympus; Taiwan Instrument, Tainan, Taiwan). Ionic currents in GH3 or HL-1 cells were measured with an RK-400 operational patch-clamp amplifier (Bio-Logic, Claix, France) or an Axopclamp-2B amplifier (Molecular Devices, Sunnyvale, CA, USA), which was equipped with a Digidata 1440A device (Molecular Devices). Ionic currents were recorded in whole-cell or cell-attached configuration of the patch-clamp technique [23,24]. By using a PP-830 vertical puller (Narishige; Taiwan Instrument, Taipei, Taiwan) or a Flaming-Brown P97 horizontal puller (Sutter, Novato, CA, USA), the recording pipettes were pulled from Kimax-51 (#34500) borosilicate glass capillaries (Kimble; Dogger, New Taipei City, Taiwan), and they had tip resistances of 3–5 MΩ in situations when filled with internal pipette solutions stated above. All measurements were undertaken at room temperature (20–25 °C) on the stage of an inverted DM-II fluorescence microscope (Leica; Major Instruments, Kaohsiung, Taiwan). Data acquisition with varying voltage-clamp waveforms (i.e., analog-to-digital and digital-to-analog) was performed using the pClamp 10.7 software suite (Molecular Devices). The liquid junction potentials were zeroed immediately before seal formation was made, and the whole-cell data were corrected.

The signals were monitored and digitally stored on-line at 10 kHz in an ASUS ExpertBook laptop computer (P2451F; Yuan-Dai, Tainan, Taiwan). During the measurements, the Digidata 1440A was operated using pClamp 10.7 software run on Microsoft Windows 7 (Redmond, WA, USA). The laptop computer was placed on the top of an adjustable Cookskin stand (Ningbo, Zhejiang, China) to enable efficient operation during the measurements.

2.4. Whole-Cell Data Analyses

To determine concentration-dependent stimulation of apocynin on the transient (peak) or late $I_{Na}$, we kept cells bathed in Ca$^{2+}$-free Tyrode’s solution. During the measurements, we voltage-clamped the examined cell at −80 mV and the brief depolarizing pulse to −10 mV was applied to evoke $I_{Na}$. The late $I_{Na}$ in response to 100 µM aPO was taken as 100% and those (i.e., peak and late $I_{Na}$) during exposure to different aPO concentrations (0.3–30 µM) were thereafter compared. The concentration-response data for stimulation of peak or late $I_{Na}$ in pituitary GH3 cells were least-squares fitted to the Hill equation. That is,

$$\text{percentage decrease(%) } = \frac{E_{max}\times[aPO]^{nH}}{EC_{50}^{nH} + [aPO]^{nH}}$$

In this equation, $[aPO]$ is the aPO concentration used, $n_H$ the Hill coefficient, $EC_{50}$ the concentration needed for a 50% inhibition of peak or late $I_{Na}$ and $E_{max}$ the maximal stimulation of peak or late $I_{Na}$ caused by the addition of aPO.$k$. 

The stimulatory effect of aPO on $I_{Na}$ is thought to be explained by a state-dependent activator that binds preferentially to the open state of the NaV channel. From a simplifying assumption, the first-order binding scheme was given as follows:

$$
\begin{array}{c}
C & \xrightarrow{\alpha} & \beta \\
O & \xrightarrow{k_+} & \{aPO\} \\
O\cdot\{aPO\} & \xleftarrow{k_-} & 0
\end{array}
$$

or

$$
\frac{dC}{dt} = O \times \beta - C \times \alpha
$$

$$
\frac{dO}{dt} = C \times \alpha + O \times [aPO] \times k_+ - O \times \beta - O \times k_+ [aPO]
$$

$$
\frac{d(O \cdot [aPO])}{dt} = O \times k_+ [aPO] - O \cdot [aPO] \times k_-
$$

where $[aPO]$ is the aPO concentration applied, and $\alpha$ or $\beta$ the voltage-gated rate constant for the opening or closing of the Na$_V$ channels, respectively. $k_+$ or $k_-$ represents the forward (i.e., on or bound) or reverse (i.e., off or un-bound) rate constant of aPO, respectively, while C, O, or O·[aPO] in each term denotes the closed (resting), open, or open-[aPO] state, respectively.

Forward or backward rate constants, $k^*_+$ or $k^*_-$, were respectively determined from the time constants of current decay activated by the brief step depolarization from $-80$ to $-10$ mV. The time constants of $I_{Na}$ inactivation were estimated by fitting the inactivation trajectory of each current trace with a double exponential curve (i.e., fast and slow components of current inactivation). These rate constants would be evaluated using the following equation:

$$
\frac{1}{\Delta \tau} = k^*_+ [aPO] + k_-
$$

where $k^*_+$ or $k_-$, respectively, are ascribed from the slope or from the y-axis intercept at $[aPO] = 0$ of the linear regression in which the reciprocal time constant (i.e., $1/\Delta \tau$) versus varying aPO concentrations was interpolated. $\Delta \tau$ indicates the difference in the slow component of current inactivation ($\tau_{\text{inact(S)}}$) obtained when the $\tau_{\text{inact(S)}}$ value during exposure to each concentration (0.03–30 μM) was subtracted from that in the presence of 100 μM aPO (Figure 1C).

The quasi-steady-state inactivation curve of peak $I_{Na}$ with or without the aPO addition identified in GH$_3$ cells was established on the basis of a simple Boltzmann distribution (or the Fermi–Dirac distribution):

$$
I = \frac{I_{\text{max}}}{1 + e^{\frac{(V-V_{1/2})st}{RT}}}
$$

where $I_{\text{max}}$ is the maximal peak $I_{Na}$ in the absence or presence of 10 μM aPO; $V$ the conditioning potential in mV; $V_{1/2}$ the half-maximal inactivation in the relationship of the curve; $q$ the apparent gating charge; $F$ Faraday’s constant; $R$ the universal gas constant; and $T$ the absolute temperature.
Figure 1. Effect of aPO on the peak and late components of voltage-gated Na⁺ current (I_{Na}) identified in pituitary GH3 cells. These experiments were undertaken in cells bathed in Ca²⁺-free Tyrode's solution containing 10 mM tetraethylammonium chloride (TEA), whereas the recording pipette was filled up with Cs⁺-enriched solution. (A) Representative I_{Na} traces activated by brief depolarizing pulse (indicated in the upper part). a: control (i.e., aPO was not present); b: 3 µM aPO; c: 10 µM aPO. (B) Concentration-dependent stimulation of aPO on peak or late I_{Na} (mean ± SEM; n = 8 for each point). The peak (□) or late (■) amplitude of the current was measured at the beginning or end of a 40-ms depolarizing pulse from −80 to −10 mV. Data analysis was performed by ANOVA-1 (p < 0.05). Each continuous line illustrates the goodness-of-fit to the Hill equation, as elaborated in Materials and Methods. The vertical broken line indicates the EC_{50} value required for 50% stimulation of the current (peak or late I_{Na}). (C) The relationship of the reciprocal to the time constant (i.e., 1/τ) versus the aPO concentration was plotted (mean ± SEM; n = 7–11 for each point). From the binding scheme (indicated under Materials and Methods), the forward (k_{+1}) or backward (k_{−1}) rate constant for aPO-accentuated I_{Na} in GH3 cells was computed to be 0.00898 ms⁻¹µM⁻¹ or 0.0303 ms⁻¹, respectively.

2.5. Curve-Fitting Procedures and Statistical Analyses

Curve fitting (linear or non-linear (e.g., exponential or sigmoidal curve)) to various data sets was carried out with the goodness of fit by using various maneuvers, such as the Microsoft “Solver” function embedded in Excel 2019 (Microsoft) and 64-bit OriginPro® 2016 program (OriginLab; Scientific Formosa, Kaohsiung, Taiwan). The data are presented as the mean ± standard error of the mean (SEM), with sample sizes (n) indicating the number of GH3 cells. These experiments were undertaken in cells bathed in Ca²⁺-free Tyrode's solution containing 10 mM tetraethylammonium chloride (TEA), whereas the recording pipette was filled up with Cs⁺-enriched solution. (A) Representative I_{Na} traces activated by brief depolarizing pulse (indicated in the upper part). a: control (i.e., aPO was not present); b: 3 µM aPO; c: 10 µM aPO. (B) Concentration-dependent stimulation of aPO on peak or late I_{Na} (mean ± SEM; n = 8 for each point). The peak (□) or late (■) amplitude of the current was measured at the beginning or end of a 40-ms depolarizing pulse from −80 to −10 mV. Data analysis was performed by ANOVA-1 (p < 0.05). Each continuous line illustrates the goodness-of-fit to the Hill equation, as elaborated in Materials and Methods. The vertical broken line indicates the EC_{50} value required for 50% stimulation of the current (peak or late I_{Na}). (C) The relationship of the reciprocal to the time constant (i.e., 1/τ) versus the aPO concentration was plotted (mean ± SEM; n = 7–11 for each point). From the binding scheme (indicated under Materials and Methods), the forward (k_{+1}) or backward (k_{−1}) rate constant for aPO-accentuated I_{Na} in GH3 cells was computed to be 0.00898 ms⁻¹µM⁻¹ or 0.0303 ms⁻¹, respectively.

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of GH3 or HL-1 cells from which the data were collected. The Student’s t-test (paired or unpaired) and the analyses of variance (ANOVA-1 or ANOVA-2) with or without repeated measures followed by post-hoc Fisher’s least-significant different test were performed. The analyses were performed using SPSS version 20.0 (Asia Analytics, Taipei, Taiwan). A p value of less than 0.05 was considered to indicate the statistical difference.

3. Results

3.1. Effect of aPO on the Voltage-Gated Na+ Current (I_Na) Recorded from Pituitary GH3 Cells

In the first stage of measurements, we kept cells immersed in a Ca^{2+}-free Tyrode’s solution containing 0.5 mM CdCl₂, the composition of which was stated in Materials and Methods, and we filled up the pipette by using the Cs⁺-containing solution. As the whole-cell configuration was firmly established, we voltage-clamped the tested cell at the level of −80 mV and a brief step depolarization to −10 mV was delivered to activate I_{Na} with a rapid activation and inactivation [23,25,26]. Of interest, one minute after cells were whole-cell configuration was firmly established, we voltage-clamped the tested cell at the value of less than 0.05 was considered to indicate the statistical difference.

A

The analyses were performed using SPSS version 20.0 (Asia Analytics, Taipei, Taiwan).

of

aPO potentials during cell exposure to pulse. The EC_{50} on the steady-state inactivation curve of I_{aPO} depicted in Figure 2A, the I-V relationship of I_{Na} was shifted slightly to more negative potentials during cell exposure to aPO (10 µM). Additionally, the stimulatory effect of aPO on the steady-state inactivation curve of I_{Na} was further characterized (Figure 2B).

of

the concomitant inactivation time course of the current slowed (Figure 1A). In the presence of 10 µM aPO, the peak I_{Na} amplitude in response to rapid depolarizing pulse from −80 to −10 mV was significantly increased to 445 ± 31 pA (n = 9, p < 0.05) from a control value of 315 ± 22 pA. Additionally, the slow component of the inactivation time constant of I_{Na} activated by brief membrane depolarization was conceivably prolonged to 65.1 ± 10.2 ms (n = 9, p < 0.05) from a control value of 11.3 ± 2.3 ms (n = 9), although the fast component of the inactivation time constant did not differ significantly between absence and presence of aPO. After washout of aPO, the current amplitude was back to 306 ± 19 pA (n = 8, p < 0.05). Similarly, the deactivation time course of I_{Na} at −50 mV was prolonged in the presence of aPO.

The relationship between the aPO concentration and the peak or late component of I_{Na} was further analyzed and constructed in GH3 cells. Each cell was depolarized from −80 to −10 mV and current amplitudes at different concentrations (0.3–100 µM) of aPO were compared. As can be seen in Figure 1B, the application of aPO resulted in a concentration-dependent increase in peak or late I_{Na} activated by a short depolarizing pulse. The EC_{50} value for aPO-stimulated peak or late I_{Na} was 13.2 or 2.8 µM, respectively, and aPO at a concentration of 100 µM almost fully increased I_{Na}. The data, therefore, reflect that aPO has a specific stimulatory action on I_{Na} in GH3 cells, and that the late component of I_{Na} was stimulated to a greater extent than the peak component of the current.

3.2. Evaluating aPO’s Time-Dependent Slowing of I_{Na} Inactivation

It needs to be mentioned that increasing aPO not only resulted in increased amplitude in the peak I_{Na} but also caused a clear and marked retardation in the magnitude of I_{Na} inactivation in response to rapid membrane depolarization. According to the first-order reaction scheme (indicated under Materials and Methods), the relationship between 1/Δτ and [aPO] turned out to be linear (Figure 1C). The forward and backward rate constants were estimated to be 0.00898 ms⁻¹µM⁻¹ or 0.0303 ms⁻¹, respectively; thereafter, the apparent dissociation constant (i.e., K_D = k_{-1}/k_{+1}) for the binding of aPO to the Na_{v} channels was consequently yielded to be 3.4 µM, a value which was noticeably close to the estimated EC_{50} value for aPO-mediated stimulation of late I_{Na} determined from the concentration-response curve (Figure 1B).

3.3. Effect of aPO on the Current-Voltage (I-V) Relationship or Steady-State Inactivation Curve of I_{Na}

We continued to examine the stimulatory effect of aPO at different membrane potential, and an I-V relationship of I_{Na} without or with the aPO addition was constructed. As depicted in Figure 2A, the I-V relationship of I_{Na} was shifted slightly to more negative potentials during cell exposure to aPO (10 µM). Additionally, the stimulatory effect of aPO on the steady-state inactivation curve of I_{Na} was further characterized (Figure 2B).
In this stage of experiments, a 40-ms conditioning pulse to various membrane potentials (from −120 to +20 mV in 10-mV steps) was delivered to precede the test pulse (40 ms in duration) to −10 mV from a holding potential of −80 mV. Under this experimental protocol, the relationship between the conditioning potentials and the normalized amplitudes of \( I_{\text{Na}} \) with or without the addition of aPO (10 μM) was constructed and properly fitted to a Boltzmann type sigmoidal function (indicated under Materials and Methods) by using a non-linear regression analysis. In the absence and presence of 10 μM aPO, the \( V_{1/2} \) value was noticed to differ significantly (−62.6 ± 1.3 mV (in the control) versus −49.2 ± 1.4 mV (in the presence of aPO); \( n = 7, p < 0.05 \); in contrast, the value of \( q \) (apparent gating charge) did not differ significantly (2.79 ± 0.12 e (in the control) versus 2.82 ± 0.13 e (in the presence of aPO); \( n = 7, p > 0.05 \). Therefore, cell exposure to aPO not only increased the maximal conductance of \( I_{\text{Na}} \), but also shifted the inactivation curve to the rightward direction by approximately 13 mV. However, we found no evident change in the gating charge of the inactivation curve during cell exposure to aPO. As such, it is reasonable to assume that the steady-state \( I_{\text{Na}} \) inactivation curve in the presence of this compound was shifted rightward, with no clear adjustment in the gating charge of this curve.

**Figure 2.** Stimulatory effect of aPO on averaged current-voltage (I-V) relationship (A) and steady-state inactivation curve (B) of \( I_{\text{Na}} \) present in GH3 cells. Cells were kept bathed in Ca\(^{2+}\)-free Tyrode’s solution containing 10 mM TEA. (A) Averaged I-V relationships of \( I_{\text{Na}} \) in the absence (■) and presence (○) of 10 μM aPO (mean ± SEM; \( n = 8 \) for each point). The examined cell was held at −80 mV and the 40-ms voltage pulse ranging from −80 to +40 mV in 10-mV steps was delivered to it. The statistical analyses were undertaken by ANOVA-2 for repeated measures, \( p \) (factor 1, groups among data ken at different level of voltages) < 0.05, \( p \) (factor 2, groups between the absence and presence of aPO) < 0.05, \( p \) (interaction) < 0.05, followed by post-hoc Fisher’s least-significant difference test, \( p < 0.05 \). (B) Effect of aPO on the steady-state inactivation curve of \( I_{\text{Na}} \) taken without (■) or with (○) the addition of 10 μM aPO. In these experiments, the conditioning voltage pulses with a duration of 40 ms to various membrane potentials between −120 and +20 mV were applied from a holding potential of −80 mV. Following each conditioning potential, a test pulse to −10 mV with a duration of 40 ms was delivered to activate \( I_{\text{Na}} \). The normalized amplitude of \( I_{\text{Na}} \) (\( I/I_{\text{max}} \)) was constructed against the conditioning potential and the sigmoidal curves were optimally fitted by the Boltzmann equation (indicated under Materials and Methods). Each point represents the mean ± SEM (\( n = 7 \)). The statistical analyses were undertaken by ANOVA-2 for repeated measures, \( p \) (factor 1, groups among data ken at different level of conditioning potentials) < 0.05, \( p \) (factor 2, groups between the absence and presence of aPO) < 0.05, \( p \) (interaction) < 0.05, followed by post-hoc Fisher’s least-significant difference test, \( p < 0.05 \).
3.4. Effect of aPO on the Recovery from $I_{Na}$ Inactivation by Using Two-Step Voltage Protocol

We then examined whether the presence of aPO produces any effect on the recovery of $I_{Na}$ from inactivation. In a two-step voltage protocol, a 50-ms conditioning step to $-10$ mV inactivated most of the current, and the recovery from current inactivation at the holding potential of $-80$ mV was examined at different times with a test step ($-10$ mV, 50 ms), as demonstrated in Figure 3A,B. In the control period (i.e., aPO was not present), the peak amplitude of $I_{Na}$ nearly completely recovered from inactivation when the interpulse duration was set at 100 ms. The time constant course of recovery from current inactivation in the absence or presence of aPO (10 μM) was least-squares fitted to a single-exponential function with a time constant of 23.3 ± 1.1 or 11.3 ± 0.9 ms (n = 8, p < 0.05), respectively. These experimental observations indicate that cell exposure to aPO produces a significant shortening in the recovery from inactivation of $I_{Na}$ in GH3 cells.

![Figure 3](image_url)

**Figure 3.** Effect of aPO on the time course of recovery from $I_{Na}$ inactivation. The cell tested was depolarized from $-80$ to $-10$ mV with a duration of 50 ms, and voltage-clamp commands with varying durations of interpulse interval (i.e., the interval between the first and second pulses) were applied to it. (A) Superimposed $I_{Na}$ traces in the presence of 10 μM aPO. The upper part shows the voltage protocol applied. The dashed arrow indicates the trajectory of current inactivation elicited by different durations of interpulse pulse. (B) Effect of aPO on the time course of recovery from current inactivation, as the cells examined were depolarized from $-80$ to $-10$ mV. ■: control; ○: aPO (10 μM). Each smooth line was optimally fitted by a single-exponential function. The relative amplitude denotes that the peak $I_{Na}$ taken at the second pulse is divided by that at the first one. Each point represents the mean ± SEM (n = 8). The statistical analyses were undertaken by ANOVA-2 for repeated measures, $p$ (factor 1, groups among data) $< 0.05$, $p$ (factor 2, groups between the absence and presence of aPO) $< 0.05$, $p$ (interaction) $< 0.05$, followed by post-hoc Fisher’s least-significant difference test, $p < 0.05$.

3.5. Comparison among Effects of aPO, Tefluthrin (Tef), Tef Plus aPO, aPO Plus Rufinamide (RFM), and aPO Plus Ranolazine (Ran) on the Peak Amplitude of $I_{Na}$

Tef, a type-I pyrethroid insecticide, was reported to be an activator of $I_{Na}$ [23–25,27]. Ran is recognized as a late $I_{Na}$ blocker as well as an inhibitor of NOX activity [26,28–30], and RFM, known to be an antiepileptic agent, was previously demonstrated to perturb $I_{Na}$ inactivation [31,32]. For these reasons, we further examined and then compared the effects of these agents on peak $I_{Na}$ identified in GH3 cells. As demonstrated in Figure 4, in accordance with previous studies [23], one minute after Tef (10 μM) was
applied, it was effective in stimulating peak \( I_{\text{Na}} \). However, in the continued presence of Tef for two minutes, one minute after further addition of 10 \( \mu \)M \( \text{aPO} \), peak \( I_{\text{Na}} \) was not increased further. In addition, as cells were continually exposed to 10 \( \mu \)M \( \text{aPO} \), subsequent application of 10 \( \mu \)M RFM or 10 \( \mu \)M Ran was able to attenuate \( \text{aPO} \)-induced stimulation of \( I_{\text{Na}} \) one minute later. The results imply that \( \text{aPO} \) and Tef share a similarity to their stimulation of \( I_{\text{Na}} \), and that further addition of RFM or Ran is effective in attenuating \( \text{aPO} \)-stimulated \( I_{\text{Na}} \) in \( \text{GH}_3 \) cells.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Effect of \( \text{aPO} \), tefluthrin (Tef), Tef plus \( \text{aPO} \), \( \text{aPO} \) plus rufinamide (RFM), and \( \text{aPO} \) plus ranolazine (Ran) on peak amplitude of \( I_{\text{Na}} \) identified in \( \text{GH}_3 \) cells. (A) Representative \( I_{\text{Na}} \) traces activated by depolarizing pulse (as indicated in the upper part). a: control; b: 10 \( \mu \)M \( \text{aPO} \); c: 10 \( \mu \)M \( \text{aPO} \) plus 10 \( \mu \)M RFM. (B) Summary bar graph showing effect of \( \text{aPO} \), Tef, Tef plus \( \text{aPO} \), \( \text{aPO} \) plus RFM, and \( \text{aPO} \) plus Ran on peak \( I_{\text{Na}} \) (mean ± SEM; \( n = 8 \)–10 for each bar). The number of the control group is 10, while those in other groups are 8. Data analysis was performed by ANOVA-1 (\( p < 0.05 \)). * Significantly different from control (\( p < 0.05 \)) and ** significantly different from \( \text{aPO} \) (10 \( \mu \)M) alone group (\( p < 0.05 \)).

### 3.6. Stimulatory Action of \( \text{aPO} \) on \( I_{\text{Na}} \) in Methylglyoxal- (MeG-) or Superoxide Dismutase- (SOD-) Treated Cells

One would expect that the effect of \( \text{aPO} \) on \( I_{\text{Na}} \) is engaged in either its inhibition of NOX activity or the reduction in the production of reactive oxygen species. The expression of NOX was previously reported to be distributed in pituitary cells \([14,15]\). As such, the effect of \( \text{aPO} \) on \( I_{\text{Na}} \) was assessed in cells preincubated with MeG or SOD for 6 h. MeG was previously recognized to be a substrate for NOX activity \([33–35]\), while SOD, an antioxidative enzyme, was reported to reduce the production of reactive oxygen species \([36]\). However, in \( \text{GH}_3 \) cells preincubated with MeG for 6 h, the I-V relationship of peak \( I_{\text{Na}} \) with or without addition of \( \text{aPO} \) is illustrated in Figure 5. For example, in cells pretreated with MeG (10 \( \mu \)M), \( \text{aPO} \) (10 \( \mu \)M) could significantly increase the amplitude of \( I_{\text{Na}} \) measured at the level of \(-20\) mV from \( 401 \pm 31 \) to \( 511 \pm 39 \) pA (\( n = 7 \), \( p < 0.05 \)). Likewise, in SOD-preincubated cells, the addition of \( \text{aPO} \) (10 \( \mu \)M) increased \( I_{\text{Na}} \) amplitude at \(-20\) mV from \( 409 \pm 31 \) to \( 515 \pm 41 \) pA (\( n = 7 \), \( p < 0.05 \)). Therefore, these results allowed us to suggest that the stimulatory effect of \( \text{aPO} \) on \( I_{\text{Na}} \), that we obtained in these cells is unlikely to be due to changes in either the production of reactive oxygen species or cytosolic NOX activity.
The augmentation of low-threshold $I_{\text{Na}}$ at high or low threshold respectively activated by the upsloping limb, and following the downsloping limb, a clockwise direction (i.e., low-threshold loop) ensued (Figure 6B). Of particular interest, one minute after GH$_3$ cells were exposed to 30 μM aPO alone, the amplitude of $I_{\text{Na(p)}}$ at high or low threshold respectively activated by the upsloping triangular ramp voltage (forward or ascending) or downsloping (backward or descending) limb of upright triangular ramp voltage was increased. The augmentation of low-threshold $I_{\text{Na(p)}}$ produced by 30 μM aPO was observed to be greater than that in the high-threshold one (Figure 6C), for example, as the isosceles-triangular ramp pulse with a duration of 3.2 s (or ramp speed of ±0.1 mV/ms). In the presence of 30 μM aPO, the peak $I_{\text{Na(p)}}$ amplitude measured at the level of −0 mV (i.e., high-threshold $I_{\text{Na(p)}}$) during the ascending phase of triangular ramp pulse was significantly raised to...
$175 \pm 29\ mP (n = 8, p < 0.05)$ from a control value (measured at the isopotential level) of $151 \pm 18\ mP (n = 8)$. Meanwhile, during cell exposure to 30 $\mu M\ aPO$, the peak $I_{Na(P)}$ amplitude measured at $-80\ mV$ during the descending phase of such a ramp concurrently increased from $285 \pm 33$ to $393 \pm 54\ mP (n = 8, p < 0.05)$. Alternatively, the subsequent application of 10 $\mu M\ Ran$, but still in the continued presence of 30 $\mu M\ aPO$, was able to attenuate the $aPO$-mediated increase of $I_{Na(P)}$ taken at either high or low threshold amplitude in the Vhys loop. These observations, therefore, enabled us to indicate that the Vhys strength of $I_{Na(P)}$ activated by isosceles-triangular ramp pulses of varying ramp duration observed in GH3 cells was enhanced in the presence of $aPO$ (Figures 6B,C).

Figure 6. Cont.
The examined cell was voltage-clamped at −80 mV and the isosceles-triangular ramp voltage with varying duration of 0.4 to 3.2 s (i.e., ramp speed of ±0.1 to 0.8 mV/ms) to activate $I_{Na(p)}$ in response to the forward (i.e., ascending from −110 to +50 mV) and backward (descending from +50 to −110 mV) that was thereafter applied to it. (A) Representative $I_{Na(p)}$ traces obtained in the control period (upper, aPO was not present), and during cell exposure to 10 µM aPO (lower). The uppermost part shows varying durations of isosceles-triangular ramp pulse applied. Of notice, the presence of aPO can augment the $I_{Na(p)}$ amplitude elicited by the upsloping and downsloping limbs of the triangular ramp. (B) Representative instantaneous I-V relation of $I_{Na(p)}$ in response to isosceles-triangular ramp pulse (the voltage between −100 and +50 mV) with a duration of 3.2 s (as indicated in the left side of panel (B)). Current trace in the left side is control, while that in the right side was acquired from the presence of 10 µM aPO. The dashed arrows in the left side show the direction of $I_{Na(p)}$ trajectory in which time passes during the elicitation by the upright isosceles-triangular ramp pulse. Of interest, a striking figure-of-eight (or infinity-shaped: ∞) exists in the I-Vhs trajectory responding to the triangular ramp. (C) Summary bar graph demonstrating the effect of aPO and aPO plus Ran on $I_{Na(p)}$ amplitude activated by the upsloping and downsloping limbs of 3.2-s triangular ramp pulse (mean ± SEM; n = 8 for each bar). Current amplitudes in the left side were taken at the level of 0 mV in situations where the 1.6-s ascending (upsloping) end of the triangular pulse was delivered to elicit $I_{Na(p)}$ (i.e., high-threshold $I_{Na(p)}$), while those in the right side (i.e., low-threshold $I_{Na(p)}$) was at −80 mV during the descending (downsloping) end of the pulse. Current amplitude measured is illustrated in the absolute value. Data analyses were performed by ANOVA-1 ($p < 0.05$). * Significantly different from controls ($p < 0.05$) and ** significantly different from aPO (30 µM) alone groups ($p < 0.05$).

3.8. Effect of aPO on Erg-Mediated K+ Current ($I_{K(erg)}$) in GH3 Cells

Earlier studies have demonstrated that telmisartan, an activator of $I_{K(erg)}$ [22]. For this reason, we decided to investigate whether aPO exercises any perturbations on $I_{K(erg)}$. The biophysical and pharmacological properties of $I_{K(erg)}$ in GH3 cells have been previously reported [22,39–41]. In these whole-cell experiments, we bathed cells in high-K+, Ca2+-free solution, and the recording pipette was filled up with K+-containing solution. The composition of these solutions was detailed under Materials and Methods. The examined cell was voltage-clamped at −10 mV and the linear downsloping ramp pulse from −10 to −100 mV with a duration of 1 s was applied to it. As shown in Figure 7, the addition of 10 µM aPO resulted in a progressive decline in the amplitude of deactivating $I_{K(erg)}$ in response to such a downsloping hyperpolarizing ramp. However, in the continued presence of aPO, further application of E-4031, an inhibitor of $I_{K(erg)}$, was
able to decrease the current amplitude further. Therefore, unlike \( I_{Na} \) induced by \( aPO \), \( I_{K(erg)} \) residing in these cells was subject to being inhibited by its presence.

**Figure 7.** Effect of \( aPO \) on erg-mediated K+ current (\( I_{K(erg)} \)) in GH3 cells. The experiments were undertaken in cells that were bathed in high-K\(^+\), Ca\(^{2+}\)-free solution containing 1 \( \mu \)M tetrodotoxin (TTX), and the recording pipette was filled up with K\(^+\)-containing internal solution. (A) Representative \( I_{K(erg)} \) traces obtained in the control (a) and during cell exposure to 10 \( \mu \)M \( aPO \) (b). The examined cell was held at −10 mV and a downsloping ramp from −10 to −100 mV with a duration of 1 s (indicated in the inset) was applied to it. The dashed arrow indicates the direction of current trajectory in which \( \tau \) increase of peak \( I_{K(erg)} \) was noticed to attenuate. * Significantly different from control (\( p < 0.05 \)). ** Significantly different from \( aPO \) (10 \( \mu \)M) alone group (\( p < 0.05 \)).

3.9. Effect of \( aPO \) on \( I_{Na} \) Recorded from Murine HL-1 Cardiomyocytes

\( aPO \) was previously demonstrated to be a chemo-preventive agent for cardiovascular disorders though the inhibition of NOX activity \[35,42–44\]. In another set of experiments, we tested whether \( I_{Na} \) inherently in heart cells (i.e., HL-1 cardiomyocytes) could still be modified by the presence of \( aPO \). The preparation of these cells was described above under Materials and Methods. Cells were kept bathed in Ca\(^{2+}\)-free Tyrode’s solution in which 10 mM TEA was included, and the pipette was filled with Cs\(^+\)-enriched solution. Noticeably, as HL-1 cells were continually exposed to \( aPO \) at a concentration of 3 or 10 \( \mu \)M, the amplitude of peak \( I_{Na} \) activated by 50-ms depolarizing pulses from −80 to −10 mV was increased; concomitantly, progressive slowing of the inactivation time course of the current was seen (Figure 8A,B). For example, cell exposure to 10 \( \mu \)M \( aPO \) resulted in a conceivable increase of peak \( I_{Na} \) from 859 ± 56 to 1381 ± 85 pA (n = 8, \( p < 0.05 \)); concomitantly, the \( \tau_{inact} \) value was significantly raised to 56.3 ± 7.1 ms (n = 8, \( p < 0.05 \)) from a control value of 7.1 ± 1.4 ms. After washout of \( aPO \) (i.e., \( aPO \) was removed, but cells were still exposed to Ca\(^{2+}\)-free Tyrode’s solution containing 10 mM TEA), current amplitude returned 892 ± 58 pA (n = 8, \( p < 0.05 \)). Alternatively, in the continued presence of \( aPO \) (10 \( \mu \)M), further application of either ranolazine (Ran, 10 \( \mu \)M) or esaxerenone (ESAX, 10 \( \mu \)M) was noticed to attenuate \( aPO \)-mediated stimulation of \( I_{Na} \) (Figure 8B). Like Ran, ESAX was recently reported to inhibit \( I_{Na} \) [24]. Therefore, consistent to some extent with the observations done in GH3 cells, the results reflect the effectiveness of \( aPO \) in stimulating \( I_{Na} \) in response to the rapid depolarizing step in HL-1 cells.
4. Discussion

The distinctive findings in the present study are that (a) GH3-cell exposure to aPO could increase $I_{\text{Na}}$ in a concentration, time-, state-, and Vhys-dependent fashion; (b) this agent resulted in the differential stimulation of peak or late amplitude of $I_{\text{Na}}$ by abrupt step depolarization with an effective EC$_{50}$ value of 13.2 or 2.8 μM, respectively; (c) aPO mildly shifted the I-V curve of $I_{\text{Na}}$ towards the depolarized potentials (i.e., a leftward shift), and it also made a rightward shift in the steady-state inactivation curve of the current towards the right side with no changes in the gating charge of the curve; (d) the recovery of the $I_{\text{Na}}$ block was enhanced in its presence; (e) subsequent addition of rufinamide (RFM) or ranolazine (Ran) counteracted aPO-accentuated $I_{\text{Na,er}}$; (f) the stimulatory effect of aPO on $I_{\text{Na}}$ remained unaltered in cells preincubated with MeG or SOD; (g) aPO was capable of increasing the high- or low-threshold amplitude of $I_{\text{Na,p}}$ elicited by the isosceles-triangular ramp at either upsloping (ascending) or downsloping (descending) limb, respectively; (h) the $aPO$ presence mildly decreased the amplitude of $I_{\text{K,er}}$ activated by the downsloping ramp pulse; and (i) the exposure to aPO was effective at increasing the amplitude and inactivation time constant of $I_{\text{Na}}$ in HL-1 atrial cardiomyocytes. Collectively, the present results allow us to reflect that aPO-stimulated changes in the amplitude, gating, and Vhys behavior of $I_{\text{Na}}$ appear to be unlinked to and upstream of its inhibitory action on NOX activity, and that it would participate in the adjustments of varying functional activities in electrically excitable cells (e.g., GH3 or HL-1 cells), presuming that similar in vivo findings exist.

From the overall I-V relationship of $I_{\text{Na}}$ demonstrated here, there was a slight shift toward more negative potential in the presence of aPO. The steady-state inactivation curve of $I_{\text{Na}}$ in its presence of aPO was also shifted to a rightward direction with no apparent change in the gating charge of the curve. The increased recovery of the $I_{\text{Na}}$ block was...
demonstrated in its presence. As a result, the window current of $I_{Na}$ in GH3 cells was expected to be increased during cell exposure to aPO. Such a small molecule may have higher affinity to the open/inactivated state than to the resting (closed) state residing in the Nav channels, despite the detailed ionic mechanism of its stimulatory action on the channel remaining elusive.

Several lines of clear evidence have been demonstrated to indicate that aPO can inhibit NOX activity and decrease the production of superoxide oxide [2–4,16]. Pituitary cells have been previously demonstrated to be expressed in the activity of NOX [14–16]. As such, the question arises as to whether the stimulatory effect of aPO on $I_{Na}$ observed in GH3 cells may actually result from either the reduction of NOX activity or the decreased level of superoxide anions [15,16]. However, this notion appears to be difficult to reconcile with the present observations disclosing that in GH3 cells preincubated with MeG or SOD, the stimulatory effect of aPO on $I_{Na}$ was indeed observed to remain effective. It is also noted that aPO can mildly inhibit the amplitude of $I_{K(erg)}$. Therefore, under our experimental conditions, the stimulation of $I_{Na}$ caused by aPO tends to emerge in a manner largely independent of its inhibitory effect on NOX activity; hence, the aPO molecule can exert an interaction at binding site(s) inherently existing on NaV channels.

Perhaps more important than the issue of the magnitude of the aPO-induced increase in $I_{Na}$ is that we observed the non-linear Vhys of $I_{Na(p)}$ in the control period (i.e., aPO was not present) and during cell exposure to aPO or aPO plus Ran, by use of the upright isosceles-triangular ramp voltage command of varying duration through digital-to-analog conversion. In particular, when cells were exposed to aPO, the peak $I_{Na(p)}$ activated by the forward (ascending or upsloping) end of the triangular ramp of varying duration was observed to be elevated, particularly at the peak level of 0 mV, whereas the $I_{Na(p)}$ amplitude at the backward (descending or downsloping) end was increased at the peak level of −80 mV. In this respect, the figure-of-eight (i.e., infinity-shaped: ∞) configuration in the Vhys loop activated by the triangular ramp pulse was evidently demonstrated (Figure 6A,B). Additionally, there appeared to be two types of Vhys loops, that is, a low-threshold loop with a peak at −80 mV (i.e., activating at a voltage range near the resting potential) and a high-threshold loop with a peak at 0 mV (i.e., activating at a voltage range near the maximal $I_{Na}$ elicited by rectangular depolarizing step. The presence of aPO was capable of enhancing the Vhys strength of $I_{Na(p)}$ and, in its continued presence, further addition of Ran attenuated aPO-increased Vhys loop of the current. In this scenario, findings from the present observations disclosed that the triangular pulse-induced $I_{Na(p)}$ was detected to undergo striking Vhys change (i.e., initial counterclockwise direction followed by clockwise one) in the voltage-dependence and that such Vhys loops were subject to enhancement by the presence of aPO.

Pertinent to the stimulatory effect of aPO on $I_{Na}$ is that in this study, due to its effectiveness in increasing the Vhys magnitude of $I_{Na(p)}$, the voltage-dependent movement of the S4 segment residing in NaV channels is probably perturbed by this agent; consequently, the coupling of the pore domain to the voltage-sensor domain, which the S1–S4 segments comprise, tended to be facilitated [45,46]. Indeed, the voltage sensor energetically coupled to channel activation, which might be influenced by the aPO molecule, is supposed to be a conformationally flexible region of the NaV-channel protein. Therefore, these findings can be interpreted to mean either that such $I_{Na(p)}$, particularly during exposure to aPO, is intrinsically and dynamically endowed with “memory” of previous (or past) events, which is encoded in the conformational (or metastable) states of the Nav-channel protein, or that there is a mode shift of channel kinetics occurring regarding the voltage sensitivity of gating charge movement, which relies on the previous state (or conformation) of the NaV channel [37,38]. Such a striking type of Vhys natively in NaV channels would potentially play substantial roles in interfering with electrical behavior, Na$^+$ overload, and hormonal sretion in varying types of excitable cells [37]. It is also worth pointing out that the subsequent addition of Ran, still in the continued presence of aPO, did produce a considerable reduction in the aPO-mediated increase in Vhys responding to triangular ramp voltage.
From pharmacokinetic studies in mice [47], following intravenous injection of aPO (5 mg/kg), the peak plasma aPO level was detected at 1 min to reach around 5500 ng/mL (or 33.1 μM). Additionally, aPO was reportedly a selective inhibitor of NOX2 activity with an effective IC$_{50}$ of 10 μM [48]. According to the data of Figure 1, the IC$_{50}$ value required for the aPO-stimulated peak or late I$_{Na}$ was 13.2 or 2.8 μM, respectively, while the K$_{D}$ value estimated on the basis of minimal reaction scheme was 3.4 μM. It is reasonable to assume, therefore, that aPO-induced changes in the amplitude, gating or Vhys behavior of I$_{Na}$ presented herein could be highly achievable and of pharmacological relevance.

On the basis of the present experimental observations, despite the inhibitory effect on NOX activity [2–4], our results strongly suggest that the stimulatory actions of aPO on transmembrane ionic currents, particularly on Nav channels, tends to direct obligate mechanisms. Pyrethroids (e.g., permethrin and cypermethrin), known to activate I$_{Na}$, have also been reported to disrupt NOX activity in brain tissue (striatum) [49]. Therefore, through ionic mechanisms shown herein, pyrethroids or other structurally similar compounds are able to adjust the functional activities of varying types of neuroendocrine or endocrine cells, or heart cells, if similar in vivo results exist [6,7,11–13,50]. To this end, the overall findings from our study highlight an important alternative aspect that has to be taken into account, inasmuch as there is the beneficial or ameliorating effect of aPO in various pathologic disorders, such as inflammatory or neurodegenerative diseases, and heart failure [1,3,6,7,9–13,16,42].

**Supplementary Materials:** The details of cell preparation in Materials and Methods were mentioned in Supplementary Material which is available online [https://www.mdpi.com/article/10.3390/biomedicines9091146/s1](https://www.mdpi.com/article/10.3390/biomedicines9091146/s1).

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**Abbreviations**

AP, action potential; aPO (apocynin, 4′-Hydroxy-3′-methoxyacetophenone); EC$_{50}$, concentration required for 50% stimulation; erg, ether-a-go-go-related gene; ESAX, esaxerenone; I-V, current versus voltage; I$_{K(erg)}$, erg-mediated K$^+$ current; I$_{Na}$, voltage-gated Na$^+$ current; I$_{Na(P)}$, persistent Na$^+$ current; K$_{D}$, dissociation constant; MeG, methylglyoxal; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; Nav channel; voltage-gated Na$^+$ channel; NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidase; Vhys, voltage-dependent hysteresis; Ran, ranolazine; RFM, rufinamide; SEM, standard error of the mean; SOD, superoxide dismutase; $\tau_{inact(S)}$, slow component of inactivation time constant; TEA, tetraethylammonium chloride; Tef, tefluthrin; TTX, tetrodotoxin.
26. Chang, W.T.; Wu, S.N. Effectiveness of Columbianadin, a Bioactive Coumarin Derivative, in Perturbing Transient and Persistent I(Na). *Int. J. Mol. Sci.* 2021, 22, 621. [CrossRef]

27. Tan, J.; Soderlund, D.M. Actions of teffluthrin on rat Nav1.7 voltage-gated sodium channels expressed in Xenopus oocytes. *Pestic. Biochem. Physiol.* 2011, 101, 21–26. [CrossRef]

28. Chen, B.-S.; Lo, Y.-C.; Peng, H.; Hsu, T.-I.; Wu, S.-N. Effects of Ranolazine, a Novel Anti-anginal Drug, on Ion Currents and Membrane Potential in Pithitary G3H Cells and NG108-15 Neuronal Cells. *J. Pharmcol. Sci.* 2009, 110, 295–305. [CrossRef]

29. Gupta, T.; Khera, S.; Kolte, D.; Aronow, W.S.; Iwai, S. Antiarrhythmic properties of ranolazine: A review of the current evidence. *Int. J. Cardiol.* 2015, 187, 66–74. [CrossRef] [PubMed]

30. Cappetta, D.; Esposito, G.; Coppini, R.; Piegari, E.; Russo, R.; Ciuffreda, L.P.; Rivellino, A.; Santini, L.; Rafaniello, C.; Scavone, C.; et al. Effects of ranolazine in a model of doxorubicin-induced left ventricle diastolic dysfunction. *Br. J. Pharmcol.* 2017, 174, 3696–3712. [CrossRef]

31. Suter, M.R.; Kirschmann, G.; Laedermann, C.J.; Abriel, H.; Decosterd, I. Rufinamide Attenuates Mechanical Allodynia in a Model of Neuropathic Pain in the Mouse and Stabilizes Voltage-gated Sodium Channel Inactivated State. *Anesthesiology* 2013, 118, 160–172. [CrossRef]

32. Kharatmal, S.B.; Singh, J.N.; Sharma, S.S. Rufinamide Improves Functional and Behavioral Deficits via Blockade of Tetrodotoxin-Resistant Sodium Channels in Diabetic Neuropathy. *Curr. Neurovasc. Res.* 2015, 12, 262–268. [CrossRef]

33. Wintergalen, N.; Thole, H.H.; Gallia, H.-J.; Schliegel, W. Prostaglandin-E2 9-Reductase from Corpus Luteum of Pseudopregnant Rats. *J. Pharmcol. Sci.* 2012, 120, 26–35. [CrossRef]

34. Mukohda, M.; Okada, M.; Hara, Y.; Yamawaki, H. Methylglyoxal Accumulation in Arterial Walls Causes Vascular Contractile Changes, and Oxidative Stress after Neonatal Administration of Pyrethroids. *Front. Physiol.* 2016, 7, 282. [CrossRef]

35. Eid, B.G.; Abu-Sharib, A.T.; El-Bassossy, H.M.; Balamash, K.; Smirnov, S.V. Enhanced calcium entry via activation of NOX/PKC oxygen species and calmodulin kinase II signaling. *Arch. Toxicol.* 2012, 86, 713–723. [CrossRef]

36. Korman, C.E.; Mayergoyz, I.D. On hysteresis of ion channels. *Arch. Toxicol.* 2011, 267, 235–243. [CrossRef]

37. Cannio, R.; D’Angelo, A.; Rossi, M.; Bartolucci, S. A superoxide dismutase from the archaeon Sulfolobus solfataricus is an extracellular enzyme and prevents the deactivation by superoxide of cell-bound proteins. *J. Biol. Inorg. Chem.* 1995, 234, 260–270. [CrossRef] [PubMed]

38. Villalba-Galea, C.A.; Chiem, A.T. Hysteretic Behavior in Voltage-Gated Channels. *J. Physiol.* 2008, 597, 676–689. [CrossRef] [PubMed]

39. Wu, S.N.; Jan, C.-R.; Li, H.-F.; Chiang, H.-T. Characterization of Inhibition by Risperidone of the Inwardly Rectifying K-Channel in Pituitary GH3 Cells. *Neuropsychopharmacology* 2000, 23, 676–689. [CrossRef]

40. Wu, S.N.; Yang, W.-H.; Yeh, C.-C.; Huang, H.-C. The inhibition by di(2-ethylhexyl)-phthalate of erg-mediated K-I(Na). *Br. J. Pharmacol.* 2020, 21, 9369. [CrossRef] [PubMed]

41. Chang, W.-T.; Wu, S.-Y.; Wu, S.-N. High Capability of Pentagalloylglucose (PGG) in Inhibiting Multiple Types of Membrane Ionic Currents. *Int. J. Mol. Sci.* 2020, 21, 5975–5996. [CrossRef] [PubMed]

42. Yu, J.; Weïwer, M.; Linhardt, R.J.; Dordick, J.S. The Role of the Methoxyphenol Apocynin, a Vascular NADPH Oxidase Inhibitor, as a Chemopreventative Agent in the Potential Treatment of Cardiovascular Diseases. *Curr. Drug. Discov. Targets* 2008, 6, 204–217. [CrossRef] [PubMed]

43. Zhao, Z.; Fefelova, N.; Shanmugam, M.; Bishara, P.; Babu, G.J.; Xie, L.-H. Angiotensin II induces afterdepolarizations via reactive oxygen species and calmodulin kinase II signaling. *J. Mol. Cell. Cardiol.* 2011, 50, 128–136. [CrossRef] [PubMed]

44. Muñoz, M.; López-Oliva, M.E.; Rodriguez, C.; Martínez, M.P.; Sáenz-Medina, J.; Sánchez, A.; Climent, B.; Benedito, S.; García-Sacristán, A.; Rivera, L.; et al. Differential contribution of Nox1, Nox2 and Nox4 to kidney vascular oxidative stress and endothelial dysfunction in obesity. *Redox Biol.* 2020, 28, 101330. [CrossRef] [PubMed]

45. Armstrong, C.M.; Bezanilla, F. Currents Related to Movement of the Gating Particles of the Sodium Channels. *Biomedicines* 2021, 9, 1146. [CrossRef] [PubMed]

46. Stühmer, W.; Conti, F.; Suzuki, H.; Wang, X.; Noda, M.; Yahagi, N.; Kubo, H.; Numa, S. Structural parts involved in activation and inactivation of the sodium channel. *Nat. Cell. Biol.* 1999, 339, 597–603. [CrossRef]

47. Liu, F.; Fan, L.M.; Michael, N.; Li, J. In vivo and in silico characterization of apocynin in reducing organ oxidative stress: A pharmacokinetic and pharmacodynamic study. *Pharmacol. Res. Perspect.* 2020, 8, e00635. [CrossRef]

48. Henríquez-Olguín, C.; Díaz-Vegas, A.; Utreras-Mendoza, Y.; Campos, C.; Arias-Calderón, M.; Llanos, P.; Contreras-Ferrat, A.; Espinosa, A.; Altamirano, F.; Jaimovich, E.; et al. NOX2 Inhibition Impairs Early Muscle Gene Expression Induced by a Single Exercise Bout. *Front. Physiol.* 2016, 7, 282. [CrossRef]

49. Nasuti, C.; Gabbianelli, R.; Falconi, M.L.; Di Stefano, A.; Sozio, P.; Cantalamessa, F. Dopaminergic specificity and behavioral changes, and oxidative stress after neonatal administration of pyrethroids. *Toxicology* 2007, 229, 194–205. [CrossRef]