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Arecoline inhibits intermediate-conductance calcium-activated potassium channels in human glioblastoma cell lines

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ABSTRACT

Arecoline (ARE) is an alkaloid-type natural product from areca nut. This compound has numerous pharmacological and toxicological effects. Whether this agent interacts with ion channels to perturb functional activity of cells remains unknown. The effects of ARE on ionic currents were studied in glioma cell lines (U373 and U87MG) using patch-clamp technique. Like TRAM-34(1-[(2-chlorophenyl)-diphenylmethyl]pyrazole), ARE suppressed the amplitude of whole-cell voltage-gated K^+ currents in U373 cells elicited by a ramp voltage clamp. In cell-attached configuration, ARE did not modify the single-channel conductance of intermediate-conductance Ca^{2+}-activated K^+ (IK_{Ca}) channels; however, it did reduce channel activity. Its inhibition of IK_{Ca} channels was accompanied by a significant lengthening in the slow component of mean closed time of IK_{Ca} channels. Based on minimal kinetic scheme, the dissociation constant (K_D) required for ARE-mediated prolongation of mean closed time was 11.2 μM. ARE-induced inhibition of IK_{Ca} channels was voltage-dependent. Inability of ARE to perturb the activity of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels was seen. Under current-clamp recordings, ARE depolarized the membrane of U373 cells and DCEBIO reversed ARE-induced depolarization. Similarly, ARE suppressed IK_{Ca}-channel activities in oral keratinocytes. This study provides the evidence that ARE block IK_{Ca} channels in a concentration, voltage and state-dependent manner. ARE-induced block of IK_{Ca} channels is unrelated to the binding of muscarinic receptors. The effects of ARE on these channels may partially be responsible for the underlying cellular mechanisms by which it influences the functional activities of glioma cells or oral keratinocytes, if similar findings occur *in vivo*.

1. Introduction

Areca nut has long been used as a medical and psychoactive stimulant in China (Lee et al., 2014; Quinn Griffin et al., 2014) and was the leading cause for oral cancer (Nair et al., 1985; Shieh et al., 2004; Chen et al., 2008; Li et al., 2014). Arecoline (ARE; 1,2,5,6-tetrahydrol-1-methyl-3-pyridinecarboxylic acid methyl ester) is an alkaloid extracted from areca nut and is a non-selective agonist of muscarinic receptors and shown to have carcinogenicity, cytotoxicity and immunotoxicity (Dasgupta et al., 2006; Shih et al., 2010; Li et al., 2014).

The neuroprotective effects of ARE was found in Alzheimer's disease (Chandra et al., 2008; Sadashiva et al., 2009; Maezawa et al., 2011; Lee et al., 2013). It stimulates production of connective tissue growth factor in human buccal mucosal fibroblasts and induces HaCaT cell apoptosis (Deng et al., 2009; Li et al., 2014). ARE could inhibit cell cycle progression or mediate phospholipase C-dependent activation of protein kinases in glioma cells through its binding to muscarinic receptors (Tang et al., 2002; Ferretti et al., 2012, 2013). Reports showed that glioma cells possess M2 muscarinic receptors, activation by acetylcholine could stimulate small-conductance Ca^{2+}-activated K^+ (SK_{Ca}) channels in neocortical pyramidal neurons (Brombas et al., 2014; Ferretti et al., 2012, 2013). ARE has been shown to suppress ether-à-go-go-related K^+ current and stimulate muscarinic-K^+ channels or ATP-sensitive K^+ channels (Chen, 2005; Yoshizumi et al., 2008; Ling et al.,

Abbreviations: ARE, arecoline; BK_{Ca} channel, large-conductance Ca^{2+}-activated K^+ channel; CAPE, caffeic acid phenethyl ester; DCEBIO, 5,6-dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one; *I–V* relationship, current–voltage relationship; I_K, voltage-gated K^+ current; IK_{Ca} channel, intermediate-conductance Ca^{2+}-activated K^+ channel; K_D, dissociation constant; RT-PCR, reverse transcriptase-polymerase chain reaction; SK_{Ca} channel, small conductance Ca^{2+}-activated K^+ channel; TEA, tetraethylammonium chloride; TRAM-34, 1-((2-chlorophenyl) (diphenyl)methyl)-1*H*-pyrazole; V_{rest}, resting membrane potential.

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2012; Zhao et al., 2012). However, whether this compound directly interacts with ion channels to perturb ion currents and change membrane potential remains largely unexplored.

Reports showed that ion channels on malignant glioma cells was essential in influencing the functional activities of these cells (Weaver et al., 2006; Debska-Vielhaber et al., 2009). The activity of IK_{Ca} channels might interfere with the invasiveness of malignant glioma (Catacuzzeno et al., 2012; Turner et al., 2014). The IK_{Ca} channels are encoded by the KCNN4 gene. They were cloned from human, mouse and rat tissues and studied in non-excitable or neoplastic cells regarding their mechanisms linked to hormonal secretion, cell motility, cell proliferation and regulation of Ca^{2+} influx and/or K^+ efflux (Jensen et al., 2001; Shen et al., 2007; Wulff et al., 2007; Lallet-Daher et al., 2009; Ohya et al., 2011; Catacuzzeno et al., 2012; McFerrin et al., 2012; Diaz et al., 2014; Grossinger et al., 2014; Xu et al., 2014). IK_{Ca} channels have single-channel conductance of 20–60 pS and their pharmacological profile is quite distinguishable from those of BK_{Ca} or SK_{Ca} (Jensen et al., 2001; Shen et al., 2007; Wulff et al., 2007). The modulators of IK_{Ca} channels represent a potentially therapeutic approach to diseases including malignant gliomas (Yin et al., 2007; Maezawa et al., 2011; Ohya et al., 2011; Catacuzzeno et al., 2012; D'Alessandro et al., 2013).

We evaluated if ARE exerts any effects on ion currents in two glioma cell lines (U373 and U87MG) and in normal human oral keratinocytes. Our results showed that ARE is effective in suppressing the activity of IK_{Ca} channels and ARE-mediated inhibition of these channels accompanied by membrane depolarization appeared to be direct and unrelated to muscarinic receptors.

2. Materials and methods

2.1. Drugs and solutions

Arecoline hydrobromide (ARE; 1,2,5,6-tetrahydro-1-methyl-3-pyridinecarboxylic acid methyl ester, monohydrobromide; CAS-No. 300-08-3; $C_8H_{13}NO_2$·HBr), atropine, caffeine, ionomycin and tetraethylammonium chloride (TEA) were obtained from Sigma-Aldrich Inc. (St. Louis, MO), clortrimazole, DCEBIO (5,6-dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one) and TRAM-34 (1-((2-chlorophenyl) (diphenyl)methyl)-1*H*-pyrazole) were from Tocris Cookson Ltd. (Bristol, UK), verruculogen was from Alomone Labs. (Jerusalem, Israel) and caffeic acid phenethyl ester (CAPE) was from Cayman (Ann Arbor, MI). Chlorotoxin and margatoxin were provided by Dr. Woei-Jer Chuang (National Cheng Kung University), squamocin was by Dr. Yang-Chang Wu (China Medical University), and α-bungarotoxin was by Dr. Long-Sen Chang (National Sun Yat-sen University). The published mode of pharmacological actions for this study is illustrated in Table 1. Tissue culture media, fetal bovine serum, L-glutamine, penicillin-streptomycin, fungizone and trypsin were obtained from Invitrogen (Carlsbad, CA). All other chemicals were obtained from regular commercial chemicals and of reagent grade. Twice-distilled water was deionized through a Milli-Q water purification system (APS Water Services Inc., Van Nuys, CA).

The composition of bathing solution (i.e., 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 K^+ currents or membrane potential, patch pipettes were filled with an internal solution consisting of 130 mM K-aspartate, 20 mM KCl, 1 mM KH_2PO_4, 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. To avoid the contamination of Cl^- currents, Cl^- ions inside the pipette solution were replaced with aspartate. For the recordings of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) or intermediate-conductance Ca^{2+}-activated K^+ (IK_{Ca}) channels, high K^+-bathing solution was used and its composition was 145 mM KCl, 0.53 mM $MgCl_2$, and 5 mM HEPES-KOH, pH 7.2, and the pipette solution contained 145 mM KCl, 2 mM $MgCl_2$, and 5 mM HEPES-KOH, pH 7.2. In some sets of cell-attached current recordings, the recording pipettes were filled with K^+-containing solution in which 10 μM atropine or 1 μM α-bungarotoxin was included. The pipette solution was filtered on the day of use with a 0.22 μm pore size syringe filter (Millipore).

Table 1
Pharmacological actions of the compounds used in the study.

Compounds	*Published mode of pharmacological actions*
Atropine	Blocker of muscarinic receptors
α-Bungarotoxin	Blocker of α_7-nicotinic receptors
CAFE	Stimulator of BK_{Ca} channels
Caffeine	Stimulator of IK_{Ca} channels
Chlorotoxin	Blocker of chloride channels
Clotrimazole	Blocker of IK_{Ca} channels
Margatoxin	Blocker of $K_V1.3$-encoded current
Squamocin	Ca^{2+} ionophore
TEA	Non-selective blocker of I_K
TRAM-34	Blocker of IK_{Ca} channels
Verruculogen	Blocker of BK_{Ca} channels
Ionomycin	Ca^{2+} ionophore

2.2. Cell preparations

The glioblastoma cell lines (U373 and U87MG) were obtained from American Type Culture Collection (ATCC, Manassas, VA). They were grown and maintained at a density of 10^6/ml in DMEM/F12 nutrient media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. All cell lines were grown at 37 °C in a 5% CO_2 incubator as monolayer cultures and sub-cultured weekly. Fresh media were added every 2–3 days to maintain a healthy cell population. To observe cell growth, a Nikon Eclipse Ti-E inverted microscope (Li Trading Co., Taipei, Taiwan) equipped with a 5-megapixel cooled digital camera was used. In another separate series of experiments, U373 cells were incubated with chlorotoxin (1 μM), margatoxin (1 μM), or atropine (10 μM) at 37 °C for 6 h.

Primary cultures of normal human oral keratinocytes were prepared from keratinizing oral epithelial tissue collected from gingival tissue during third molar extraction. Cells were cultured in keratinocyte serum-free medium (Invitrogen), with low Ca^{2+} (0.1 mM) on flasks coated with rat-tail collagen. Cells were grown in 50-mL plastic culture flasks in a humidified environment of 5% CO_2/95% air at 37 °C (Shieh et al., 2005). All patients signed informed consent, and the study protocol was approved by a local ethical committee.

2.3. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

To detect the expression of $K_{Ca}3.1$ (KCNN4) channel mRNA in U373 and U87MG cells, a semi-quantitative RT-PCR assay was performed. Total RNA samples were extracted from cells according to TRIzol reagent protocol (Invitrogen) and reverse-transcribed into complementary DNA using Superscript II reverse-transcriptase (Invitrogen). The sequences of forward and reverse primers for KCNN4 were as follows: KCNN4-f, 5′-ACC TTT CAG ACA CAC TTT GG-3′; and KCNN4-r, 5′-TCT CTG CCT TGT TAA ACT CC-3′. The PCR cycling conditions were 35 cycles of 95 °C for 2 min, 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 10 min. The PCR products were made on 1.5% (w/v) agarose gel containing ethidium bromide and then visualized under ultraviolet trans-illumination. Optical densities of DNA bands were scanned and quantified by AlphaImager 2200 (ProteinSimple; Santa Clara, CA). The results are illustrated in Fig. 1.