British Journal of Pharmacology

DOI:10.1111/bph.13276
www.brjpharmacol.org

RESEARCH PAPER

Actions of KMUP-1, a xanthine and piperazine derivative, on voltage-gated Na^+ and Ca^{2+}-activated K^+ currents in GH_3 pituitary tumour cells

Yi-Ching Lo[1,2], Yu-Ting Tseng[1,2], Chi-Ming Liu[3], Bin-Nan Wu[1] and Sheng-Nan Wu[4,5]

[1]Department of Pharmacology, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, [2]Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan, [3]Department of Nursing, Tzu Hui Institute of Technology, Pingtung, Taiwan, [4]Department of Physiology, National Cheng Kung University Medical College, Tainan, Taiwan, and [5]Institute of Basic Medical Sciences, National Cheng Kung University Medical College, Tainan, Taiwan

Correspondence
Sheng-Nan Wu, Department of Physiology, National Cheng Kung University Medical College, No. 1, University Road, Tainan 70101, Taiwan.
E-mail: snwu@mail.ncku.edu.tw

Received
16 June 2015
Revised
6 August 2015
Accepted
7 August 2015

BACKGROUND AND PURPOSE

7-[2-[4-(2-Chlorophenyl)piperazinyl]ethyl]-1,3-dimethylxanthine (KMUP-1) is a xanthine-based derivative. It has soluble GC activation and K^+-channel opening activity. Effects of this compound on ion currents in pituitary GH_3 cells were investigated in this study.

EXPERIMENTAL APPROACH

The aim of this study was to evaluate effects of KMUP-1 on the amplitude and gating of voltage-gated Na^+ current (I_{Na}) in pituitary GH_3 cells and in HEKT293T cells expressing SCN5A. Both the amplitude of Ca^{2+}-activated K^+ current and the activity of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels were also studied.

KEY RESULTS

KMUP-1 depressed the transient and late components of I_{Na} with different potencies. The IC_{50} values required for its inhibitory effect on transient and late I_{Na} were 22.5 and 1.8 μM respectively. KMUP-1 (3 μM) shifted the steady-state inactivation of I_{Na} to a hyperpolarized potential by −10 mV, despite inability to alter the recovery of I_{Na} from inactivation. In cell-attached configuration, KMUP-1 applied to bath increased BK_{Ca}-channel activity; however, in inside-out patches, this compound applied to the intracellular surface had no effect on it. It prolonged the latency in the generation of action currents elicited by triangular voltage ramps. Additionally, KMUP-1 decreased the peak I_{Na} with a concomitant increase of current inactivation in HEKT293T cells expressing SCN5A.

CONCLUSIONS AND IMPLICATIONS

Apart from activating BK_{Ca} channels, KMUP-1 preferentially suppresses late I_{Na}. The effects of KUMP-1 on ion currents presented here constitute an underlying ionic mechanism of its actions.

Abbreviations

AC, action current; AP, action potential; BK_{Ca}, channel large-conductance Ca^{2+}-activated K^+ channel; $I_{K(Ca)}$, Ca^{2+}-activated K^+ current; I_{Na}, voltage-gated Na^+ current; *I–V*, current versus voltage; K_{ATP} channel ATP-sensitive K^+ channel; ODQ, 1*H*-[1,2,4]oxadiazolo-[4,3-*a*] quinoxalin-1-one; TEA, tetraethylammonium chloride; $\tau_{inact(S)}$, slow component of inactivation time constant for I_{Na}; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole

Tables of links

TARGETS	
Ion channels[a]	**GPCRs**[c]
BK_{Ca} channels	α_{1A} -adrenoceptors
K_{ATP} channels, $K_{ir}6.x$	α_{1D}-adrenoceptors
NaVα1.5 (SCN5A) channels	
NaV1.7 channels	
Enzymes[b]	
PKA	
PKC	
Soluble GC, guanylyl cyclase	

LIGANDS
Cirazoline
Glimepiride
ODQ
Paxilline
Phenylephrine
Ranolazine
Riluzole
YC-1

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a b c]Alexander *et al.*, 2013a, b, c).

Introduction

7-[2-[4-(2-Chlorophenyl)piperazinyl]ethyl]-1,3-dimethylxanthine (KMUP-1) is a xanthine and piperazine derivative (Figure 1) structurally with six nitrogen atoms and an ethylpiperazine moiety on position 7 of xanthine base. It has been recognized to exert a variety of actions, including stimulation of soluble GC, opening of K^+ channels, inhibition of phosphodiesterase activity and blockade of α-adrenoceptors (Wu *et al.*, 2001; Lin *et al.*, 2002; Wu *et al.*, 2004; Liu *et al.*, 2007). A recent report showed the ability of this compound to attenuate serum deprivation-induced toxicity in neuroblastoma SH-SY5Y cells (Hsu *et al.*, 2010). Whether KMUP-1 can affect other types of ion channels has been incompletely investigated, although its activation of K^+ channels and inhibition of Ca^{2+} channels have been shown previously (Wu *et al.*, 2001; Lin *et al.*, 2002; Wu *et al.*, 2004; Wu *et al.*, 2005; Chen *et al.*, 2011).

Voltage-gated Na^+ channels (Na_V channels) are essential for the generation and propagation of action potentials (APs) in excitable membranes. The Na^+ channel protein contains four homologous domains (D1–D4), each with six transmembrane segments (S1–S6). Upon depolarization, Na^+ channels go through rapid transitions from their resting to the open state and then to the inactivated state. The genetic defects in Na^+ channel inactivation that result in small sustained Na^+ currents after the AP firing have been recognized to have devastating consequences, including seizures, periodic paralysis, paramyotonia and LQT-3 syndrome (Bankton *et al.*, 2007; Webb and Cannon, 2008; George *et al.*, 2009). There are nine isoforms ($Na_V1.1$–1.9) found among mammalian excitable tissues, including central nervous system, peripheral nervous system, skeletal muscle and heart (Catterall *et al.*, 2005). Most therapeutic Na^+ channel blockers are recognized to be not isoform selective and may have more than one clinical application.

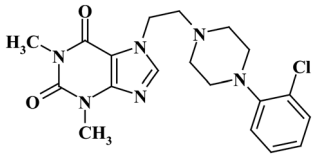

Figure 1
The chemical structure of KMUP-1.

We recently showed that riluzole or ranolazine caused a significant inhibition of non-inactivating voltage-gated Na^+ current (I_{Na}) in differentiated NG108-15 neuronal cells (Wu *et al.*, 2009a). In pituitary GH_3 cells and hypothalamic GT1-7 neurons, we also found that tefluthrin, a synthetic type I pyrethroid, increased the peak amplitude of I_{Na} together with a reduction of current inactivation (Wu *et al.*, 2009c). $Na_V1.7$ was found to be a major subfamily of Na_V channels functionally expressed in these cells (Morinville *et al.*, 2007). Regulation of these channels can be responsible for the actions on endocrine or neuroendocrine function occurring *in vivo*.

Therefore, the goal of this study was to examine the effect of KMUP-1 on ion currents in pituitary GH_3 cells. Interestingly, we provide substantial evidence that besides the activation of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels, KMUP-1 can inhibit I_{Na} in pituitary tumour (GH_3) cells in a time-dependent and concentration-dependent fashion at micromolar concentrations. These inhibitory effects on I_{Na} are thought to be direct and not mediated by activation of soluble GC and might contribute to its pharmacological actions *in vivo*. The Na_V channels can thus be an important target for the action of this agent or for structurally related compounds.

Methods

Cell preparations

GH_3, a clonal cell line derived from a rat prolactin-secreting pituitary tumour, was obtained from the Bioresources Collection and Research Center (BCRC-60015; Hsinchu, Taiwan),

and the detailed methodology has been described previously (Wu *et al.*, 2009b). Cells were cultured in Ham's F-12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% heat-inactivated horse serum (v/v), 2.5% fetal calf serum (v/v) and 2 mM L-glutamine in a humidified environment of 5% CO_2/95% air. The culture medium was changed every 2–3 days and cells underwent passaged when they reached confluence. In order to promote cell differentiation, GH_3 cells were transferred to a serum-free, Ca^{2+}-free medium. Under these conditions, cells remained 80–90% viable for at least 2 weeks. In another set of experiments, GH_3 cells were incubated with 1*H*-[1,2,4]oxadiazolo-[4,3-*a*] quinoxalin-1-one (ODQ) (10 μM) at 37°C for 6 h.

HEK (HEK293T) cells were obtained from the American Type Culture Collection (CRL-11268; Manassas, VA, USA). They were grown in DMEM (Invitrogen) supplemented with 10% heat-inactivated FBS (v/v), 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in a 5% CO_2 atmosphere. For transfection of HEK293T cells, cells at a number of 2–6 × 10^5 were seeded on the 6 cm culture dish for 24 h before transfection. Cell viability was evaluated using WST-1 assay (Roche Diagnostics, Taipei, Taiwan). In the experiments of observing cell growth and differentiation, a Nikon Eclipse Ti-E inverted microscope (Li Trading Co., Taipei, Taiwan) equipped with a 5 megapixel cooled digital camera was used. The digital camera was connected to a personal computer running NIS-Elements BR3.0 software (Nikon, Kanagawa, Japan).

Transfection

The $Na_V\alpha1.5$ DNA construct (*SCN5A*; GenBank™ accession number M77235) was previously subcloned into a modified pSP64T vector. This plasmid pSP64T-WT-SCN5A was a kind gift from Dr. Ru-Chi Shieh (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan). The plasmid was transfected into HEKT293T cells for transient expression with the use of polyethylenimine (PEI) reagents (ExGen 500; MBI Fermentas, Hanover, MD, USA). After plasmid was diluted in 150 mM NaCl, it was mixed with PEI and incubated for 10 min at room temperature. The plasmid–PEI mixture solution was added to the 24 well plate and then centrifuged at 280× *g* for 3 min. After centrifugation, cells were incubated at 37°C for an additional 48 h. The expression of *SCN5A* was determined by immunofluorescence staining and electrophysiological measurements (Wu *et al.*, 2009b).

Electrophysiological measurements

GH_3 or HEK293T cells were dissociated with 1% trypsin/EDTA solution, and an aliquot of cell suspension was transferred to a recording chamber mounted on the stage of a DM-IL inverted microscope (Leica Microsystems, Wetzlar, Germany) for experiments. Cells were bathed at room temperature (20–25°C) in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Patch pipettes were made from Kmax-51 glass capillaries (Kimble Glass, Vineland, NJ, USA) using a PP-830 puller (Narishige, Tokyo, Japan) and fire polished with an MF-83 microforge (Narishige). When the electrodes were filled with the internal solution, their resistance ranged between 3 and 5 MΩ. Patch-clamp recordings were obtained in cell-attached, inside-out or whole-cell configurations using an RK-400 (Bio-Logic, Claix, France) or Axopatch-200B (Molecular Devices, Sunnyvale, CA, USA) amplifier (Wu *et al.*, 2008).

Data recordings and analyses

The signals were displayed on a digital oscilloscope (model 1602; Gould, Chandler, AZ, USA) and on a liquid crystal projector (PJ550-2; ViewSonic, Walnut, CA, USA). The data were online stored in a TravelMate-6253 laptop computer (Acer, Taipei, Taiwan) at 10 kHz through a Digidata-1322A interface (Molecular Devices). The latter device was equipped with an Adaptec SlimSCSI card (Milpitas, CA, USA) via a PCMCIA card slot and controlled by pCLAMP 9.2 (Molecular Devices). Ion currents were low pass filtered at 1–3 kHz. The signals recorded during whole-cell or single-channel experiments were offline analysed using pCLAMP 9.2 (Molecular Devices), Origin 7.5 (OriginLab, Northampton, MA, USA) or custom-made macros built in Excel 2007 spreadsheet running on Windows 7 (Microsoft, Redmond, WA, USA). A family of voltage steps generated by pCLAMP 9.2 were generally used to evaluate the current versus voltage (*I–V*) relationships for ion currents [e.g. I_{Na} or Ca^{2+}-activated K^+ current ($I_{K(Ca)}$)].

To calculate the percentage inhibition of KMUP-1 on the transient and late component of I_{Na}, GH_3 cells were bathed in normal Tyrode's solution, which contained 1.8 mM $CaCl_2$. Each cell was depolarized from −80 to −20 mV at a rate of 0.5 Hz, and the amplitude of transient and late I_{Na} was, respectively, measured at the beginning and end of the depolarizing pulses. The concentration–response relationship for KMUP-1-induced inhibition of transient and late I_{Na} in GH_3 cells was determined by the Hill equation using a nonlinear regression analysis, that is,

$$\text{percentage decrease} = \frac{E_{max} \times [C]^{n_h}}{IC_{50} + [C]^{n_h}} \quad (1)$$

where [C] is the KMUP-1 concentration; IC_{50} and n_h are the half-maximal concentration of KMUP-1 and the Hill coefficient respectively; and E_{max} is the maximal inhibition of transient or late I_{Na} induced by this compound.

To determine the effect of KMUP-1 on the steady-state inactivation curve of I_{Na}, the relationship between the membrane potential and the normalized amplitude of I_{Na} with or without addition of KMUP-1 (3 μM) were fitted by the Boltzmann function:

$$\frac{I_{max}}{1 + \exp\left((V - V_{1/2})/k\right)} \quad (2)$$

where I_{max} represents the maximal activated I_{Na}; V is the membrane potential in mV; $V_{1/2}$ is the membrane potential for a half-maximal inactivation; and k is the slope factor of I_{Na} inactivation curve. Curve fitting to the data presented here was generally performed by means of Origin 7.5 (OriginLab) or GraphPad Prism 5 (La Jolla, CA, USA).

The inhibitory effects of KMUP-1 on I_{Na} can be explained by a state-dependent blocker that binds to the open state of the channel according to a minimal kinetic scheme (Wu *et al.*, 2009b).

$$C \underset{\beta}{\overset{\alpha}{\rightleftarrows}} O \underset{k-1}{\overset{k+1}{\rightleftarrows}} O{\cdot}B \quad (3)$$

where α and β are the voltage-dependent rate constants for the opening and closing of Na_V channel; k_{+1} and k_{-1} are those for blocking and unblocking by KMUP-1 (i.e. the on-rate and off-rate constants for KMUP-1); and [B] is the blocker (i.e. KMUP-1) concentration. C, O and O•B are the closed, open and open-blocked states respectively.

The blocking and unblocking rate constants, k_{+1} and k_{-1}, were determined from the slow component of inactivation time constants for I_{Na} ($\tau_{inact(S)}$) evoked by the depolarizing pulses from −80 to −20 mV. Blocking and unblocking rate constants were estimated using the following relation:

$$\frac{1}{\tau_b} = k_{+1} \times [B] + k_{-1} \quad (4)$$

where the slope is equal to k_{+1} and the y-intercept is equal to k_{-1}, both of which can be determined by fitting the data using linear regression. The regression then interpolates the reciprocal time constants ($1/\tau_b$) versus different KMUP-1 concentrations ([B]).

Action currents (ACs) that can represent APs were measured in GH_3 cells by means of cell-attached voltage-clamp recordings as described previously (Costantin and Charles, 1999; Wu *et al.*, 2006; Wu *et al.*, 2009c). Specifically, AC measurements were used to allow quantification of the underlying AP frequency under the condition where the intracellular contents were left intact (Costantin and Charles, 1999; Wu *et al.*, 2006; Wu *et al.*, 2009c). The AC waveform is mainly due to the capacitive current, which is shaped as the first derivative of the AP. The capacitive current, which can be measured when the cell fires an AP, appears as a brief spike in the downward direction.

Data analysis

Results are presented as means ± SEM with the sample sizes (*n*) indicating the number of cells from which the results were taken. The paired or unpaired *t* test and one-way ANOVA with the least significance difference method for multiple comparisons were used for statistical evaluation of the differences among the mean values. Statistical analyses were performed using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). Differences between values were considered significant when $P < 0.05$.

Materials

Cirazoline, ranolazine and riluzole were obtained from Tocris Cookson Ltd. (Bristol, UK). Glimepiride, ODQ, phenylephrine and tetraethylammonium chloride (TEA) were from Sigma Chemical (St. Louis, MO, USA), and tetrodotoxin and paxilline were from Alomone Labs (Jerusalem, Israel). YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole) was kindly provided by Professor Che-Ming Teng, Pharmacology Institute, National Taiwan University Medical Center. KMUP-1 was chemically synthesized as described previously (Wu *et al.*, 2001) and dissolved in 10% absolute alcohol, 10% propylene glycol and 2% 1N HCl. The stock solution of KMUP-1 (100 mM) was made in distilled water and diluted to final concentration (0.1–100 μM) in the bath solution. All other chemicals were commercially available and of reagent grade. The double-distilled water that was de-ionized through a Millipore-Q system (Bedford, MA, USA) was used in all experiments.

The composition of normal Tyrode's solution was 136.5 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.53 mM $MgCl_2$, 5.5 mM glucose and 5.5 mM HEPES-NaOH buffer, pH 7.4. To measure I_{Na}, KCl inside the pipette solution was replaced with equimolar CsCl, and pH was adjusted to 7.2 with CsOH. To record K^+ currents or membrane potential, a patch pipette was filled with a solution consisting of 140 mM KCl, 1 mM $MgCl_2$, 3 mM Na_2ATP, 0.1 mM Na_2GTP, 0.1 mM EGTA and 5 mM HEPES-KOH buffer, pH 7.2. For single-channel recordings, high K^+-bathing solution contained 145 mM KCl, 0.53 mM $MgCl_2$ and 5 mM HEPES-KOH, pH 7.4; and pipette solution contained 145 mM KCl, 2 mM $MgCl_2$, and 5 mM HEPES-KOH, pH 7.2.

Results

Inhibitory effect of KMUP-1 on INa in pituitary GH3 cells

In the initial set of experiments, a whole-cell configuration was used to investigate the effect of KMUP-1 on macroscopic ion currents in GH_3 cells. Cells were bathed in Ca^{2+}-free Tyrode's solution containing 10 mM TEA, and patch pipettes were loaded with a Cs^+-containing solution. It is notable that the magnitude of KMUP-1-mediated inhibition of I_{Na} measured at the beginning and end of the depolarizing pulses was found to be distinguishable. The presence of KMUP-1 was found to suppress the transient and late components of I_{Na} in a concentration-dependent manner (Figure 2A). The relationships between the KMUP-1 concentration and the percentage inhibition for transient and late components of I_{Na} are illustrated in Figure 2B. The IC_{50} values required for the inhibitory effects of KMUP-1 on transient and late components of I_{Na} were calculated to be 22.5 and 1.8 μM respectively. Therefore, it is clear that KMUP-1 exerts a significant action on the inhibition of transient and late I_{Na} in these cells. Moreover, during exposure to this agent, late $I_{Na,L}$ is subject to inhibition to a greater extent than transient I_{Na}. However, no discernible change in the overall *I–V* relationship of transient I_{Na} can be detected in the presence of 3 μM KMUP-1 (Figure 2C).

Evaluation of time-dependent block of I_{Na} inactivation in the presence of KMUP-1

It is also notable that when the depolarizing pulses from −80 to −20 mV were applied to evoke I_{Na}, application of KMUP-1 diminished the peak amplitude of I_{Na} (i.e. $I_{Na,T}$) together with a progressive increase in current decay (Figure 3A). For example, when cells were depolarized from −80 to −10 mV, cell exposure to KMUP-1 (3 μM) caused a significant reduction in the peak amplitude of I_{Na} by 73 ± 6% from 287 ± 15 to 208 ± 12 pA (n = 8). Moreover, in the presence of KMUP-1 (3 μM), the time constants in the slow component of current inactivation (i.e. $\tau_{inact(S)}$) were significantly reduced to 18.3 ± 0.5 ms from a control value of 28.4 ± 0.6 ms (n = 7, $P <$

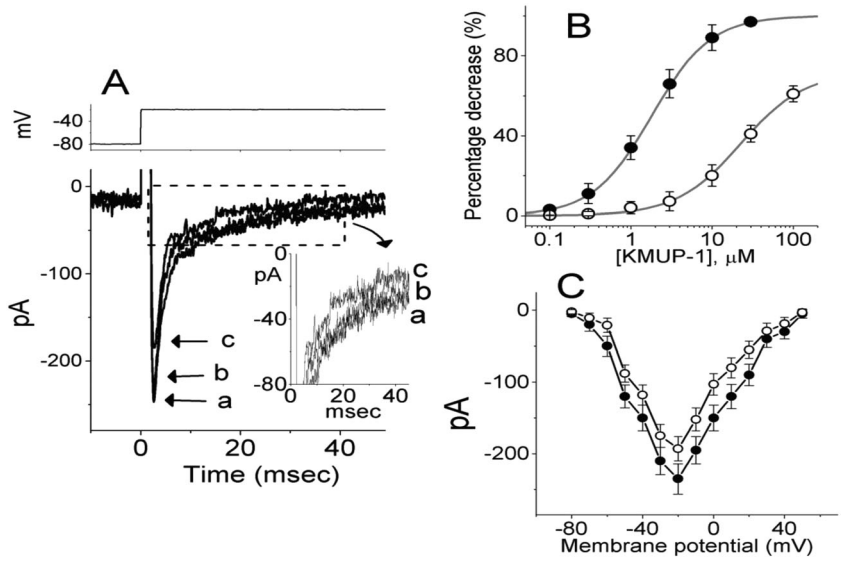

Figure 2

Inhibitory effects of KMUP-1 on I_{Na} in pituitary GH_3 cells. In these experiments, each pipette was loaded with a Cs^+-containing solution, and cells were bathed in Ca^{2+}-free Tyrode's solution containing 10 mM TEA. The cell was depolarized from −80 mV to different membrane potentials with a duration of 50 ms at a rate of 0.5 Hz. (A) Superimposed traces of I_{Na} obtained with or without KMUP-1 (1 or 3 μM). The insets show expanded records of late I_{Na} as indicated in the dashed box. The uppermost part shown here and the following figures generally indicates the voltage protocol used. (B) Concentration–response relationships for KMUP-1-induced inhibition of I_{Na} measured at the beginning and end of the depolarizing pulses (mean ± SEM; n = 5–10 for each point). Smooth lines represent the best fit to eq. 1, as described in *Methods*. (C) Averaged *I–V* relationships of I_{Na} obtained in control and during the exposure to 3 μM KMUP-1 (mean ± SEM; n = 7–10 for each point). Notably, KMUP-1 does not alter the overall *I–V* relationship of I_{Na}.

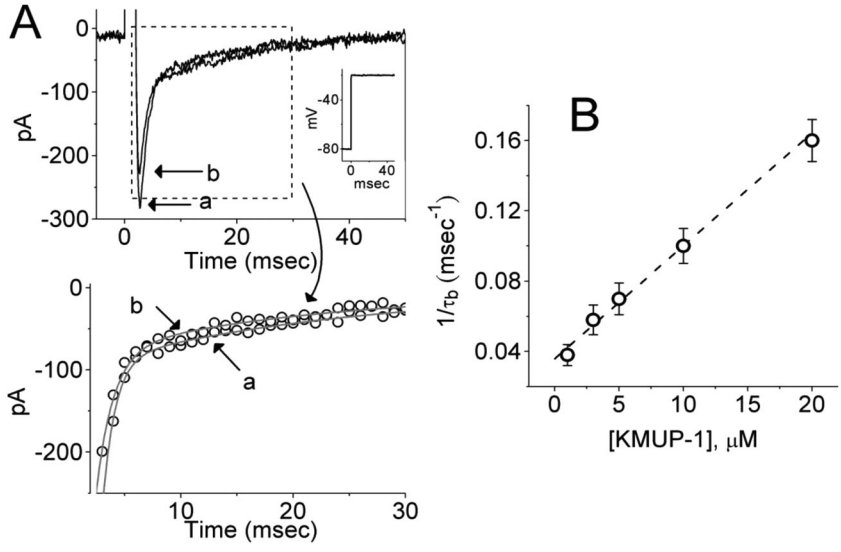

Figure 3

Evaluation of the kinetics of KMUP-1-induced block of I_{Na} in GH_3 cells. In (A), typical I_{Na} was elicited by depolarizing pulse from −80 to −20 mV with or without KMUP-1 (3 μM). The time courses of current decay in the absence and presence of 3 μM KMUP-1 were fitted by a two-exponential as indicated in smooth lines. The values of $\tau_{inact(S)}$ measured in the control and during exposure to KMUP-1 are 24.8 and 16.4 ms respectively. Inset in the upper part of (A) indicated the voltage protocol used. The lower part of (A) shows the expanded record as indicated by the dashed box in the upper part. In (B), the reciprocal of the time constants of the rate of block ($1/\tau_b$), obtained by exponential fits to the slow component of I_{Na} inactivation ($\tau_{inact(S)}$), was plotted against the KMUP-1 concentrations. Data points were fitted by a linear regression (dashed line), reflecting that such a block occurs with a one-step binding reaction. Block (k_{+1}) and unblock (k_{-1}) rate constants, given by the slope and the y-axis intercept of the interpolated line, were estimated to be 0.00642 $ms^{-1}\cdot\mu M^{-1}$ and 0.0359 ms^{-1} respectively. Each point represents mean ± SEM (n = 6–9).

0.05), although there is no change in the time course of the fast component of I_{Na} inactivation during cell exposure to this compound.

During cell exposure to KMUP-1, in addition to the decreased amplitude of $I_{Na,T}$, the degree of I_{Na} inactivation during exposure to this compound tends to be increased. To provide more evidence for its block of I_{Na}, the time courses of current inactivation in the absence and presence of different KMUP-1 concentrations were fitted by a two-exponential process (i.e. fast and slow components). The effects of this agent on I_{Na} were found to result in a concentration-dependent reduction of $\tau_{inact(S)}$ (Figure 3), although no significant changes in the fast component of current inactivation can be demonstrated in the presence of KMUP-1. Therefore, increasing KUMP-1 concentration not only diminished the peak amplitude of I_{Na} but also increased the inactivation rate of the current. Based on the first-order blocking scheme (eq. 4, as described in *Methods*), the relationship between $1/\tau_b$ and [B] was found to be linear with a correlation coefficient of 0.97 (Figure 2D), indicating that there is a one-step binding reaction. The blocking (i.e. on) and unblocking (i.e. off) rate constants obtained from six to nine different cells were calculated to be 0.0064 ± 0.0004 $ms^{-1}\cdot\mu M^{-1}$ and 0.036 ± 0.005 ms^{-1} respectively. On the basis of these rate constants in eq. 3 as described in *Methods*, the apparent dissociation constant ($K_D = k_{-1}/k_{+1}$) for the binding of KMUP-1 to Na_V channels was thus estimated to be 5.6 μM. Notably, this value is close to the estimated IC_{50} value for KMUP-1-mediated inhibition of late I_{Na} determined from the concentration–response curve (Figure 2B). However, the rate constant of the unblocking reaction (i.e. k_{-1}) showed little dependence on the KMUP-1 concentration: k_{-1} was 0.038 ± 0.002 ms^{-1} (n = 6) at 1 μM and 0.037 ± 0.002 ms^{-1} (n = 6) at 3 μM.

Effect of KMUP-1 on the steady-state inactivation of I_{Na}

To characterize the inhibitory effect of KMUP-1 on I_{Na}, we also evaluated its effect on the steady-state inactivation curve of I_{Na}. Figure 4 shows the steady-state inactivation curve of I_{Na} obtained in the absence and presence of KMUP-1 (3 μM). A two-step voltage pulse protocol was applied to the cells. In these experiments, a 60 ms conditioning pulse to different membrane potentials preceded the depolarizing pulse (60 ms in duration) to −20 mV from a holding potential of −80 mV. The interval between two sets of voltage pulses was 10 s to prevent incomplete recovery of I_{Na}. The relationships between the conditioning potentials and the normalized amplitude of I_{Na} with or without addition of KMUP-1 (3 μM) were plotted and fitted with eq. 2, as described in *Methods*. In control, $V_{1/2}$ = −46.8 ± 3.1 mV, k = 11.4 ± 1.6 mV (n = 7), whereas, in the presence of KMUP-1 (3 μM), V = −57.9 ± 3.8 mV, k = 11.1 ± 1.5 mV (n = 7). Thus, cell exposure to KMUP-1 not only suppresses the maximal conductance of I_{Na} but also shifts the inactivation curve to a hyperpolarized potential by about −10 mV. In contrast, no discernible change in the slope (i.e. k value) of I_{Na} inactivation curve was observed in the presence of KMUP-1. Taken together, it is clear that KMUP-1 is capable of modifying the steady-state inactivation of I_{Na} in these cells.

Lack of effect of KMUP-1 on the recovery of I_{Na} from inactivation

The effect of KMUP-1 on the recovery of I_{Na} from inactivation was studied with a double-pulse protocol. The recovery of I_{Na} from inactivation at a holding potential of −80 mV was examined at different interpulse intervals with a test step (−20

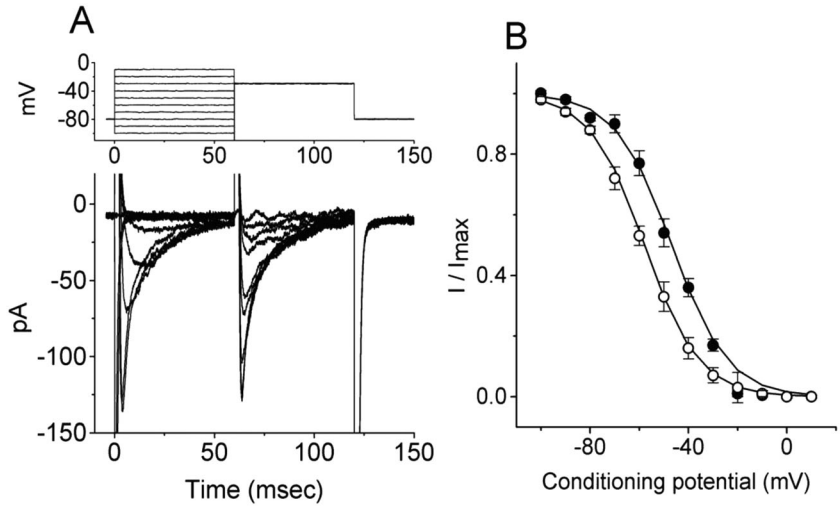

Figure 4

Steady-state inactivation curve of I_{Na} in the absence and presence of KMUP-1. With the aid of a double-pulse protocol, the steady-state inactivation parameters of I_{Na} were obtained with or without application of KMUP-1 (3 μM). As indicated in the upper part of (A), the conditioning voltage pulses with a duration of 60 ms to various membrane potentials were applied from a holding potential of −80 mV. Following a family of conditioning pulses, a test pulse to −20 mV with a duration of 60 ms was applied to evoke I_{Na}. (A) Superimposed traces of I_{Na} in the control. (B) Steady-state inactivation curves of I_{Na} obtained with or without KMUP-1 (3 μM; means ± SEM; n = 6–9 for each point). The normalized amplitude of I_{Na} (I/I_{max}) was constructed against the conditioning potential, and each smooth curve was fitted by eq. 2 as described in *Methods*.

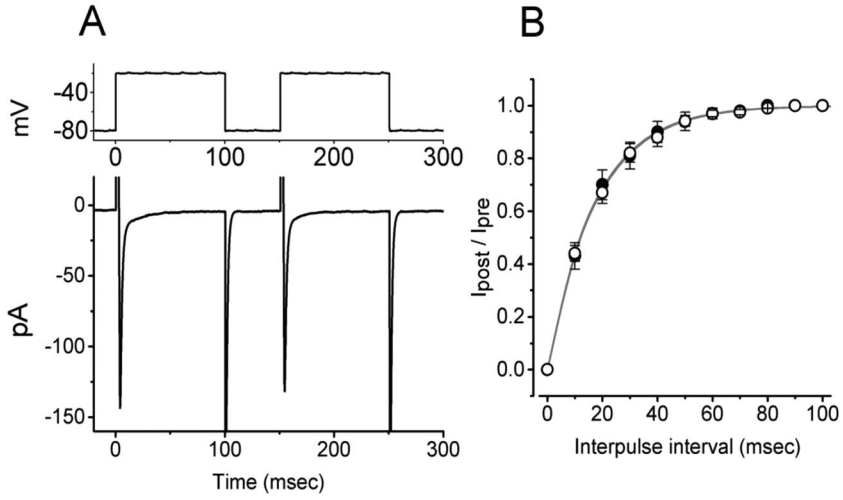

Figure 5

Lack of effect of KMUP-1 on the recovery of I_{Na} from inactivation in GH_3 cells. In these experiments, cells, bathed in Ca^{2+}-free Tyrode's solution containing 10 mM TEA, were depolarized from −80 to −20 mV with a duration of 100 ms, and different interpulse durations were then applied. An example of current trace obtained by a two-pulse protocol is illustrated in (A). (B) Recovery of I_{Na} with or without KMUP-1 (3 μM; means ± SEM; n = 5–8 for each point). Note that after application of KMUP-1, the time course of recovery from inactivation in these cells remains unchanged.

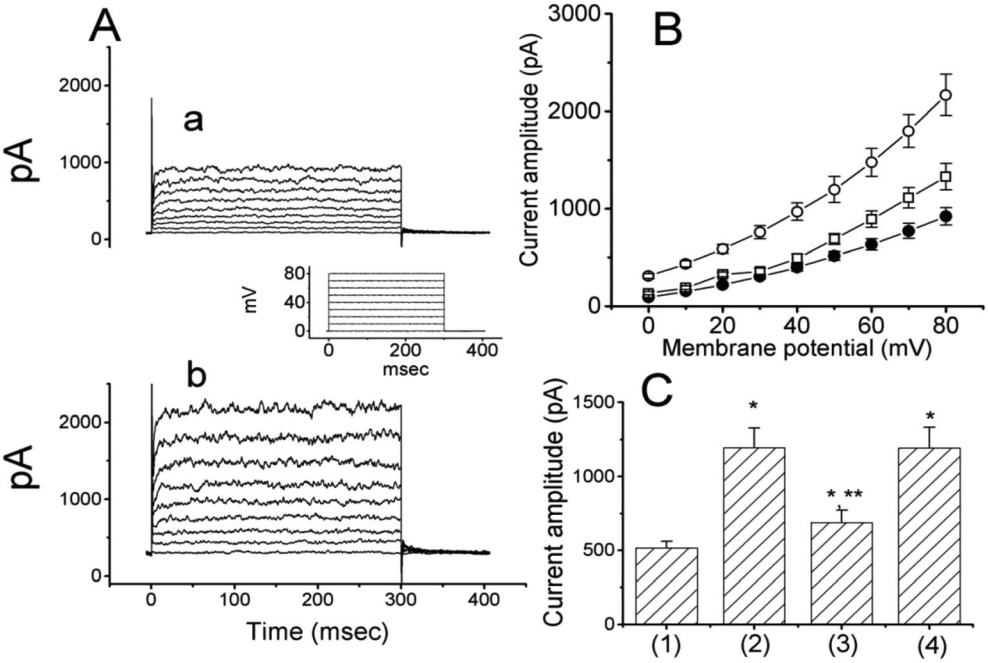

Figure 6

Effect of KMUP-1 on $I_{K(Ca)}$ in GH_3 cells. These experiments were conducted in the cells that were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Each cell was held at 0 mV, and the depolarizing steps to different potentials ranging from 0 to +80 mV at a rate of 0.1 Hz were delivered. (A) Superimposed current traces obtained with or without KMUP-1 (3 μM). Inset indicates the voltage protocol used. (B) I–V relationships of $I_{K(Ca)}$ obtained in the control, during exposure to 3 μM KMUP-1 and washout of KMUP-1 (mean ± SEM; n = 6–8 for each point). (C) Effect of paxilline and glimepiride on KMUP-1-stimulated $I_{K(Ca)}$ (means ± SEM; n = 6–9 for each bar). *P< 0.05, significantly different from control group. **P< 0.05, significantly different from KMUP-1 (3 μM) alone group. Note that unlike paxilline, glimepiride is not capable of reversing KMUP-1-stimulated $I_{K(Ca)}$.

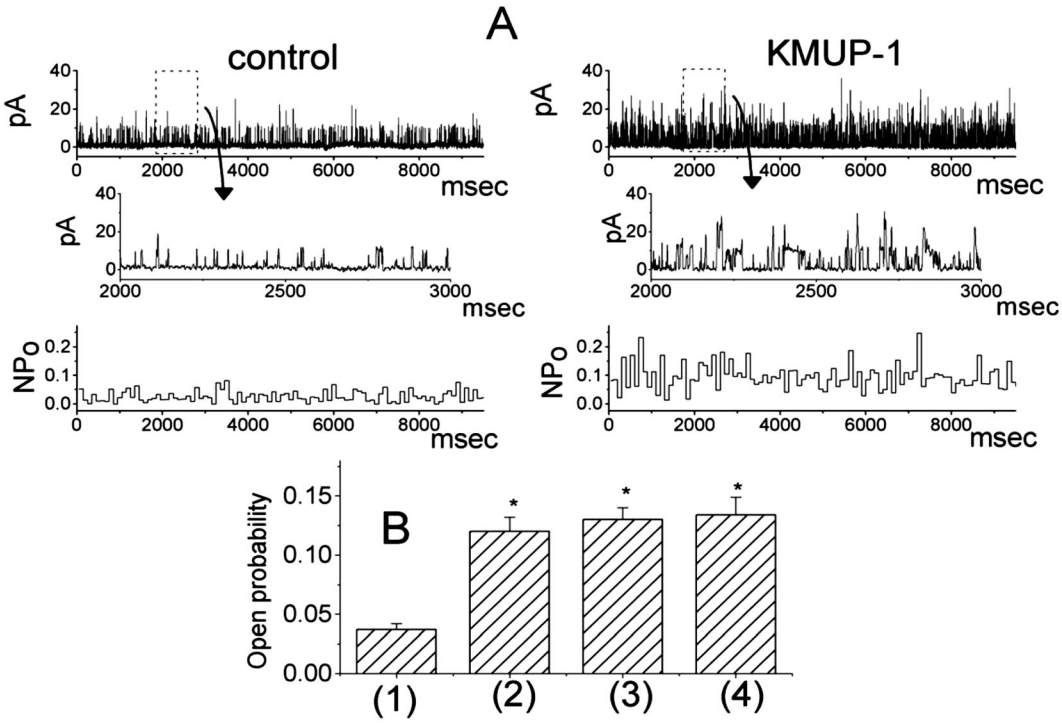

Figure 7

Effect of KMUP-1 on BK_{Ca}-channel activity recorded from GH_3 cells. In these experiments, cells were bathed in a high-K^+ solution (145 mM) containing 1.8 mM $CaCl_2$ and single-channel recordings from cell-attached patches at +60 mV were made. (A) The activity of BK_{Ca} channels recorded before (left) and during the application (right) of 3 μM KMUP-1 into the bath. The middle part in (A) shows the expanded record as indicated in the upper part (dashed box). Upward deflections represent the opening events of the channel. The lowest part in (A) shows the time course of change in the open probability (NP_o) of unitary outward currents before (left) and during the application (right) of 3 μM KMUP-1. (B) Summary of data showing effect of KMUP-1, YC-1 and KMUP-1 plus YC-1 on the open probability of BK_{Ca} channels recorded from GH_3 cells (means ± SEM; n = 6–9 for each bar). In KMUP-1 plus YC-1 experiments, YC-1 (3 μM) was subsequently applied after the cells were exposed to 3 μM KMUP-1. $^*P<$ 0.05, significantly differently different from control group.

mV for 100 ms) to determine the effect of KMUP-1 on $I_{Na,}$ recovery. As shown in Figure 4, the amplitude of I_{Na} almost completely recovered from inactivation when the recovery time was about 80 ms. The time course of recovery from inactivation in the control was fitted to a single exponential function with a time constant of 17.3 ± 0.5 ms (n = 9). However, in the presence of KMUP-1 (3 μM), the recovery from inactivation remained unaltered with a time constant of 17.6 ± 0.5 ms (n = 7, $P > 0.05$). Therefore, the recovery of I_{Na} from inactivation tends to be unaffected by the presence of KMUP-1 (Figure 5).

Stimulatory effect of KMUP-1 on $I_{K(Ca)}$ in GH_3 cells

Previous reports have shown the ability of KMUP-1 to increase K^+ outward current in vascular myocytes (Wu *et al.*, 2005). We further evaluated the possible effect of KMUP-1 on $I_{K(Ca)}$ in GH_3 cells. To measure $I_{K(Ca)}$, cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$, and the pipette solution contained a low concentration (0.1 mM) of EGTA and 3 mM ATP. When the cell was held at 0 mV, $I_{K(Ca)}$ was elicited in response to a series of voltage pulses from 0 to +80 mV in 10 mV increments at a rate of 0.1 Hz. As depicted in Figure 6, KMUP-1 (3 μM) applied to the bath produced a drastic increase in the amplitude of $I_{K(Ca)}$ throughout the entire voltage-clamp step. For example, when the depolarizing pulses from 0 to +50 mV were applied, KMUP-1 (3 μM) significantly increased $I_{K(Ca)}$ to 1195 ± 134 pA (n = 7) from a control value of 516 ± 45 pA (n = 7, $P < 0.05$). After KMUP-1 was removed, $I_{K(Ca)}$ almost returned to the control level. The averaged I–V relationship of $I_{K(Ca)}$ in the control, during cell exposure to KMUP-1 and after washout of KMUP-1, is illustrated in Figure 6B.

The next series of experiments was made to investigate whether the stimulatory effect of KMUP-1 on $I_{K(Ca)}$ can be altered by further application of paxilline or glimepiride. Paxilline is a blocker of BK_{Ca} channels, whereas glimepiride can suppress the activity of ATP-sensitive K^+ (K_{ATP}) channels, thus influencing stimulus-secretion coupling (Morishita *et al.*, 2000; Wu *et al.*, 2000b; Wu, 2003; Wu and Chang, 2006). As shown in Figure 6C, paxilline (1 μM) was effective in reducing the amplitude of $I_{K(Ca)}$ stimulated by KMUP-1; however, glimepiride (3 μM) was found to have minimal effect on it. The KMUP-1-stimulated $I_{K(Ca)}$ observed in these cells is thus sensitive to block by paxilline.

Effect of KMUP-1 on the activity of BK_{Ca} channels expressed in GH_3 cells

To further evaluate how KMUP-1 can affect $I_{K(Ca)}$, single-channel recordings were performed in these cells. In cell-attached configuration, bath application of KMUP-1 (3 μM)

could progressively increase the activity of BK_{Ca} channels. As shown in Figure 7, when KMUP-1 (3 μM) was applied to the bath, the probability of channel openings at the level of +60 mV significantly increased from 0.037 ± 0.005 to 0.121 ± 0.012 ($n = 8$, $P < 0.01$). However, no significant difference in single-channel amplitude in the absence and presence of KMUP-1 [9.7 ± 0.4 vs. 9.8 ± 0.4 pA ($n = 8$), $P > 0.05$] can be detected.

>A previous study reported that depletion of Ca^{2+} stores facilitated Ca^{2+} influx, which had been induced by the elevation of intracellular cyclic GMP in GH_3 cells (Willmott *et al.*, 1996). We further test the hypothesis that KMUP-1-stimulated channel activity is associated with its activation of soluble GC (Wu *et al.*, 2001; Liu *et al.*, 2007). As shown in Figure 7C, when YC-1 (3 μM) was applied to the bath, BK_{Ca}-channel activity was elevated. YC-1, known to potently activate soluble GC, has been reported to increase the amplitude of $I_{K(Ca)}$ in GH_3 cells (Ko *et al.*, 1994; Wu *et al.*, 2000a; Wu, 2003; Liu and Wu, 2005). However, further application of YC-1 (3 μM) in continued presence of KMUP-1 did not produce an additional rise in channel activity. Furthermore, in inside-out patches from GH_3 cells, KMUP-1 (10 μM) applied to the intracellular side of detached channels was not found to have any effect on BK_{Ca}-channel activity (data not shown). Therefore, in close similarity to previous observations made in basilar arterial myocytes (Wu *et al.*, 2005), our findings indicate that KMUP-1 can interact with BK_{Ca} channels to increase the amplitude of $I_{K(Ca)}$ in these cells. This action is closely associated with a mechanism linked to its activation of soluble GC.

Effect of KMUP-1 on ACs and BK_{Ca} channels elicited by triangular voltage pulses in GH_3 cells

Numerous reports have demonstrated that changes in the magnitude of late I_{Na} might affect the generation of neuronal APs (Catterall *et al.*, 2005; George *et al.*, 2009; Wu *et al.*, 2009a). The questions thus arise how KMUP-1 affects the subthreshold depolarization associated with the initiation of excitation in GH_3 cells. In this series of experiments, cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$, and cell-attached current recordings were made to measure AC and BK_{Ca}-channel activity simultaneously (Wu *et al.*, 2006; Wu *et al.*, 2009a). When the cell was held at −100 mV, the triangular voltage pulses with a duration of 800 ms were applied. The corresponding current traces in response to this voltage-clamp protocol are illustrated in Figure 8A. Notably, the currents consist of two components, that is, AC and BK_{Ca}-channel activity. The AC appears during the rising ramp, while the activity of BK_{Ca} channels, which appears as downward deflections, can be seen at the level of −100 mV. When cells were exposed to KMUP-1, the latency of AC generation in response to triangular voltage ramps was progressively prolonged. Similar results were also obtained when cells were exposed to ranolazine (3 μM) or riluzole (3 μM) (Figure 8B). Ranolazine and riluzole were recently reported to be blockers of late I_{Na} (Wu *et al.*, 2009c; Wu *et al.*, 2009a). In addition to the slowing in the initiation of AC, a significant increase in BK_{Ca}-channel activity was seen in the presence of KMUP-1.

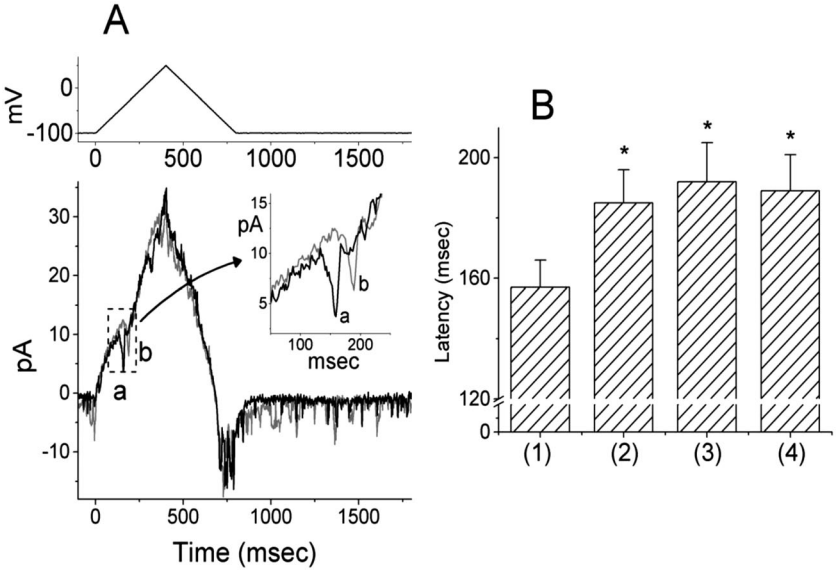

Figure 8

Effect of KMUP-1 on action currents and BK_{Ca}-channel activity in response to triangular voltage ramps. In these experiments, cell-attached current recordings were made in cells bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The potential was held at −100 mV so that, under cell-attached single-channel recordings, membrane potential with respect to resting potential was around +30 mV. The triangular ramp pulses with a duration of 800 ms at a rate of 0.05 Hz were then delivered. In (A), current traces in response to triangular voltage ramps as indicated in the upper part were obtained in the control (a) and during cell exposure to 3 μM KMUP-1 (b). Inset indicates expanded records of action currents, which represent APs (dashed box) and appear as a brief spike in the downward direction. Notably, the activity of BK_{Ca} channels at −100 mV appearing in downward deflections was elevated in the presence of KMUP-1. (B) Summary of data showing effect of KMUP-1 on the latency in the generation of action currents elicited by triangular voltage ramps (means ± SEM; $n = 6$–9 for each bar). *$P < 0.05$, significantly different from control group.

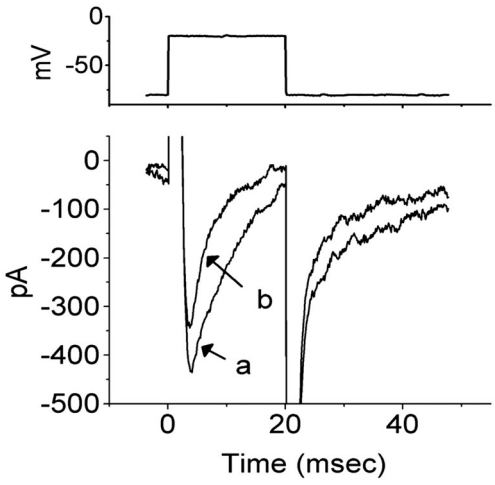

Figure 9

Inhibitory effect of KMUP-1 on HEK293T cells expressing *SCN5A*. In these experiments, cells were bathed in Ca^{2+}-free Tyrode's solution. Each cell was depolarized from −80 to −20 mV with a duration of 20 ms. (A) Original current traces obtained with or without KMUP-1 (10 μM).

Inhibitory effects of KMUP-1 on I_{Na} in SCN5A-expressing HEK293T cells

In a final set of experiments, we evaluated whether KUMP-1 exerts any effect on the amplitude of I_{Na} in HEK293T cells transfected with *SCN5A*. Under our experimental conditions, the transfection of *SCN5A* into HEK293T cells could result in the appearance of I_{Na}. When tetrodotoxin (1 μM) was applied, I_{Na} recorded in transfected cells could be suppressed. When KMUP-1 (10 μM) was applied to the bath, the peak amplitude of I_{Na} was significantly diminished. In addition, within 2 min of exposing the cells to KMUP-1 (10 μM), the amplitude of I_{Na} was reduced, along with the increased rate of current inactivation (Figure 9). When cells were exposed to KMUP-1 (10 μM), the value of the slow components of I_{Na} inactivation was significantly decreased to 8.5 ± 0.3 ms from a control of 14.1 ± 3.4 ms ($n = 7$, $P < 0.05$). The time constant of I_{Na} deactivation was also reduced from 6.2 ± 0.3 to 4.6 ± 0.2 ms ($n = 7$, $P < 0.05$). Our experimental results indicate that *SCN5A*-encoded I_{Na} can be functionally expressed in HEK293T cells. Similar to the observations made in GH_3 cells, the exposure to KMUP-1 is able to reduce the peak amplitude and the inactivation time course of I_{Na} in HEK293T cells expressing *SCN5A*.

Discussion and conclusion

The present results demonstrate that in pituitary GH_3 cells, KMUP-1 produces a depressant action on I_{Na} in a concentration-dependent and state-dependent fashion. KMUP-1 was noted to preferentially inhibit transient over late I_{Na} in a concentration range from 0.1 to 30 μM (IC_{50} = 1.8 vs. 22.5 μM), indicating that there is a 10-fold selectivity for its block of late versus transient I_{Na}. In particular, these IC_{50} values are of the same order of magnitude as the concentrations used to stimulate soluble GC in other preparations (Wu *et al.*, 2001; Wu *et al.*, 2004; Wu *et al.*, 2005; Liu *et al.*, 2007). Therefore, besides GC activation, KMUP-induced block of I_{Na} could be a potential mechanism through which it may depress the excitability of neurons, or neuroendocrine or endocrine cells *in vivo*. However, the differential effects by this agent of transient and late I_{Na} observed in this study suggest that this compound at a low concentration might increase the repolarization phase of neurons and endocrine or neuroendocrine cells following an AP to a greater extent, as compared with the fact that it depresses AP amplitude. Consequently, as cells are exposed to relatively low concentrations of this agent, the frequency for repetitive or bursting firing of these cells can be raised, despite a lowering of AP amplitude.

In our study, the effects of KMUP-1 on I_{Na} were not limited to its inhibition of the peak amplitude of these currents. A notable feature of the block of I_{Na} by KMUP-1 observed in GH_3 cells is that the decay phase of the current (i.e. slow component of inactivation time course) were shortened in the presence of this agent, while no change in the initial rising phase of the current was seen. The rate of I_{Na} inactivation was elevated as the KMUP-1 concentration increased. In addition to the decreased amplitude of transient I_{Na}, the value of $\tau_{inact(S)}$ was significantly decreased, suggesting that the KMUP-1 molecule is unable to reach the binding site unless the Na_V channel is in the open state. A hyperpolarizing shift in the steady-state inactivation of I_{Na} can also be detected during exposure to KMUP-1. However, this agent did not alter the time course in the recovery of I_{Na} from inactivation. These results thus lead us to suggest that block by KMUP-1 of I_{Na} presented here tends to be located at the open state of Na_V channels. This feature can be incorporated in a minimal binding scheme (i.e. closed ↔ open ↔ open-blocked) as described in *Methods* (Wu *et al.*, 2009b). As a result, KMUP-1-induced block apparently is not instantaneous but develops with time after the channel becomes opened, thereby producing an increase in current inactivation. $Na_V1.7$ was found to be a major subfamily of Na_V channels functionally expressed in the pituitary gland (Morinville *et al.*, 2007). It still remains to be determined whether other isoforms of Na_V channels can be differentially subject to block by this agent or other structurally related compounds.

Previous studies made in arterial myocytes and isolated vessel preparations (Wu *et al.*, 2001; Lin *et al.*, 2002) have reported that KMUP-1 was able to increase the activity of K_{ATP} channels, thereby resulting in its vasorelaxant actions. However, the pipette solution used in our experiments contained 3 mM ATP, a value that is high enough to abolish K_{ATP}-channel activity (Wu *et al.*, 2000a; Wu and Chang, 2006). Moreover, KMUP-1-induced increase in K^+ outward currents was not altered by further application of glimepiride, a blocker of K_{ATP} channels. Therefore, it seems unlikely that the observed increase in outward currents caused by KMUP-1 in GH_3 cells arises primarily from the activation of these channels.

In cell-attached recordings, bath application of KMUP-1 can increase the activity of BK_{Ca} channels; however, in the experiments with excised membrane patches, KMUP-1, when applied intracellularly, was not noted to exert any effects on channel activity. Additionally, subsequent application of YC-1, another activator of soluble GC (Ko *et al.*, 1994), was

not found to increase BK_{Ca}-channel activity further. YC-1 was reported to stimulate BK_{Ca}-channel activity in GH_3 cells (Wu *et al.*, 2000a). Taken together, in pituitary GH_3 cells, KMUP can interact with BK_{Ca} channels to increase the amplitude of $I_{K(Ca)}$ probably through its GC activation.

Previous report demonstrated the ability of KMUP-1 to antagonize the phenylephrine-stimulated contractility in prostate (Liu *et al.*, 2007), suggesting that it can act as a blocker of α-adrenoceptors. In this study, we were able to demonstrate that KMUP-1 can differentially produce an inhibitory effect on transient and late I_{Na} in GH_3 cells. However, these inhibitory effects observed in this study were not reversed by subsequent application of either phenylephrine or cirazoline (data not shown). Cirazoline is a compound known to be a selective blocker of α_1-adrenoceptors. Therefore, it is conceivable that KMUP-1-induced inhibition of transient or late I_{Na} described here is direct and independent of the binding to α_{1A}/α_{1D}-adrenoceptors.

Sustained I_{Na} might be modulated in certain conditions by the activity of protein kinase(s)-dependent pathways (Tateyama *et al.*, 2003). Similar to YC-1 and Bay 41-2272, KMUP-1 was previously reported to be an activator of soluble GC (Ko *et al.*, 1994; Wu *et al.*, 2001; Lin *et al.*, 2002; Wu *et al.*, 2004Liu and Wu, 2005). The GH_3 cell line used in this study has been recognized to express the activity of GC and protein kinase A or C (Willmott *et al.*, 1996; Wu *et al.*, 2000a; Liu and Wu, 2005; Song *et al.*, 2008). One may then argue that KMUP-1-mediated inhibition of I_{Na} is influenced by any changes in these signal-transduction pathways. However, KMUP-1-induced inhibition of I_{Na} could not be reversed by further addition of ODQ (10 μM), an inhibitor of soluble GC. In GH_3 cells preincubated with ODQ (10 μM) for 6 h, this agent still was also effective at suppressing I_{Na} amplitude and at enhancing I_{Na} inactivation (data not shown). Additionally, in our study, worthy of noting is that KMUP-1 can diminish the peak amplitude of I_{Na} with a concomitant increase of current inactivation in HEK293T cells expressing *SCN5A*. Taken together, these results can be explained by the possibility that the ability of this compound to suppress I_{Na} is direct and unrelated to any changes in the activity of protein kinase(s). It is also likely that the hydrophobic domain of this molecule does not bind to the Na^+ channels, while its diethylpirazine domain may interact with the pore region of Na_V channel, although the detailed binding mechanism(s) need to be further elucidated.

In this study, we also used cell-attached current recordings to simultaneously examine the ACs and the activity of BK_{Ca} channels in response to triangular ramp pulse (Costantin and Charles, 1999; Wu *et al.*, 2006; Wu *et al.*, 2009c). Notably, KUMP-1 applied to the bath significantly prolonged the latency of AC generation elicited by triangular voltage. Concomitant with this change, the open probability of BK_{Ca} channels was elevated in the same cell attached. These results suggest that KMUP-1-induced effects on neurons or neuroendocrine cells are attributable in part to its activation of BK_{Ca} channels as described previously in vascular myocytes (Lin *et al.*, 2002; Wu *et al.*, 2005). However, besides that, the ability of this compound to inhibit I_{Na} has important implications in understanding its depressant action on cell excitability. Due to its effects on BK_{Ca} channels and I_{Na}, it is expected that this agent may influence stimulus-secretion coupling, thereby depressing hormonal secretion in endocrine cells like GH_3 cells, if similar results were found *in vivo*.

The possible role of $Na_V1.7$ channels in vascular smooth myocytes has been recently reported (Meguro *et al.*, 2009). It has also been shown that prostate cancer (PC-3) cells can functionally express $Na_V1.7$ channels, and the activity of these channels appears to be linked to cancer invasiveness (Nakajima *et al.*, 2009). More recently, the studies also showed that ranolazine, a selective blocker of late I_{Na}, can be efficacious in improving endothelial function and myocardial perfusion (Deshmukh *et al.*, 2009; Venkataraman *et al.*, 2009). Therefore, from a pharmacological standpoint, it will be of importance to determine to what extent the actions of this compound on ion currents observed in this study are responsible for its effects on corpus cavernosum, blood vessels, trachea and neurons (Lin *et al.*, 2002; Wu *et al.*, 2004; Liu and Wu, 2005; Wu *et al.*, 2005; Liu *et al.*, 2007; Hsu *et al.*, 2010). KMUP-1 and its structurally related compounds may be considered as useful pharmacological probes for gaining insights in the possible mechanisms controlling the gating of Na_V channels. Our results suggest that they might also be useful for treating the aberrant secretion of prolactin existing in different types of pituitary disorders.

Acknowledgements

This research project was funded by the National Science Council (NSC-98-2320-B-006-027-MY3 and NSC-102-2628-B-037-001-MY3) and the Program for Promoting Academic Excellence and Developing World Class Research Centers, Ministry of Education, Taiwan. The authors are grateful to Hsien-Ching Huang and Chia-Chen Yeh for their assistances.

Author Contributions

Y. C. L. and S. N. W. conceived and designed the experiments. Y. C. L., Y. T. T., C. M. L., B. N. W. and S. N. W. performed experiments and analysed the data. Y. C. L. and S. N. W. contributed reagents/materials/analysis tools. Y. C. L. and S. N. W. wrote the paper.

Conflict of Interest

None of the authors have any potential conflict of interest nor financial interests to disclose.

References

Alexander SP, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Catterall WA *et al.* (2013a). The concise guide to pharmacology 2013/14: Ion channels. Br J Pharmacol 170 1607–1651.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al* (2013b). The Concise Guide to PHARMACOLOGY 2013/14: Enzymes. Br J Pharmacol 170: 1797–1867.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013c). The Concise Guide to PHARMACOLOGY 2013/14: G Protein-Coupled Receptors. Br J Pharmacol 170: 1459–1581.

Bankton JR, Sampson KJ, Kateriya S, Glaaser IW, Malito DL, Chung WK *et al.* (2007). A novel LQT-3 mutation disrupts an inactivation gate complex with distinct rate-dependent phenotypic consequences. Channels 1: 273–280.

Catterall WA, Goldin AL, Waxman SG (2005). International Union of Pharmacology. XLVII. Nomenclature and structure–function relationships of voltage-gated sodium channels. Pharmacol Rev 57: 397–409.

Chen JY, Jiang MC, Chu LW, Hsieh SL, Chen IJ, Wu BN (2011). KMUP-1 inhibits L-type Ca(2)(+) channels involved the protein kinase C in rat basilar artery myocytes. Kaohsiung J Med Sci 27: 538–543.

Costantin JL, Charles AC (1999). Spontaneous action potentials initiate rhythmic intercellular calcium waves in immortalized hypothalamic (GT1-1) neurons. J Neurophysiol 82: 429–435.

Deshmukh SH, Patel SR, Pinassi E, Mindrescu C, Hermance EV, Infantino MN *et al.* (2009). Ranolazine improves endothelial function in patients with stable coronary artery disease. Coron Artery Dis 20: 343–347.

George J, Dravid SM, Prakash A, Xie J, Peterson J, Jabba SV *et al.* (2009). Sodium channel activation augments NMDA receptor function and promotes neurite outgrowth in immature cerebrocortical neurons. J Neurosci: Official J Soc Neurosci 29: 3288–3301.

Hsu YY, Liu CM, Tsai HH, Jong YJ, Chen IJ, Lo YC (2010). KMUP-1 attenuates serum deprivation-induced neurotoxicity in SH-SY5Y cells: roles of PKG, PI3K/Akt and Bcl-2/Bax pathways. Toxicology 268: 46–54.

Ko FN, Wu CC, Kuo SC, Lee FY, Teng CM (1994). YC-1, a novel activator of platelet guanylate cyclase. Blood 84: 4226–4233.

Lin RJ, Wu BN, Lo YC, Shen KP, Lin YT, Huang CH *et al.* (2002). KMUP-1 relaxes rabbit corpus cavernosum smooth muscle *in vitro* and *in vivo*: involvement of cyclic GMP and K(+) channels. Br J Pharmacol 135: 1159–1166.

Liu CM, Lo YC, Wu BN, Wu WJ, Chou YH, Huang CH *et al.* (2007). cGMP-enhancing- and alpha1A/alpha1D-adrenoceptor blockade-derived inhibition of Rho-kinase by KMUP-1 provides optimal prostate relaxation and epithelial cell anti-proliferation efficacy. Prostate 67: 1397–1410.

Liu YC, Wu SN (2005). BAY 41-2272, a potent activator of soluble guanylyl cyclase, stimulates calcium elevation and calcium-activated potassium current in pituitary GH cells. Clin Exp Pharmacol Physiol 32: 1078–1087.

Meguro K, Iida H, Takano H, Morita T, Sata M, Nagai R *et al.* (2009). Function and role of voltage-gated sodium channel NaV1.7 expressed in aortic smooth muscle cells. Am J Physiol Heart Circ Physiol 296: H211–219.

Morinville A, Fundin B, Meury L, Jureus A, Sandberg K, Krupp J *et al.* (2007). Distribution of the voltage-gated sodium channel Na(v)1.7 in the rat: expression in the autonomic and endocrine systems. J Comp Neurol 504: 680–689.

Morishita M, Iwasaki Y, Yamamori E, Nomura A, Mutsuga N, Yoshida M *et al.* (2000). Antidiabetic sulfonylurea enhances secretagogue-induced adrenocorticotropin secretion and proopiomelanocortin gene expression *in vitro*. Endocrinology 141: 3313–3318.

Nakajima T, Kubota N, Tsutsumi T, Oguri A, Imuta H, Jo T *et al.* (2009). Eicosapentaenoic acid inhibits voltage-gated sodium channels and invasiveness in prostate cancer cells. Br J Pharmacol 156: 420–431.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP *et al.* (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledge base of drug targets and their ligands. Nucl Acids Res 42: D1098–1106.

Song T, Hatano N, Kambe T, Miyamoto Y, Ihara H, Yamamoto H *et al.* (2008). Nitric oxide-mediated modulation of calcium/calmodulin-dependent protein kinase II. Biochem J 412: 223–231.

Tateyama M, Kurokawa J, Terrenoire C, Rivolta I, Kass RS (2003). Stimulation of protein kinase C inhibits bursting in disease-linked mutant human cardiac sodium channels. Circulation 107: 3216–3222.

Venkataraman R, Belardinelli L, Blackburn B, Heo J, Iskandrian AE (2009). A study of the effects of ranolazine using automated quantitative analysis of serial myocardial perfusion images. JACC Cardiovasc Imaging 2: 1301–1309.

Webb J, Cannon SC (2008). Cold-induced defects of sodium channel gating in atypical periodic paralysis plus myotonia. Neurology 70: 755–761.

Willmott NJ, Asselin J, Galione A (1996). Calcium store depletion potentiates a phosphodiesterase inhibitor- and dibutyryl cGMP-evoked calcium influx in rat pituitary GH3 cells. FEBS Lett 386: 39–42.

Wu BN, Lin RJ, Lin CY, Shen KP, Chiang LC, Chen IJ (2001). A xanthine-based KMUP-1 with cyclic GMP enhancing and K(+) channels opening activities in rat aortic smooth muscle. Br J Pharmacol 134: 265–274.

Wu BN, Lin RJ, Lo YC, Shen KP, Wang CC, Lin YT *et al.* (2004). KMUP-1, a xanthine derivative, induces relaxation of guinea-pig isolated trachea: the role of the epithelium, cyclic nucleotides and K+ channels. Br J Pharmacol 142: 1105–1114.

Wu BN, Tu HF, Welsh DG, Chen IJ (2005). KMUP-1 activates BKCa channels in basilar artery myocytes via cyclic nucleotide-dependent protein kinases. Br J Pharmacol 146: 862–871.

Wu SN (2003). Large-conductance Ca^{2+}-activated K+ channels: physiological role and pharmacology. Curr Med Chem 10: 649–661.

Wu SN, Chang HD (2006). Diethyl pyrocarbonate, a histidine-modifying agent, directly stimulates activity of ATP-sensitive potassium channels in pituitary GH(3) cells. Biochem Pharmacol 71: 615–623.

Wu SN, Chen BS, Hsu TI, Peng H, Wu YH, Lo YC (2009a). Analytical studies of rapidly inactivating and noninactivating sodium currents in differentiated NG108-15 neuronal cells. J Theor Biol 259: 828–836.

Wu SN, Chen BS, Wu YH, Peng H, Chen LT (2009b). The mechanism of the actions of oxaliplatin on ion currents and action potentials in differentiated NG108-15 neuronal cells. Neurotoxicology 30: 677–685.

Wu SN, Hwang T, Teng CM, Li HF, Jan CR (2000a). The mechanism of actions of 3-(5′-(hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1) on Ca(2+)-activated K(+) currents in GH(3) lactotrophs. Neuropharmacology 39: 1788–1799.

Wu SN, Li HF, Chiang HT (2000b). Characterization of ATP-sensitive potassium channels functionally expressed in pituitary GH3 cells. J Membr Biol 178: 205–214.

Wu SN, Peng H, Chen BS, Wang YJ, Wu PY, Lin MW (2008). Potent activation of large-conductance Ca^{2+}-activated K+ channels by the

diphenylurea 1,3-bis-[2-hydroxy-5-(trifluoromethyl)phenyl]urea (NS1643) in pituitary tumor (GH3) cells. Mol Pharmacol 74: 1696–1704.

Wu SN, Wu AZ, Lin MW (2006). Pharmacological roles of the large-conductance calcium-activated potassium channel. Curr Top Med Chem 6: 1025–1030.

Wu SN, Wu YH, Chen BS, Lo YC, Liu YC (2009c). Underlying mechanism of actions of tefluthrin, a pyrethroid insecticide, on voltage-gated ion currents and on action currents in pituitary tumor (GH3) cells and GnRH-secreting (GT1-7) neurons. Toxicology 258: 70–77.