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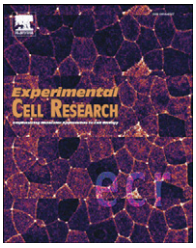

Research Article

KCa2.3 channel-dependent hyperpolarization increases melanoma cell motility

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ABSTRACT

Cell migration and invasion are required for tumour cells to spread from the primary tumour bed so as to form secondary tumours at distant sites. We report evidence of an unusual expression of KCa2.3 (SK3) protein in melanoma cell lines but not in normal melanocytes. Knockdown of the KCa2.3 channel led to plasma membrane depolarization, decreased 2D and 3D cell motility. Conversely, enforced production of KCa2.3 protein in KCa2.3 non-expressing cells led to the plasma membrane becoming hyperpolarized, and enhanced cell motility. In contrast, KCa3.1 channels had no effect on cell motility despite an active role in regulating membrane potential. Our data also suggest that membrane hyperpolarization increases melanoma cell motility and that this occurs through the KCa2.3 channel. Our findings reveal a previously unknown function of the KCa2.3 channel, and suggest that the KCa2.3 channel might be the only member of the Ca^{2+}-activated K^+ channel family involved in melanoma cell motility pathways.

Introduction

The KCa2.3 channel (SK3) is a small-conductance potassium channel (SKCa) belonging to calcium-activated potassium channel (KCa) family that comprises three isoforms: KCa2.1–3 [1]. The KCa2.3 channel controls the excitability of a nerve cell of the central nervous system by mediating after-hyperpolarization [2]. We recently suggested that the KCa2.3 channel participates in cancer cell migration, but the mechanisms were not fully characterized [3]. In view of the other physiological functions of KCa2.3, its involvement in cancer cell migration was unexpected and therefore needs to be confirmed. The KCa2.3 channel has several functional features that may contribute to cancerous cell migration: i) cyclical activation and inhibition of the KCa2.3 channel, following intracellular Ca^{2+} oscillations and/or cell volume changes, allow changes of cell shape ii) the KCa2.3 channel, an activated-membrane molecule, routinely adopts a polarized cell location, especially at protrusions (filopodia or lamellipodia) [4,5] and iii) the activity of the KCa2.3 channel is sensitive to variations in the free Ca^{2+} concentration often

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associated with cell motility/migration [1]. Thus, although the KCa2.3 channel had not previously been physiologically related to any cell migration process, its physical and functional features are compatible with an involvement in cancer cell migration.

One main cause for the malignancy of a cancer is the ability of tumour cells to spread from the primary tumour to form secondary tumours at distant sites. Before the formation of metastasis, cancer cells have to go through a series of sequential steps. First they invade adjacent tissues. Next, they pass through blood and lymph vessels and spread to distant permissive sites. Several mechanical processes (e.g. cell adhesion and motility) and various molecular events (e.g. acto-myosin contraction) govern tumour cell dissemination. Despite growing research efforts, the molecular mechanisms underlying metastasis formation are still fragmentary. Discovering and understanding the molecular mechanisms underlying metastasis formation will help in the design of innovative therapeutic strategies. This is of particular importance for melanoma because the metastatic forms of this cancer are largely refractory to existing therapies. The ability of melanoma to spread was simply thought to be a consequence of melanocytes originating from highly motile cells with enhanced survival properties [6].

Here, we report that KCa2.3 protein, which is produced in melanoma cells but not in untransformed melanocytes, forms functional KCa2.3 channels which hyperpolarize the plasma membrane and promote cell motility. In contrast, KCa3.1 channels had no effect on cell motility despite actively regulating membrane potential. Our data show that KCa2.3-dependent hyperpolarization increases melanoma cell motility.

Materials and methods

Cell culture

Four human metastatic melanoma cell lines were used in this study 518A2 (a gift from Dr. van der Minne L., Leiden, The Netherlands), SKmel28, HBL, and Bris (a gift from Dr. S. Priest, IGR, Paris, France). All cell lines were maintained in Dulbecco's modified Eagle's medium except for HBL that was maintained in Ham's F10 medium. Culture media were supplemented with 10% (v/v) foetal bovine serum. Normal human epithelial melanocyte (a gift from Dr. J.E. Branka, Effiscience, Rennes, France) were maintained in serum-free Melanocyte growth medium M2 according to the manufacturer's protocol (PromoCell, Heidelberg, Germany). Cells were grown in a humidified atmosphere at 37 °C (95% air, 5% CO_2). The absence of mycoplasma contamination was verified regularly using the Mycotech kit (Invitrogen).

Constructs, transfection, and transduction

To inhibit KCa2.3 expression, we constructed a lentiviral vector encoding a short hairpin RNA (shRNA) specifically targeting the human KCa2.3. The sequence encoding shKCa2.3 was obtained by PCR elongation of two partially complementary primers; this also allowed us to introduce two restriction enzyme sites to facilitate manipulations. Forward primer: shKCa2.3-BamH I 5′ ggATCCCCCCATTCCTggCgAgTACAATTCAAgAgATT; shKCa2.3-Hind III 5′ AAgCTTAAAAACCATTCCTggCgAgTACAATCTCTTgAATT, as a reverse primer. The underscored sequences are complementary. The shKCa2.3 fragment was inserted into pGEMT easy vector (Promega) and transferred into pH1, a plasmid expressing the shRNA from RNA pol III promoter (a gift from Anne Galy, Généthon-Evry), at Bgl II/Hind III sites. The H1 promoter linked to the shKCa2.3 sequence was then inserted between the Spe I and Cla I sites in a pHR'-derived vector [7], expressing the GFP from a CMV promoter and an IRES. For control experiments we constructed, following the same protocol as above, a lentiviral vector expressing an untargeted shRNA (pLenti-shRD). For this, we used the following primers, shRD1_S: 5′ggATCCCCgCCgACCAATTCACggCCgTTCAAgAgACg 3′ and shRD1_AS: 5′AAgCTTAAAAAgCCgACCAATTCACggCCgTCTCTTgAACg 3′.

For KCa2.3 over-expression, the full length rat KCa2.3 cDNA was cloned by recombination with the system developed by Invitrogen, a shuttle vector was generated containing the PCR product of the full length cDNA: forward primer: ggCCCCAAgATggACACTTCTgggCACTTC and reverse primer: TTAgCAACTgCTTgAACTTgTg.

293T cells were plated at 3×10^6 cells per 100 ml culture and grown overnight before transfection. Transfection was performed with 10 μg of pCMV-8.74 encoding HIV1 Gag-Pol and all accessory proteins, 5 μg of pH-CMV-G encoding the VSV-G and 5 μg of the lentiviral vector (pLenti-shKCA2.3 or pLenti-shRD), using a calcium phosphate transfection kit (Invitrogen) according to the manufacturer's instructions. After 24 h, the medium was removed and the cells were washed with PBS, then fresh medium was added. Supernatants were harvested 48 and 72 h later and filtered through a 0.45 μm pore-size Millex HA filter (Millipore). The lentiviral vectors were then concentrated on 20% sucrose cushion by centrifugation at 26 krpm for 90 min (Sorvall, Discovery 90SE). Viral titres were determined by transducing 293T cells with serial dilutions of the viral stock, and then counting GFP cells using FACS analysis.

Melanoma cells were transduced with lentiviral vector at multiplicities of infection (MOI) of 1 to 3 in the presence of polybrene (4 μg/ml, Sigma). The transduction rate determined by counting GFP cells was close to 90%.

Cell proliferation assays

Cell proliferation was determined using the tetrazolium salt reduction method (MTT), as described elsewhere [8]. Cells were seeded on 24-well plates at a density of 20,000 cells per well and measurements were performed in triplicate daily for 5 days. Note that drugs and high external potassium concentration used in trans-well migration assays had no effect on cell proliferation/viability (48 h).

Two-dimensional (2D) and 3D motility assays

Cell motility was analyzed in 24-well plates receiving 8-μm pore-size polyethylene terephthalate membrane cell culture inserts (Becton Dickinson, France), as previously described [8]. Briefly, 4×10^4 cells were seeded in the upper compartment with medium culture supplemented with 10% of FBS (±drugs/high external potassium concentration). The lower compartment was filled with medium culture supplemented with 20% FBS (±drugs/high external potassium concentration) as a chemoattractant. Two-dimensional motility assays were performed without coating except for HBL cell lines for which bovine fibronectin and gelatin (0.05%; 0.02%) were used (Sigma-Aldrich). Three-dimensional