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## **Epidermal growth factor upregulates motility of Mat-LyLu rat prostate cancer cells via voltage-gated Na<sup>+</sup> channel activity**

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Running title: EGF regulation of sodium channel in prostate cancer

Keywords: Voltage-gated sodium channel; prostate cancer; metastasis; Mat-LyLu cells; epidermal growth factor; nerve growth factor; migration.

## **ABSTRACT**

The main aim of this investigation was to determine whether a functional relationship existed between epidermal growth factor (EGF) and voltage-gated sodium channel (VGSC) upregulation, both associated with strongly metastatic prostate cancer cells. Incubation with EGF for 24 h more than doubled VGSC current density. Similar treatment with EGF significantly and dose-dependently enhanced the cells' migration through Transwell filters. Both the patch clamp recordings and the migration assay suggested that endogenous EGF played a similar role. Importantly, co-application of EGF and tetrodotoxin, a highly selective VGSC blocker, abolished 65% of the potentiating effect of EGF. It is suggested that a significant portion of the EGF-induced enhancement of migration occurred via VGSC activity.

## **ABBREVIATIONS**

I-V, current-voltage; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FBS, foetal bovine serum; MiI, migration index; NGF, nerve growth factor; PCa, prostate cancer; SEM, standard error; TTX, tetrodotoxin; VGSC, voltage-gated Na<sup>+</sup> channel.

## **INTRODUCTION**

Ionic activity plays a significant role in intracellular homeostasis under both physiological and pathophysiological conditions. Abnormally high levels of voltage-gated Na<sup>+</sup> channels (VGSCs) have been detected in rat and human metastatic prostate cancer (PCa) in vitro and in vivo (Grimes et al., 1995; Laniado et al., 1997; Smith et

al., 1998; Bennett et al., 2004; Diss et al., 2005) and also occurs in human glioblastomas (Labrakakis et al., 1997), oligodendrogliomas (Patt et al., 1996), melanomas (Allen et al., 1997), lung cancer (Blandino et al., 1995; Onganer and Djamgoz, 2005), medullary thyroid carcinomas (Klugbauer et al., 1995), neoplastic mesothelia (Fulgenzi et al., 2006), cervical cancer (Diaz et al., 2007) and breast cancer (Roger et al., 2003; Fraser et al., 2005). As regards the functional consequences of these VGSCs, most work has been done on PCa and it has been shown that VGSC activity enhances a range of metastatic cellular behaviours, including directional motility (Djamgoz et al., 2001; Fraser et al., 2003) and invasiveness (Grimes et al., 1995; Laniado et al., 1997). However, the mechanism(s) responsible for the VGSC upregulation in PCa is not known. VGSC expression generally is well known to be dynamic (Diss et al., 2004). In PCa, VGSC plasticity has been demonstrated by the sensitivity to external serum concentration, although the serum factor(s) responsible was not determined (Ding and Djamgoz, 2004). VGSC regulation by growth factors has been shown for a variety of cells, including pheochromocytoma PC12 cells (Toledo-Aral et al., 1995) and neurones (Blum et al., 2002). The role of growth factors has also been emphasised in PCa, as regards both androgen sensitivity (Culig et al., 1996) and progression from invasive to metastatic carcinoma (Culig et al., 1994). We have shown previously that nerve growth factor (NGF) upregulated functional VGSC expression and transwell migration in Mat-LyLu rat PCa cells but the two effects were not connected (Brackenbury and Djamgoz, 2007).

In the present study, we aimed to evaluate the role of epidermal growth factor (EGF) in this regard. EGF has recently been shown to potentiate VGSC currents in guinea

pig ventricular myocytes via tyrosine phosphorylation (Liu et al., 2007). Expressed prostatic fluid contains the highest concentration of EGF in the body (175.5 ng/ml; Russell et al., 1998; Gann et al., 1999), and EGF has been demonstrated to enhance cellular invasiveness, thus suggesting that it could also play a role in metastatic PCa (Turner et al., 1996; Kim et al., 1999; Montano and Djamgoz, 2004). These data collectively would suggest that EGF could underlie the VGSC upregulation in PCa. In the present study, we tested this hypothesis, again, using the Mat-LyLu rat Dunning cell model of PCa. These cells induce metastases in >90% of cases when injected into syngeneic rats (Isaacs et al., 1986) and express ~1,800× more VGSC/Nav1.7 mRNA, compared with their weakly/non-metastatic counterparts (Diss et al., 2001), and generate functional VGSCs (Grimes et al., 1995).

## **MATERIALS AND METHODS**

### **Cell culture**

Mat-LyLu cells were maintained in RPMI medium containing 1% heat-inactivated foetal bovine serum (FBS; Invitrogen/Gibco, Paisley, UK), supplemented with 250 nM dexamethasone. Medium was supplemented with 2 mM glutamine, 1 mM sodium pyruvate, and 100 IU/ml penicillin/streptomycin (Invitrogen/Gibco) (Grimes et al., 1995).

### **Pharmacology**

Mat-LyLu cells were plated for 24 h, serum starved for another 24 h and then treated with pharmacological agent(s) for a further 24 h. The agents used, their working concentrations and suppliers were as follows: EGF, 100 ng/ml (Calbiochem,

Nottingham, UK); AG1478, 1  $\mu$ M (Calbiochem), an inhibitor of EGF receptor (EGFR) tyrosine kinase (e.g. Liu et al., 1999); tetrodotoxin (TTX), 500 nM (Alomone, Jerusalem, Israel); and EGFR antibody, 1  $\mu$ g/ml (Oncogene Research Products/Calbiochem, Nottingham, UK).

### **Electrophysiology**

Patch-clamp recordings were performed as detailed before (e.g. Grimes and Djamgoz, 1998; Ding and Djamgoz, 2004). Patch pipettes were pulled from borosilicate glass capillaries (Clarke Electromedical GC100F) and typically had resistances of 5–10 M $\Omega$  when filled with intracellular solution containing: 145 mM CsCl; 5 mM NaCl; 2 mM MgCl<sub>2</sub>; 1 mM CaCl<sub>2</sub>; 11 mM EGTA and 10 mM HEPES (pH 7.2 adjusted with 1 M CsOH). The extracellular solution contained 144 mM NaCl; 5.4 mM KCl; 1 mM MgCl<sub>2</sub>; 5 mM CaCl<sub>2</sub>; 5 mM HEPES and 5.6 mM glucose (pH 7.2 adjusted with 1 M NaOH). VGSC currents were recorded by pulsing membrane potentials from –50 to +70 mV in 10 mV increments, from a holding potential of –100 mV.

### **Migration assay**

Details of this were described before (Fraser et al., 2005). Briefly, cells were seeded in multi-well dishes in tissue culture medium. Following drug treatment, cells were re-suspended using trypsin-EDTA and put onto 12  $\mu$ m-pore Transwell filters (Corning, MA) at a density of  $2 \times 10^5$  cells/well. Following 6 h incubation, MTT assay was performed to determine the number of migrated cells. The optical density of the coloured reaction was measured at 570 nm on a plate reader. These measurements were plotted as a percentage of the fluorescence readings for migrated cells/original cell number plated in the upper chamber, giving migration index (MiI).

In the text, MiI values given were normalized with respect to the control value (corresponding to untreated cells in 0% FBS) as 100%.

### **Data analysis**

All data were analysed as means  $\pm$  standard errors (SEM). For statistical comparisons, Student's *t*-test or ANOVA with Newman–Keuls post hoc analysis were used, as appropriate (Brackenbury and Djamgoz, 2006).

## **RESULTS**

Initial observations suggested that Mat-LyLu cells grown in 0% FBS were viable for at least 24 h, the monolayer appearing flat and most cells having extended pseudopodia (Ding and Djamgoz, 2004). Treatment with EGF (100 ng/ml) for 24 h presented a more rounded and refractive form.

### **EGF increased VGSC current amplitude**

Whole-cell patch clamp recordings showed that treatment of cells with EGF for 24 h increased the VGSC current amplitude (Fig. 1). Similar treatment for only 5 min had no effect. The effect of EGF was blocked completely by co-incubation with AG1478, an inhibitor of EGFR tyrosine kinase (Fig. 1). The current–voltage (*I*–*V*) relationships showed that activation voltages and voltages for peak were similar, around  $-40$  and  $-10$  mV, respectively, under all three conditions tested: 0% FBS, EGF- and AG1478-treated (Fig. 1B). However, the mean peak current density of cells grown in the presence of EGF ( $68.1 \pm 4.7$  pA/pF;  $n = 19$ ) was significantly (two- to sevenfold) greater than in 0% FBS ( $31.3 \pm 2.8$  pA/pF;  $n = 18$ ;  $P < 0.01$ ), anti-EGFR antibody

( $9.4 \pm 2.3$  pA/pF;  $n = 5$ ;  $P < 0.001$ ) or EGF + AG1478 ( $20.6 \pm 2.2$  pA/pF;  $n = 9$ ;  $P < 0.001$ ; Fig. 1C). Thus, EGF upregulated VGSC functional expression and this was dependent on tyrosine kinase activity. Importantly, EGF + AG1478, or application of the anti-EGFR antibody by itself, reduced the VGSC current amplitude to levels significantly less than the control (Fig. 1A,C). The latter effects were consistent with biochemical data (not shown) in suggesting that some basal EGFR activity occurred.

### **EGF enhanced cellular migration via VGSC activity**

Pre-treatment with 100 ng/ml EGF for 24 h significantly increased Mat-LyLu cell migration by  $26 \pm 4\%$  ( $n = 14$ ;  $P < 0.001$ ; Fig. 2). This effect was dose-dependent (Fig. 2A). Interestingly, following treatment with AG1478 (alone or with EGF), migration was significantly less than in 0% FBS, the control value ( $P < 0.01$  and  $P < 0.05$ , respectively). These results would indicate possible involvement of endogenous EGF in migration, also apparent from the electrophysiology. There was no difference in the values of MiI for AG1478 and EGF + AG1478 ( $86 \pm 3\%$  and  $89 \pm 3\%$ , respectively;  $P = 0.28$ ;  $n = 14$ ; Fig. 2B). This suggested that AG1478 blocked completely the effect of exogenous (and endogenous) EGF. TTX (500 nM) applied by itself during the assay, suppressed migration by  $18 \pm 3\%$  ( $P < 0.001$ ;  $n = 14$ ; Fig. 2B). Importantly, in the presence of TTX (500 nM), exogenous EGF (100 ng/ml) still caused an increase in migration but this was ~65% less than the effect of EGF alone ( $P < 0.05$ ;  $n = 14$ ; Fig. 2B). This result suggested that a significant portion of EGF signalling operated upstream of the VGSC in the same pathway controlling migration.



## DISCUSSION

Whereas advanced PCa initially responds to androgen ablation therapy, most patients eventually develop androgen-independent cancer, which often leads to metastatic disease (Kreis, 1995). The transition of androgen-dependent to independent status could be associated with increased EGF signalling (Limonta et al., 1995; Sherwood and Lee, 1995). Indeed, it is well established that EGF promotes migration of PCa cells (Zolfaghari and Djakiew, 1996; Kim et al., 1999). Also, in primary corneal epithelial cells disoriented in an external direct-current electric field when grown in serum free medium, directional motility was restored by addition of EGF (Zhao et al., 1999). Thus, EGF and VGSC activity could both contribute to directional movement (Djamgoz et al., 2001; Fraser et al., 2003). The present study is the first to provide evidence in support of a functional relationship between EGF signalling and VGSC activity in PCa cells. Thus, adding EGF to Mat-LyLu cells serum-starved for 24 h increased VGSC current density and this was strongly EGFR-mediated. These effects were seen at a concentration of EGF, ca. 100 ng/ml, very similar to that found in expressed prostatic fluid (Gann et al., 1999). The somewhat limited increase in migration caused by EGF application may have been caused by the presence of endogenously secreted EGF (Fig. 2B). In agreement with this, and consistent with the electrophysiology (Fig. 1A,B), AG1478 alone also slightly but significantly reduced migration. The effect of TTX was also less than previously reported value of 40–50% for Mat-LyLu cells (Grimes et al., 1995). A likely cause of this is the serum-free condition used, which could have limited the involvement of VGSC activity in migration. Nevertheless, co-application of TTX with EGF blocked >50% of the EGF-

induced increase in migration, suggesting that the enhancing effect of EGF occurred significantly via VGSC activity.

Upregulation of VGSC activity by EGF may be through a direct interaction with channel protein, for example tyrosine phosphorylation (Liu et al., 2007). It is also possible that the effect may be indirect and involve mechanisms in addition to VGSC. Indeed, EGF is likely to regulate a multiplicity of cellular components in metastatic PCa cells, which in turn may also influence migration. For example, EGF has been reported to cause system-wide changes in actin cytoskeleton extracellular matrix,  $\text{Ca}^{2+}$  signalling, pH, and transcription factor expression (Schalkwijk et al., 1995; Citri and Yarden, 2006; Lopez-Perez and Salazar, 2006; Mimura et al., 2006; Neumann-Giesen et al., 2007). Further work is required to evaluate these possibilities and to determine the signal transduction pathway involved in the EGF-induced VGSC upregulation. There are two other issues worthy of discussion.

First, although both NGF and EGF upregulated VGSC expression, their effects upon migration were different, the EGF-induced effect involved VGSC activity (this study), the NGF effect did not (Brackenbury and Djamgoz, 2007). This is a clear demonstration of the growth factor multiplicity and diversity of metastatic cell behaviour control, in part involving upregulation of VGSC expression/activity. Such a situation could have consequences for treatment modes for metastatic disease (Onganer et al., 2005).

Second, since (i) VGSC activity was shown earlier to control secretory membrane activity in PCa cells (Abdul and Hoosein, 2001; Mycielska et al., 2003; Krasowska et

al., 2004) and (ii) PCa cells have been reported to secrete EGF (Connolly and Rose, 1990, 1991; also inferred in the present study from the effects of AG1478), it is possible that there is a positive feed-back loop between VGSC activity/upregulation and EGF release (Montano and Djamgoz, 2004). Such a mechanism could have a significant accelerating effect upon metastatic PCa progression.

Finally, EGF-induced upregulation of VGSC activity could also occur in other cancers, especially metastatic breast cancer which is known to be associated with both expression of EGF/EGFR (Atalay et al., 2003) and VGSC upregulation (Fraser et al., 2005; Brackenbury et al., 2007). Hence, suppressing VGSC expression/activity, alongside the EGF system could have added therapeutic value in clinical management of metastatic disease.

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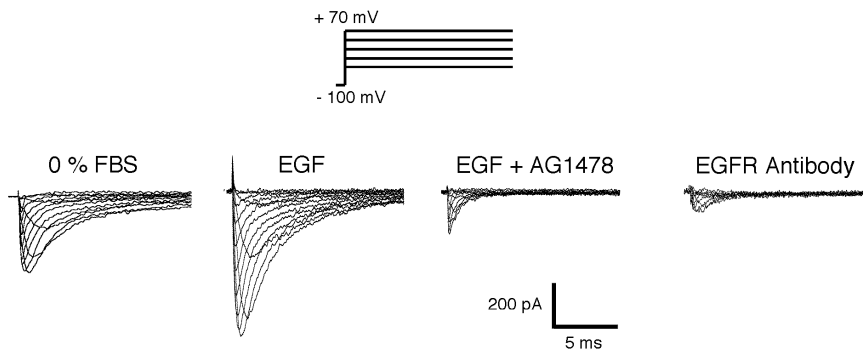
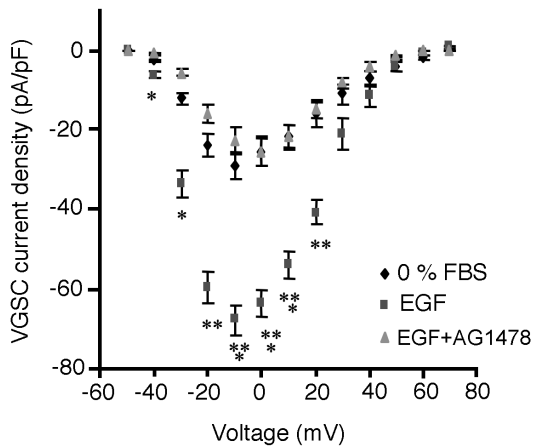
## LEGENDS TO FIGURES

### Figure 1

Upregulation of VGSC activity in Mat-LyLu cells by treatment for 24 h with exogenous EGF. **A:** Typical VGSC current traces recorded from Mat-LyLu cells in different culture conditions: 0% FBS, EGF (100 ng/ml), EGF + AG1478 (1  $\mu$ M), and anti-EGFR antibody (1  $\mu$ g/ml). VGSC currents were recorded by pulsing membrane potentials from  $-50$  to  $+70$  mV in 10 mV increments, from a holding potential of  $-100$  mV. **B:** Effects of EGF and EGF + AG1478 (as in A) on current–voltage relationship. Peak values of VGSC current density were plotted against membrane potential, showing EGF-stimulated VGSC functional activity. **C:** Histograms showing mean values of peak VGSC current density recorded in different conditions (as in A). All data points shown are mean and SEM. Significance:  $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ .

### Figure 2

Effects of EGF, AG1478 and TTX on Transwell migration of Mat-LyLu cells. Migration index (MiI) values were expressed as percentages of the basal value in 0% FBS. **A:** Dose–response relationship for the effect of EGF (1–100 ng/ml) on percentage increase in MiI ( $\Delta$ MiI). **B:** Effects of EGF (100 ng/ml), AG1478 (1  $\mu$ M) and TTX (500 nM), and their specific combinations on MiI. Data are shown relative to the control value for 0% FBS (100%). Bars represent means and SEMs ( $n = 14$ ). These data were consistent with the following: (1) Both EGF/EGFR and VGSC activity were involved in potentiating Mat-LyLu cell migration. (2) The EGF-stimulated enhancement of motility occurred partially via VGSC activity.

**A****B****C**