Voltage-independent inhibition of the TTX-sensitive Na⁺ currents by oxotremorine and angiotensin II in rat sympathetic neurons

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Voltage-independent inhibition of Na⁺ currents

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ATP, GTP, TTX, PTX, PLC, PZ, MT-7, BIS-I, IBIS-V, Cal-C

Nonstandard abbreviations used in the paper:

Angio II, angiotensin II; GPCR, G protein coupled receptors; I₉, sodium current; Oxo-M, oxotremorine M; PIP₂, phosphatidylinositol 4,5-bisphosphate; PLL, poly-L-lysine; PTX,
pertussis toxin; SCG, superior cervical ganglion neurons; t_{INa50}, time at 50% of I_{Na}; WMN, wortmannin.
ABSTRACT

TTX-sensitive Na\(^+\) currents have been extensively studied as they play a major role in neuronal firing and bursting. Here, we show that voltage-dependent Na\(^+\) currents are regulated in a slow manner by oxotremorine (Oxo-M) and angiotensin II (Angio II) in rat sympathetic neurons. We found that these currents can be readily inhibited through a signaling pathway mediated by G proteins and PLC\(\beta 1\). This inhibition is slowly established, PTX-insensitive, is partially reversed within tens of seconds after Oxo-M washout and is not relieved by a strong depolarization, suggesting a voltage-insensitive mechanism of inhibition. Specificity of the M1 receptor was tested by the MT-7 toxin. Activation and inactivation curves show no shift on the voltage-dependency under the inhibition by Oxo-M. This inhibition is blocked by a PLC inhibitor (U73122), and the recovery from inhibition is prevented by wortmannin (WMN), a PI3/4 kinase inhibitor. Hence, the pathway involves G\(_{q/11}\) and is mediated by a diffusible second messenger. Oxo-M inhibition is occluded by screening PIP\(_2\) negative charges with poly-L-lysine (PLL) and prevented by intracellular dialysis with a PIP\(_2\) analog. In addition bisindolylmaleimide I (BIS-I), a specific ATP-competitive PKC inhibitor, rules out that this inhibition may be mediated by this protein kinase. Furthermore, Oxo-M-induced suppression of Na\(^+\) currents remains unchanged when neurons are treated with calphostin C (Cal-C), a PKC inhibitor that targets the diacylglycerol-binding site of the kinase. These results support a general mechanism of Na\(^+\) current inhibition that is widely present in excitable cells through modulation of ion channels by specific G protein-coupled receptors (GPCR).
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Introduction

Voltage-gated ion channels are inhibited by a broad range of neurotransmitters or hormones acting through GPCRs. Visionary initial experiments on Na⁺ currents showed them as a feasible target for modulation by neurotransmitter receptors (Cantrell et al., 1996; Mantegazza et al., 2005). Neurotransmitter modulation has been divided into voltage-dependent (reversed by a strong depolarization) and voltage-independent (Hille, 1994). The former modulation has been widely studied, particularly on Ca²⁺ channels. This modulation is mediated by Gβγ subunits diffusing along the plane of the cell membrane to produce a fast, membrane-delimited inhibition (<1 s) (Hille, 1994). In contrast, voltage-independent inhibition shows a slow time course and cannot be reversed by strong membrane depolarization (Vivas et al., 2012). There are a number of agonists that may exert voltage-independent inhibition on voltage-gated ion channels (Suh and Hille, 2002), including Oxo-M, Angio II and substance P. Similar to M current inhibition, voltage-insensitive inhibition of calcium channels is also resistant to pertussis toxin (PTX). This inhibition is slowly established and mediated by a second messenger cascade that include Gq/11 proteins (Berstein et al., 1992). Increasing evidence shows that slow modulation of calcium channels require PIP₂ (Suh et al., 2010). Recently, we showed evidence that PIP₂ is the molecule responsible for the voltage-insensitive inhibition of CaV2.2 channels (Vivas et al., 2013). In addition, we showed that all Gβ subunits exhibit a differential voltage-insensitive component (Hernández-Castellanos et al., 2014). TTX-sensitive sodium currents can be modulated by voltage-sensitive and voltage-insensitive pathways. Pieces of evidence indicate that these currents are inhibited by thyrotropin releasing hormone (Lopez-Barneo
et al., 1990) and that this hormone receptor is coupled to G_q/11 proteins (Aragay et al., 1992). We have found that voltage-independent inhibition of TTX-sodium currents can be elicited by Angio II (Hernández-Ochoa et al., 2007). In addition, voltage-gated Na\(^+\) channels have been shown to be modulated by G proteins (Ma et al., 1994; Saab et al., 2003). However, the mechanism underlying voltage-independent modulation of sodium currents (\(I_{\text{Na}}\)) by GPCR remains unknown. The purpose of this study was to investigate whether a signaling pathway activated by putative neurotransmitters is able to inhibit \(I_{\text{Na}}\) in a voltage-independent fashion. Our results using rat superior cervical ganglion (SCG) neurons show that \(I_{\text{Na}}\) are slowly inhibited by Oxo-M or Angio II, and this inhibition acts through a signaling pathway involving G proteins and phospholipase C (PLC) (Smrcka et al., 1991). It is slow, PTX-insensitive and partially reversibly upon washout. The inhibition is blocked by a PLC inhibitor (U73122), and its recovery from inhibition is prevented by WMN. It is occluded by PLL and prevented by an intracellular dialysis with diC8-PIP\(_2\), a PIP\(_2\) analog. Furthermore, this inhibition cannot be relieved by a strong depolarization indicating that it is voltage-independent. Taking together, these results support a model to explain the modulation of the typically TTX-sensitive voltage-dependent \(I_{\text{Na}}\) by a voltage-independent mechanism, triggered by neurotransmitter receptors and GPCR activation.

Materials and Methods

Cell culture. SCG neurons were isolated from 5-week old male Wistar rats (García-Ferreiro et al., 2001). Rats were obtained from the School of Medicine at the Universidad Nacional Autonoma de Mexico (UNAM) animal breeding facility and were handled according to the Mexican Official Norm for Use, Care and Reproduction of Laboratory Animals (NOM-062-Z00-1999). Rats were euthanized with CO\(_2\) and decapitated using a guillotine. After
Dissection, each ganglion was desheathed and sliced into eight pieces. Whole ganglia tissue was transferred to a tube containing modified Hank’s solution supplemented with 20 U/ml papain. After 20 min at 37 °C, the tissue was transferred to a solution containing 1 mg/ml collagenase type I and 10 mg/ml dispase. Whole ganglia tissue was incubated for 20 minutes before mechanically disaggregating the tissue. The cell suspension was centrifuged at 180 g for 3 minutes, washed twice with Leibovitz’s L-15 medium and once with Dulbecco’s modified Eagle’s medium (DMEM), both supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. Cells were then plated on polystyrene culture dishes coated with poly-L-lysine (≥300,000 MW) and incubated in a humidified atmosphere of 95% air and 5% CO2 at 37 °C. Cells were grown for 12 h after plating before recording. Reagents were obtained as following: L-15 and DMEM (Invitrogen Corp., Carlsbad, CA, USA), U73122 and U73343 (Merck KGaA, Darmstadt, Germany), diC8-PIP2 (Echelon, Salt Lake Cty, UT, USA), MT-7 was obtained from Peptides International (Louisville, KY), BIS-I (HCl salt) was from Calbiochem (La Jolla, CA), Cal-C was purchased from Cayman chemical. All other reagents were obtained from Sigma (St. Louis, MO, USA).

Electrophysiology. I_{Na} were recorded in the whole-cell configuration of the patch-clamp method (Hamill et al., 1981) with a HEKA EPC-9 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) at room temperature (22-24 °C). We used borosilicate glass pipettes (Kimble-Chase, Vineland, NJ, USA) with a resistance of 1.8-2.2 MΩ. The series resistance was 3-5 MΩ and compensated to >70%. Steady-state currents were sampled at 10 kHz. Current recordings were filtered at 5 kHz. Cells were continuously bathed with control or test solutions at a 2 ml/min flow rate. The control bath solution contained (in
mM): 63.6 NaCl, 103 TEA-Cl, 10 HEPES, 8 glucose, 0.3 CaCl$_2$, 2.9 MgCl$_2$, pH adjusted to 7.4 with TEA-OH. Oxo-M was diluted in bath solution to a final concentration of 10 µM. The pipette solution contained (in mM): 5 NaCl 125 CsCl, 40 TEA-Cl, 10 HEPES, 1 BAPTA-4 Cs, 5 MgCl$_2$, 3 Na$_2$ATP, 0.3 Na$_2$GTP, and 0.1 leupeptin, pH adjusted to 7.4 with CsOH. Cal-C, BIS-I, IBIS-V and WMN were dissolved in DMSO, to a final concentration of DMSO that never exceeded 0.3%. U73122 and U73343 were dissolved in chloroform and then was evaporated under nitrogen and stored at -20 °C, so that the day of the experiment was reconstituted with DMSO. TTX-sensitive $I_{Na}$ were defined as the component of the Na$^+$ currents sensitive to 100 nM TTX. $I_{Na}$ were elicited with a pulse protocol applied every 5 s. Peak current amplitude was defined as the maximal excursion from the baseline holding current. To avoid one source of systematic bias, experimental and control measurements were alternated whenever possible, and concurrent controls were always performed. Where appropriate, data are expressed as the mean ± SEM. Student’s unpaired $t$ test was used to test for statistical significance (*$p$ < 0.05).

Results

M1 receptor activation elicited a voltage-independent slow inhibition on the TTX-sensitive Na$^+$ currents.

M1 receptor activation has been established as a key molecular mechanism to engage a slow signaling pathway, utilizing a diffusible second messenger, and culminating in the inhibition of the M-current (Selyankto et al., 1992). We have found evidence that voltage-gated Na$^+$ channels, may be also modulated in a slow manner by a diffusible second messenger (Hernández-Ochoa et al., 2007). In a recent study, we showed that M1 receptor
activation inhibits CaV2.2 channels in a voltage-independent manner by a slowly-activating pathway (Vivas et al., 2013). These observations prompted us to study a general mechanism in which muscarinic activation also modulates INa through a diffusible cytoplasmic second messenger. Typical experiments were conducted with a pulse protocol starting from a -100 mV holding potential and depolarizing to -30 mV with voltage steps delivered 5 s apart to avoid cumulative inactivation of the Na+ channels. As shown in Fig. 1A, INa inhibition by Oxo-M exhibited two components, fast (F) and slow (S). The fast component showed an inhibition of 22.05% ± 11.69% of the whole INa while the slow component showed an inhibition of 28.37% ± 3.9% of the remaining current after fast inhibition (Fig. 1C). Steady-state inhibition showed no change in Na+ channel voltage-dependency, as no shift in the I-V relationship was observed (Fig. 1D) (n = 6). Furthermore the activation curve, as well as the h-infinity curve showed no changes in their voltage-dependency (Fig. 1E, 1G). These results are consistent with a voltage-insensitive mechanism eliciting a slow modulation of voltage-gated Na+ channels. In a previous paper, we showed evidence that INa can also be inhibited by Angio II with similarly slow kinetics (Hernández-Ochoa et al., 2007). We wanted to test whether the slow inhibition of INa by Oxo-M could be mimicked by Angio II. In Fig 1H, it can be observed that the INa decrease had two components (fast and slow). The fast component showed an inhibition of 28.32% ± 11.86%, while the slow component inhibited 17.0% ± 3.14% of the current remaining after the fast inhibition (Fig. 1J) (n = 6). This is consistent with our preliminary report that showed slow kinetics of INa inhibition by Angio II (Hernández-Ochoa et al., 2007). Similarly to Oxo-M, the inhibition by Angio II did not change any voltage-dependent parameter of the current (Fig. 1K) (n = 6). Current inhibition elicited by either Oxo-M or Angio II stimulation were qualitatively similar; however, these differences statistically
significantly in percentage of inhibition for the slow component might be explained by
differences in the specificity of the signaling cascade to Oxo-M or Angio II. These results
led us to propose that there is a common molecule involved in the slow inhibition by GPCR
activated pathways, a second messenger hydrolyzed following stimulation with Oxo-M or
Angio II, and resynthesized afterwards and eliciting a long-lasting inhibition of $I_{Na}$.

The M1 slow inhibition of sodium currents is isolated by pertussis toxin (PTX).
It is well-documented for calcium currents that voltage-dependent modulation of certain
GPCR-activated pathways is blocked by PTX (Beech et al., 1992). To determine the PTX
sensitivity of the fast and the slow components of $I_{Na}$ inhibition by Oxo-M and Angio II, we
recorded neurons incubated overnight with 500 ng/ml PTX (Shapiro et al., 1994). PTX
treatment eliminated the fast inhibition of $I_{Na}$ elicited by both Oxo-M and Angio II.
Conversely, the slow inhibition of the $I_{Na}$ by Oxo-M was 26.39% ± 11.04% (Fig. 2I) ($n =
6$), similar to the slow inhibition in non-PTX pretreated neurons (28.37% ± 3.9%) (Figs.
1A, 1C). These findings confirm the observation that $I_{Na}$ modulation uses a slow-activating
pathway, presumably mediated by Gq/11 and not by a Gi/o-mechanism. Similarly to Oxo-
M, after PTX treatment, Angio II elicited only the slow component of inhibition (19.04% ±
11.66%) (Figs. 2B, 2J) ($n = 6$). The slow component inhibition in non PTX-treated cells
was similar (17.0% ± 3.14%) (Figs. 1H, 1J) once the fast component was subtracted. That
is, once it was normalized back to original current before the fast component. In order to
further support that slow inhibition is voltage-insensitive, we used the prepulse protocol
(Fig. 2C, 2G). This maneuver revealed that the inhibition by Oxo-M remains unchanged
after a strong depolarization, consistent with a voltage-independent mechanism. These
results support the hypothesis that a common diffusible second messenger may be involved
in the slow modulation of \( I_{Na} \) by Oxo-M or Angio II, similar to \( L \) and \( N \) type voltage-gated Ca\(^{2+} \) channels (Mathie et al., 1992). In order to assess the magnitude of the Oxo-M inhibition, we developed a concentration-response curve (Fig. 2D, 2H). It revealed an EC\(_{50} \) of 64 µM and a Hill Coefficient of 1. The concentration of Oxo-M we typically used in these experiments was of 100 µM, which inhibits a 71% of the whole TTX-sensitive Na\(^{+} \) currents in SCG neurons.

M1 inhibition of \( I_{Na} \) is blocked by either pirenzepine or MT-7 toxin.

Next, we wanted to test the specificity of the signaling cascade triggered by the M1 receptor and to document the effective concentration of Oxo-M to inhibit TTX-sensitive sodium channels. Therefore, SCG neurons were challenged with MT-7 toxin, a specific M1 receptor antagonist. In addition, in another group of cells pirenzepine was tested. We found that both antagonists abolished Oxo-M inhibition of the TTX-sensitive Na\(^{+} \) currents (Fig.3A, 3B). Data show that Oxo-M significantly inhibited \( I_{Na} \) with a percentage of 41.07 ± 22.79 (\( n = 5 \)) compared to Pz (6.84 ± 6.76) (\( n = 5 \)) or MT-7 (8.72 ± 12.57) (\( n = 6 \)) which revealed no significant changes compared to Oxo-M. These results confirm the specificity of M1 receptor activation in the \( I_{Na} \) slow inhibition.

M1 inhibition of sodium currents is blocked by U73122 and recovery is prevented by wortmannin (WMN).

Muscarinic receptors are coupled to G protein signaling pathways to modulate voltage-gated ion channels. Some of these pathways use a diffusible second messenger to inhibit voltage-gated ion channels. Examples include the M-type K\(^{+} \) channel (Suh and Hille, 2002) and, to a lesser extent the L-type Ca\(^{2+} \) channel (Mathie et al., 1992). This slow modulation
has been shown to involve Gq/11 and PLC (Berstein et al., 1992). To test whether PLC is involved in the slow GPCR signaling pathway that inhibits I_Na, we used U73122 as a PLC inhibitor (Suh and Hille, 2002). Consistently, after bath application of 3 µM U73122, we observed reduced inhibition by Oxo-M (4.19% ± 5.89%) (Figs. 4A, 3H) (n = 6). Conversely, exposure of the cells to its inactive analogue, U73343, did not reduce muscarinic inhibition (26.88% ± 2.27%) (Figs. 4B, 3H) (n = 6). Recovery from muscarinic inhibition of the M-current has been shown to depend strongly on PI3 and PI4 kinases, to replenish PIP2 levels (Suh and Hille, 2002). This prompted us to apply WMN, which blocks PI3 and PI4 kinases at micromolar concentrations (50 µM) (Suh and Hille, 2002), to determine if PI kinases were necessary for I_Na recovery from Oxo-M inhibition (Fig. 4C).

We pre-incubated the cells overnight with PTX to specifically isolate the slow component. WMN significantly diminished I_Na recovery from inhibition by Oxo-M (3.49% ± 0.01%) (Fig. 4D) (n = 6) compared to control (23.07% ± 8.60%) (Fig. 4D) (n = 6). These results indicate that PIP2 resynthesis is necessary for I_Na recovery from muscarinic inhibition.

The slow inhibition by M1 activation is occluded by Poly-L-lysine (PLL) and prevented by intracellular dialysis with a PIP2 analog.

It has been proposed that intracellularly diffusible PLL (30,000-70,000 MW) and other polyvalent cations inhibit the M-current by electrostatic interaction with a diffusible second messenger produced by PLC (Suh and Hille, 2007). PLL should inhibit I_Na by sequestering PIP2 away from the channels and this inhibition could then occlude further inhibition of I_Na by Oxo-M. This observation led us to test whether PLL, a polybasic polypeptide that binds to the membrane producing a local positive electrostatic potential, was able to diminish I_Na inhibition by Oxo-M. It is hypothesized that PLL strongly attracts and effectively
sequesters multivalent PIP$_2$ molecules (Suh and Hille, 2007). Hille and coworkers have documented interactions between PIP$_2$ and the M-channel (Suh and Hille, 2007). To dialyze PLL or PIP$_2$ we used a microflow method for rapidly changing the intracellular solution under whole-cell patch-clamp conditions. An air bubble was used to separate two solutions and to produce rapid "step" changes of solution without affecting the seal resistance (Kakei and Ashcroft, 1987). This maneuver allowed us to observe $I_{Na}$ before and after the application of intracellular PLL or PIP$_2$ to the same cell. We observed that dialyzing with 50 µM PLL from a patch pipette solution slowly decreased $I_{Na}$ by 50% ($t_{Na50}$) in approximately 5 min ($n = 6$) (data not shown). Oxo-M $I_{Na}$ inhibition decreased from $13.96\% \pm 2.8\%$ in control to $6.46\% \pm 1.7\%$ in the presence of PLL at $t_{Na50}$ (Figs. 5A, 5I) ($n = 6$). These observations are in agreement with a slow modulation of $I_{Na}$ involving a diffusible second messenger into the cell membrane that is affected by highly charged molecules rather than affecting the channel pore (Suh and Hille, 2007). Muscarinic inhibition of $I_{Na}$ may be regulated by a $G_{q/11}$ signaling pathway involving PLC$\beta$ and PIP$_2$ hydrolysis. Thus, a feasible mechanism involves voltage-insensitive regulation by PIP$_2$ on the voltage-gated $I_{Na}$. Therefore, diC8-PIP$_2$ was used to flood the cell to prevent Oxo-M mediated PIP$_2$ depletion that is required for inhibition. Thus, PLL occludes Oxo-M inhibition of $I_{Na}$ while PIP$_2$ prevents Oxo-M inhibition. Cells were dialyzed using the above described conditions with 100 µM of diC8-PIP$_2$, an improved hydrolyzable and diffusible PIP$_2$ analog, through the patch pipette. Pretreatment with diC8-PIP$_2$, starting from 3 min (Sohn et al., 2007) prior to Oxo-M treatment, abolished the slow muscarinic inhibition of $I_{Na}$. In contrast, intracellular dialysis without diC8-PIP$_2$ did not prevent the slow muscarinic inhibition of $I_{Na}$ by Oxo-M (Figs. 5B, 5J). It is likely that additional substrate for PLC$\beta$1 hampers the hydrolysis of endogenous PIP$_2$ in the plasma membrane, preventing inhibition...
of $I_{\text{Na}}$ by Oxo-M. These results showed that in diC8-PIP2-dialyzed neurons, Oxo-M application did not inhibit $I_{\text{Na}}$ (0.02% ± 0.04%) (Fig. 5B, 5J) ($n = 8$) compared to control-dialyzed neurons (26.5% ± 2.4%) (Fig. 5B, 5J) ($n = 5$). Abolition of the muscarinic $I_{\text{Na}}$ inhibition by diC8-PIP2 dialysis supports the hypothesis that PIP2 hydrolysis is responsible for the slow muscarinic inhibition of voltage-gated sodium channels. We also wanted to test whether this inhibition might be due to PKC activation. To evaluate this possibility, we used BIS-I, a specific PKC inhibitor (Garcia et al., 1998). Pre-incubation and superfusion of the cells with this inhibitor, or its inactive analog (IBIS-V), had no effect on the inhibition elicited by Oxo-M (Fig. 5C, 5K). In order to further support non PKC-mediated M1-induced suppression of Na$^+$ currents, neurons were preincubated with calphostin C, a PKC inhibitor that targets the diacylglycerol-binding site of the kinase (Hoshi et al., 2010), for 10 min before starting the experiment according to Boehm (Boehm et al., 1996). Data revealed no significant changes in the inhibition of $I_{\text{Na}}$ by Oxo-M (54.48 ± 8.64%) ($n = 3$) compared to the $I_{\text{Na}}$ Inhibition by Oxo-M in cells treated with Cal-C (47.32 ± 10.50%) ($n = 6$). These results confirm that PKC is not involved in the M1 inhibition of $I_{\text{Na}}$.

Discussion

Antecedents of the molecular mechanisms and biophysical properties of the voltage-insensitive modulation of voltage-gated ion channels have been reported (Rodriguez-Menchaca et al., 2012; Suh et al., 2010; Vivas et al., 2013). Several reports have shown that a variety of ion channels are modulated by PIP2 and that PLCβ1 depletes this essential PIP2 from the plasma membrane (Suh and Hille, 2002; Suh and Hille, 2005). We chose SCG neurons for this study as they have the advantage of natively expressing a robust number of TTX-sensitive sodium channels, mostly NaV1.7, and a minimal amount of NaV1.1 (Jia et
We used muscarinic M1 activation, PLCβ1 inhibition, PIP2 neutralization by polycations and an inexhaustible source of PIP2 to support the hypothesis that PIP2 hydrolysis modulates the slow inhibition of TTX-sensitive Na⁺ channels. Slow inhibition by Oxo-M remained unchanged after a strong depolarization, further supporting that the slow modulation occurs via a voltage-insensitive mechanism. Furthermore, I_Na recovery from muscarinic inhibition appears to require PIP2 resynthesis. The decrease in I_Na with WMN suggests ongoing PIP2 synthesis is necessary to maintain I_Na. However, dissimilarly to M-current, I_Na do not exhibit PIP2 dependence to support channel opening, rather to modulating I_Na amplitude. It has been hypothesized that polycations might act through electrostatic screening of PIP2 such as PLL-PIP2 complexes neutralize some of the charges on five negative charges of PIP2 (McLaughlin et al., 2002). Finally, the lack of effect of BIS-I on M1-mediated slow inhibition of I_Na suggests that PKC is not involved in this inhibition. We used BIS-I, a specific ATP-competitive PKC inhibitor, in order to discard that this inhibition is mediated by protein phosphorylation by this protein kinase. However, for further support of this conclusion another group of neurons was treated with calphostin C, a PKC inhibitor that targets the diacylglycerol-binding site of the kinase. Under these conditions, Oxo-M-induced suppression of I_Na remain unchanged. These results confirm that PKC is not involved in M1 slow inhibition of I_Na. This is the first report showing that TTX-sensitive Na⁺ channels require the phospholipid PIP2 for proper function. There may be a critical site for PIP2-dependent coupling at the interface between the voltage-sensing domain and the pore domain. This site is a conserved lipid-binding site present in many different ion channels, suggesting that phosphoinositide derivatives play a critical role in mediating a variety of cell-membrane functions. Available data lead us to propose that phospholipids stabilize interactions between a positively charged voltage-sensor and
negatively charged lipid phosphodiester groups. Thus, PIP_2 provides an appropriate
environment for the energetic stability and operation of the voltage-sensing machinery.
Currently, a variety of ion channels share a PIP_2 requirement, including M-current, most
inward rectifier potassium channels, TRP channels, ENaC channels, HCN channels and
Ca^{2+} channels. There are a number of PIP_2 requiring channels where the mechanism of PIP_2
modulation is clearly distinct from the fast voltage-dependent modulation and from the
strongly rectifying pore block found in many channels. PIP_2 is a minor, although essential,
signaling lipid that is needed for many plasma membrane processes, including ion channel
function, transporters (Hilgemann et al., 2001) and endocytosis (McLaughlin et al., 2002).
Many ligands acting through GPCRs will stimulate PIP_2 hydrolysis and alter the electrical
excitability of neurons as well as non-neuronal, but excitable cells. Despite numerous
experimental challenges, it appears that a variety of voltage-gated ion channels, including
Na^+, K^+ and Ca^{2+} channels, are modulated by PIP_2. In all of these cases, voltage-insensitive
modulation seems to be a key regulator of the voltage-gated ion channel pore-opening
mechanism by G protein coupled receptors. Diverse evidence indicates that this form of
modulation involves a diffusible second messenger produced at the cell membrane, but
requiring release into the cytoplasm, where it then acts on a variety of proteins, transporters
and enzymes. Taken together, our results highlight the physiological role of a PIP_2 in I_{Na}
regulation by showing a significant inhibition of the TTX-sensitive voltage-gated Na^+
channels by M1 receptor activation. This observation enables the field to address
unanswered questions regarding the long-term modulation of spike firing patterns in
neurons and hormone release from excitable cells. Our results help to bridge the gap
between voltage-sensitive and voltage-insensitive modulation of ion channels.
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Authorship Contribution:

Research design and participated in experiments: DEG

Conducted experiments and contributed to the writing of the manuscript: EIP

Contributed new reagents or analytic tools: DEV

Performed data analysis: EIP, IA, LDC

Wrote the manuscript: DEG
REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. M1 slow inhibition of TTX-sensitive Na⁺ currents did not change voltage-dependency. (A) Fast and slow components of $I_{\text{Na}}$ by Oxo-M inhibition in: 1. control, 2. 100 µM Oxo-M, 3. 100 nM TTX. Bars indicate the time of bathing the recording cell with agonist. F denotes fast inhibition and S, slow inhibition for both agonists. The fast component is established in <1 s whereas the slow component >100 s. (B upper) Pulse protocol elicited every 5 s for all experiments. (B lower) Typical traces of $I_{\text{Na}}$ from time course shown in (A) at the numbers indicated. (C) Fast Oxo-M inhibition of $I_{\text{Na}}$ was $22.05 \pm 11.69\%$, while slow inhibition of $I_{\text{Na}}$ by Oxo-M was $28.37 \pm 3.90\%$ ($n = 6$). (D) I-V relationship of $I_{\text{Na}}$ in control and in the presence of Oxo-M ($n = 6$). (E) Steady-state activation curve fitted to the Boltzmann function (control, open circles, $n = 6$ versus Oxo-M filled circles, $n = 7$) no significant changes were observed. Parameters for the Boltzmann fitting were: control, $V_{1/2} = -35.44 \pm 0.96$ mV while Oxo-M, $V_{1/2} = -31.99 \pm 0.56$ mV; control, slope $k = 1.5 \pm 0.31$ mV while Oxo-M $5.75 \pm 0.40$ mV; no significant changes were observed. (F) Protocol for the steady state h-infinity curve. (G) Steady-state inactivation curves during control (open circles, $n = 7$) and with Oxo-M (filled circles, $n = 8$). Boltzmann fitting parameters were: $V_{1/2} = -90.27 \pm 2.21$ mV for control whereas $-96.24 \pm 1.02$ mV for Oxo-M, slope $k$ for control was $18.57 \pm 1.58$ mV whereas for Oxo-M $9.1 \pm 0.82$ mV; no significant changes were observed. (H) Fast and slow components of $I_{\text{Na}}$ by Angio II inhibition in: 1. control, 2. 500 nM Angio II, 3. 100 nM TTX. (I) Typical traces of $I_{\text{Na}}$ from time course shown in (H) at the numbers indicated. (J) Fast inhibition of Na⁺ current induced by Angio II was $28.32 \pm 11.86\%$, while slow inhibition of Na⁺ current was $17.00 \pm 3.14\%$ ($n = 6$). (K) I-V relationship of $I_{\text{Na}}$ in control and in the presence of Angio II ($n = 6$). Unpaired $t$-test and mean values ± S.E.M. (*$p < 0.05$).

Fig. 2. The slow inhibition by Oxo-M of TTX-sensitive Na⁺ currents was isolated by PTX and was not relieved by a strong depolarization. (A, B) Time course of TTX-sensitive Na⁺ currents in SCG neurons treated overnight with 500 ng/ml PTX. 1. control, 2. 100 µM Oxo-M or 500 nM Angio II, 3. 100 nM TTX. (E, F) Typical traces of $I_{\text{Na}}$ from time course at the numbers indicated. (I, J) Oxo-M inhibition of $I_{\text{Na}}$ in cells pre-incubated with PTX was $26.39 \pm 11.04\%$ ($n = 6$). PTX selectively blocks the fast component inhibition by Oxo-M or Angio II. On the other hand by subtracting the fast component inhibition in non-PTX treated cells the slow inhibition was $26.15 \pm 1.05\%$ ($n = 6$). For Angio II, cells pre-incubated with PTX exhibited an inhibition of $I_{\text{Na}}$ of $19.04 \pm 11.66\%$ ($n = 6$). Also after subtraction of the fast component inhibition, cells in the absence of PTX exhibited a slow component of $17.55 \pm 2.20\%$ ($n = 6$). No significant changes were observed in the slow component inhibition in cells treated with PTX or after subtraction of the fast component. (C) Prepulse protocol at +125 mV. Current traces below the protocol are illustrated in control or following treatment with 100 µM Oxo-M. (G) Time course from a cell challenged with the prepulse. Open circles represent the current in P1 while filled circles in P2. Aggregated data showing facilitation ratio (P2/P1) (control, open bars, $n = 18$; Oxo-M,
filled bars, \( n = 10 \), no significant changes were observed. (D) Dose-response curve for Oxo-M (in \( \mu M \)): (0.000001), (0.1), (100) and (1000 \( \mu M \)) for every measured point, respectively, the curve was fitted to the Hill equation; parameters were: \( EC_{50} = 64 \, \mu M \), 67.5% with 100 \( \mu M \) and a Hill coefficient of 1.0. (H) Representative traces for each dose. Unpaired \( t \)-test and mean values ± S.E.M. (\( p < 0.05 \)).

Fig. 3. MT-7 or pirenzepine blocked M1 receptor activation. (A, B) Time course of \( I_{Na} \) preincubated with 10 \( \mu M \) Pirenzepine or 100 nM MT-7. (C, D) Representative traces with the indicated treatments at the time indicated for A and B. (E, F) Oxo-M inhibition (41.07 ± 22.79) \( (n = 5) \) versus Pz (6.84 ± 6.76) \( (n = 5) \) or MT-7 (8.72 ± 12.57) \( (n = 6) \). Pz and MT-7 were statistically different compared to Oxo-M. Unpaired \( t \)-test and mean values ± S.E.M. (*\( p < 0.05 \)).

Fig. 4. M1 \( Na^+ \) currents inhibition was blocked by U73122 while its recovery from inhibition was blunted by WMN. (A, B, C) Time course of TTX-sensitive \( I_{Na} \) in: 100 \( \mu M \) Oxo-M, 3 \( \mu M \) U73122 or U73343, 50 \( \mu M \) WMN. (A, B) the fast inhibition of \( I_{Na} \) was subtracted for U73122 and U73343. (E, F, G) Typical \( I_{Na} \) traces: 1. control, 2. Oxo-M with U73122, U73343 or WMN, 3. TTX. (H) Inhibition of \( I_{Na} \) by Oxo-M in the presence of U73122 was 4.19 ± 5.89%, while in the presence of its inactive analog, U73343, 26.88 ± 2.27% \( (n = 6) \). Cells treated overnight with 500 ng/ml PTX and challenged with Oxo-M exhibited a \( I_{Na} \) recovery from inhibition of 23.07 ± 8.60% \( (n = 6) \) whereas those treated with 50 \( \mu M \) WMN exhibited a recovery of 0.12 ± 9.30%. Unpaired \( t \)-test and mean values ± S.E.M. (*\( p < 0.05 \)).

Fig. 5. PIP2 or PLL intracellular dialysis occluded M1 TTX-sensitive \( Na^+ \) currents inhibition while BIS-I or calphostin C did not prevent this inhibition. (A) Time course of TTX-sensitive \( I_{Na} \) with 50 \( \mu M \) PLL and 100 \( \mu M \) Oxo-M, the fast component of \( I_{Na} \) inhibition by Oxo-M was subtracted. (B) \( I_{Na} \) recorded with 100 \( \mu M \) diC8-PIP2 and Oxo-M and. (C) SCG neurons pre-incubated with 100 nM Cal-C and 100 \( \mu M \) Oxo-M, the fast component of \( I_{Na} \) inhibition by Oxo-M was subtracted. (D) SCG neurons pre-incubated with 100 nM bisindolylmaleimide I (BIS-I) or V (IBIS-V). (E, F and H) Typical traces of \( I_{Na} \) from the time course for each condition showed in A, B, C and D. (I) Oxo-M inhibited \( I_{Na} \) 6.46 ± 1.70% in the presence of PLL, while Oxo-M in absence of PLL inhibited \( I_{Na} \) 13.96 ± 2.84% \( (n = 6) \). (J) \( I_{Na} \) Inhibition by Oxo-M was 26.30 ± 3.40% \( (n = 5) \) while \( I_{Na} \) inhibition by Oxo-M after dialyzed diC8-PIP2 was 0.98 ± 0.04% \( (n = 8) \). (K) Inhibition of \( I_{Na} \) by Oxo-M was 52.89 ± 14.50% \( (n = 4) \) \( I_{Na} \) Inhibition by Oxo-M was unchanged with BIS-I (41.42 ± 9.66%) \( (n = 8) \) or IBIS-V (34.42 ± 11.02%) \( (n = 6) \). (L) Inhibition of \( I_{Na} \) by Oxo-M was 54.48 ± 8.64% \( (n = 3) \) \( I_{Na} \) Inhibition by Oxo-M was unchanged with Cal-C (47.32 ± 10.50%) \( (n = 6) \). Unpaired \( t \)-test and mean values ± S.E.M. (*\( p < 0.05 \)).
Figure 1
Figure 2

A. Normalized $I_{Na}$ vs. Time (s) for Oxo-M, TTX, and PTX.

B. Normalized $I_{Na}$ vs. Time (s) for Angio II and TTX with PTX.

C. Graph showing inhibition (%) vs. Oxo-M concentration (μM).

D. Graph showing inhibition (%) vs. Oxo-M concentration (μM).

E. Graph showing current (pA/pF) vs. time (2 ms).

F. Graph showing current (pA/pF) vs. time (2 ms).

G. Graph showing normalized $I_{Na}$ vs. time (s) with points P1 and P2.

H. Graph showing current (pA/pF) vs. time (4 ms) for Control, 10^-6 mM, 0.1 mM, and 1 mM.

I. Bar chart showing inhibition (%) for Oxo-M Slow, PTX + Oxo-M, and TTX.

J. Bar chart showing inhibition (%) for Angio II Slow, PTX + Angio II, and TTX.

Figure 2
Figure 3
Figure 4
Figure 5