An acetylcholinesterase-derived peptide inhibits endocytic membrane activity in a human metastatic breast cancer cell line

Pinar U. Onganer a, Mustafa B.A. Djamgoz a,*, Kathryn Whyte b, Susan A. Greenfield b

a Neuroscience Solutions to Cancer Research Group, Division of Cell and Molecular Biology, Sir Alexander Fleming Building, Imperial College London, South Kensington Campus, London SW7 2AZ, UK
b Department of Pharmacology, University of Oxford, Mansfield Rd., Oxford OX1 3QT, UK

Received 15 September 2005; received in revised form 14 December 2005; accepted 19 December 2005
Available online 11 January 2006

Abstract

Acetylcholinesterase (AChE) is well established as having non-cholinergic functions and is also expressed in breast tumours where its function(s) is not known. Recently, a candidate peptide sequence towards the C-terminal of the AChE molecule has been identified, as the salient site remote from normal catalysis in neurons, and possibly other cells. The main aim of this study was to explore the possibility that ‘AChE-peptide’ might also affect human breast cancer cells. Uptake of the non-cytotoxic tracer horseradish peroxidase (HRP) was used as an index of endocytosis, a key component of the metastatic cascade, representing exocytosis/secretory membrane activity and/or plasma membrane protein turnover. AChE-peptide had no affect on the weakly metastatic MCF-7 human breast cancer cell line. By contrast, application of AChE-peptide to the strongly metastatic MDA-MB-231 cells resulted in a dose-dependent inhibition of HRP uptake; treatment with a scrambled variant of the peptide of comparable amino acid length was ineffective. The action of AChE-peptide was suppressed by lowering the extracellular Ca2+ concentration and co-applying a selective antagonist of α7, but not α4/β2, nicotinic receptor. The results suggest that AChE-peptide has a novel, selective bioactivity on breast cancer cells and can potentiate metastatic cell behaviour.

Keywords: Acetylcholinesterase; Breast cancer; HRP; Metastasis; MDA-MB-231; MCF-7

1. Introduction

Although best known as the enzyme that hydrolysates acetylcholine (ACh), acetylcholinesterase (AChE) is now widely accepted as having additional, non-enzymatic functions, in relation to stress, development and neurodegeneration [1–4]. Interestingly, various observations have also indicated the possibility of a comparable, novel role for AChE in non-neuronal tissue, including tumours [5,6]. Thus, there appears to be a clear association of AChE with tumourigenesis in a variety of cancers including P19 cells [7], human meningioma [8], glioma [9] and breast cancer [10,11]. However, these data relate mostly either to changes in the characteristics of the relevant genes, including the splice variants of mRNA or to the expression of different forms of the AChE protein itself [12]. There are no data reported to date regarding the non-catalytic effects that AChE might play in metastatic cell behaviour.

Recently, a 14mer peptide has been identified towards the C-terminus of the AChE molecule, which could be responsible for at least some of its non-catalytic actions [4,13,14]. Indeed, this ‘AChE-peptide’ appears to have bioactivity on neurons [15], stimulating the α7 nicotinic receptor [16], which consequently enhances Ca2+ influx [17] that can in turn induce trophic or toxic effects [18] depending on dose and length of exposure to the peptide [16].

In this study, we have explored whether AChE-peptide might have a comparable action on human breast cancer cells, and if so whether a similar Ca2+-dependent mechanism might operate as for neurons. Evidence suggests that strongly metastatic cancer cells may have ‘neuronal’ characteristics [19]. Two human breast cancer cell lines, MDA-MB-231 and MCF-7, were used. The MDA-MB-231 cell line is strongly tumourigenic and metastatic in athymic nude mice and exhibits high activity in Matrigel-based invasion assays [20]. In contrast, the MCF-7 cell...
line is only weakly metastatic [21]. AChE-peptide was tested on each of these cell lines, whilst a scrambled variant of the peptide [15] served as a negative control for any non-specific effect. Uptake of the tracer horseradish peroxidase (HRP) was used as a measure of endocytic membrane activity [22–24]. Endocytosis is a critical component of metastasis in at least two respects. First, it is a reflection of exocytosis, i.e., vesicular secretion, possibly of growth factors and cytokines that cancer cells may release [25,26]. Second, endocytosis is a means of regulating protein expression in the plasma membrane, which is important to cellular signalling, for example, as in the case of epidermal growth factor receptor activity [27,28].

In order to test for possible mediation of any effect by Ca\textsuperscript{2+} influx, the experiments were repeated in conditions where extracellular Ca\textsuperscript{2+} was omitted from the incubation medium. Possible involvement of α7 nicotinic receptors was tested by application of AChE-peptide in the presence of subtype-specific nicotinic receptor blockers.

2. Materials and methods

2.1. Peptide preparation

Peptides were synthesised (Dr. M. Pitkeathley, Dyson Perrins Laboratory for Organic Chemistry, University of Oxford) using an Applied Biosystems 430A automated peptide synthesizer with standard Fmoc protein synthesis methodology. AChE-peptide and variants thereof were purified by HPLC (>95%) (Mr. K. Pryor, Department of Pharmacology, University of Oxford), freeze-dried and maintained in aliquots [1 mM in phosphate buffered saline (PBS)] and serially diluted to the required concentration in fresh filtered serum-free medium. Cultures were treated with synthetic AChE-peptide (AEFHRWSSYMVHWK) or scrambled AChE-peptide (“control-peptide”) (HSWRAEVFHKYWSM) over a wide concentration range (1 nM–10 μM).

2.2. Cell line maintenance

MDA-MB-231 and MCF-7 cell lines were maintained in a Minimum Essential Medium (Eagle), containing 10% foetal calf serum (FCS) and 1% l-glutamine, in a 37 °C incubator supplied with 5% CO\textsubscript{2}. Cells were used in experiments 2–5 days after plating.

2.3. Measurement of endocytosis

Cells were seeded in 12-well dishes at a density of $5 \times 10^4$ cells in 1 ml of tissue culture medium in each well. Before the start of the endocytosis experiment, the medium was replaced by mammalian Krebs solution and the cells were washed 2–3 times with this solution. The cells were incubated in Krebs solution containing horseradish peroxidase (HRP; type IV; Sigma); ionic composition of the solution varied depending on the experiment. The HRP concentration used ranged from 0.1 to 1 mg/ml and incubation time from 5 min to 2 h (Fig. 1A and B). During all experimental treatments, the cells were kept sterile in the incubator at 37 °C, except for the experiments at 4 °C when the cells were kept in a cold room. After the HRP incubation, the cells were rinsed three times with Krebs solution to remove any residual external tracer. RIPA lyses buffer was applied to disrupt cell membranes and dianimobenzidine (0.5 mg/ml) + hydrogen peroxide (0.01%) in 1 M Tris buffer (pH 7.4) were added. The optical density of the coloured reaction was measured at 540 nm (OD\textsubscript{540}) on a plate reader. The HRP uptake being due to endocytosis, rather than passive, was confirmed by testing the effect of lowering the incubation temperature to 4 °C. As a control of endogeneous peroxidase activity, in every experiment, cells were treated identically, but without prior exposure to HRP. The difference was assumed to represent true endocytosis and was denoted by E\textsubscript{540}. Cell viability was monitored using trypan blue staining.

3. Results

3.1. Optimization of HRP uptake protocol

Vesicular uptake of HRP was observed in MDA-MB-231 and MCF-7 cells microscopically under control conditions.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Time (A) and concentration (B) dependence of HRP uptake by MDA-MB-231 and MCF-7 human breast cancer cell lines. OD\textsubscript{540} (defined in Materials and methods) denotes the total peroxidase activity (mainly the endocytic HRP uptake) of MDA-MB-231 (light triangles) and MCF-7 (dark squares) cells.}
\end{figure}
When the incubation was carried out at 4 °C, no HRP was detected in either cell line, consistent with uptake representing endocytosis, as in our earlier studies [22–24]. There was a rapid increase in HRP uptake during the first 40 min; longer incubations, up to 2 h, led much smaller but still significant effects (P<0.016; Fig. 1A). Uptake of HRP into both cell lines also increased as the concentration was increased, up 1.0 mg/ml (Fig. 1B). At 0.1 mg/ml, the HRP uptake was 92% higher than the level of endogeneous peroxidase (P<0.001) but this was still lower than the uptake for 1.0 mg/ml (P=0.003). Thus, the uptake was concentration- and time-dependent, and 15 min of 0.1 mg/ml HRP uptake was taken as optimal and used in all subsequent experiments.

3.2. Effects of AChE-peptide on endocytic membrane activities of MDA-MB-231 and MCF-7 cells

Uptake of HRP into strongly metastatic MDA-MB-231 cells was reduced by the AChE-peptide in the concentration range 1 pM–1 μM (Fig. 2A), whereas no comparable effect was seen with the control, scrambled variant (Fig. 2A). Importantly, the AChE-peptide had no effect on HRP uptake into MCF-7 cells and the control-peptide was equally ineffective (Fig. 2B). The effect of the AChE-peptide on the MDA-MB-231 cells was broadly concentration dependent. The effect increased up to 0.1 nM (P<0.001 cf. 1 pM) and then gradually reversed as the concentration was increased to 100 nM (P<0.001 cf. 0.1 nM; Fig. 3A).

3.3. Ca2+ dependency of AChE-peptide

Endocytosis (HRP uptake) was partly dependent on extracellular Ca2+. In ‘Ca2+-free’ solutions, and independent of peptide application, HRP uptake into MDA-MB-231 cells was significantly (P<0.001) reduced by 30%, relative to respective controls. Similar treatment had no effect on MCF-7 cells (not illustrated). In the remainder of the experiments, therefore, only MDA-MB-231 cells were studied.

In Ca2+-free medium, AChE-peptide decreased HRP uptake into MDA-MB-231 cells, as under normal conditions. However, the dose–response curve was shifted to the right, with the peak effect occurring at 100 nM (cf. 100 pM under control conditions); also, the reduced effect seen normally at the high end of the concentration range used was less prominent (Fig. 3A). Interestingly, the control-peptide was also effective under this condition, but tended to increase the HRP uptake (Fig 3B). Relative to the Ca2+-free control ("control-2" in Fig. 3B), the effects of the control-peptide were significant for all the
3.4. Effects of subtype-specific nicotinic receptor blockers

Application of 100 nM methyllycaconitine (MLA), a specific neuronal $\alpha 7$ nAChR antagonist, had no effect on basal HRP uptake (Fig. 4B). However, in the presence of MLA, the inhibitory effect of AChE-peptide was significantly reduced: AChE-peptide (0.1 nM) + MLA (100 nM) reduced HRP uptake by only 27±6% ($P=0.002$; $n=6$), compared with 59±2% reduction by equimolar AChE-peptide alone (Fig. 4A and B). A similar experiment was performed using 100 nM dihydro-$\beta$-erythroidine (DHE), an antagonist of $\alpha 4/\beta 2$ nAChR (Fig. 4C). By itself, DHE had no effect on HRP uptake. Interestingly, the working concentration of AChE-peptide was still effective in suppressing endocytosis in the presence of DHE. Thus, AChE-peptide (0.1 nM) + DHE (100 nM) caused a reduction of 54±4%, which was statistically the same as the effect of AChE-peptide alone ($P=0.2$; $n=3$) (Fig. 4A and C). The control-peptide combined with MLA or DHE remained ineffective (Fig. 4B and C).

4. Discussion

4.1. Characteristics of the preparations and the endocytosis assay used

AChE has been shown previously to be expressed in human breast cancer [10,11]. The two human breast cancer cell lines, MDA-MB-231 and MCF-7, adopted here differ markedly in their metastatic potential (strong and weak, respectively) and, corresponding, electrophysiological characteristics. In particular, the strongly metastatic MDA-MB-231 cells express functional voltage-gated $Na^+$ channels, which enhance the cells’ metastatic behaviour, including directional motility, endocytosis and invasiveness [23,29].

We used HRP as a non-cytotoxic tracer to optimally measure endocytic membrane activity. Previously, a similar method was used to determine endocytosis in a variety of cell types, such as pancreatic acinar cells [30] and amphibian photoreceptor synaptic terminals [31]. We have also applied this technique to cancer cells and have shown that uptake is high in strongly vs. weakly invasive cell lines of prostate cancer [22], small-cell lung carcinoma [24] and breast cancer [23]. Basically, the cells take up this non-cytotoxic tracer from extracellular space during endocytic membrane retrieval; this can readily be quantified and has proven to be a very useful tool for studying endocytosis. Endocytosis would reflect at least two major cellular functions: (i) exocytosis and hence vesicular secretion and (ii) plasma membrane protein turn-over. Both these aspects would be involved in the metastatic cascade. For example, storage/release of growth factors and peptide hormones appear to be $Ca^{2+}$ sensitive and vesicular [32–34] and, this would be in balance with endocytosis [35], as measured here. Second, endocytic internalization/recycling of membrane proteins is well known to play a significant role in the signalling associated with metastatic cell behaviour. For example, epidermal growth factor receptors, which can have a significant role in breast cancer [27], undergo endocytosis as a part of their regulatory activity [28].

4.2. The effect AChE-peptide: possible mechanisms and $Ca^{2+}$ dependence

The inhibitory action seen on HRP uptake under normal incubation conditions was specific to AChE-peptide, and not replicated by the scrambled peptide variant. This suggested that AChE-peptide had a (patho)physiological action that could not, for example, be attributed to mechanical disruption of the cells, e.g., due to the formation of fibrils [36]. The effective doses seen here, in the nM range, are comparable with the specific effects of AChE-peptide seen in neurons [e.g., 15 16 17]. The reduced efficacy of AChE-peptide tested on MDA-MB-231 cells at high doses could be explained by the aggregation of the peptide out of solution. Fibrils are formed from the peptide and its variant at high (mM) concentrations [37] and sustained exposure to these in neuronal preparations can lead to non-specific effects [16].

The uptake of HRP into MDA-MB-231 cells was found to be partially $Ca^{2+}$-dependent. Thus, in $Ca^{2+}$-free medium, endocytosis was reduced, the AChE-peptide dose–response curve shifting to higher concentrations by some 3 log units (Fig. 3A). Any remaining effect in $Ca^{2+}$-free medium may be attributable to residual levels of endogenous $Ca^{2+}$ inherent in the preparation. Such $Ca^{2+}$ dependency of the effect of AChE-peptide has been seen previously in ‘excitable’ cells [e.g., 38]. This could, at least in part, be due to the well known $Ca^{2+}$ dependence of exocytosis, in balance with endocytosis [35],
presumably lower Ca$^{2+}$ levels requiring a higher dose of peptide for a similar effect. The Ca$^{2+}$ dependency of the effect of AChE peptide was consistent with its selective sensitivity to blockade of the α7 receptor, a highly efficient Ca$^{2+}$ ‘ionophore’ [38]. The enhanced HRP uptake, seen under Ca$^{2+}$-free conditions as the concentration of scrambled peptide increased, was most likely due to a non-specific membrane effect resulting from possible fibril formation [36].

4.3. Concluding remarks: possible pathophysiological role of AChE-peptide

The main findings of this study are as follows. First, AChE-peptide has a selective action upon the strongly metastatic human breast cancer cell line MDA-MB-231, significantly reducing its basal endocytic membrane activity. Second, the action of the AChE-peptide is at least partially Ca$^{2+}$ dependent. The latter result is consistent with the mode of action of the AChE-peptide suggested earlier for neurons [16].

The bioactivity of AChE-peptide upon the strongly metastatic cell line used here would raise the possibility that the peptide, and the associated Ca$^{2+}$ flux, might be linked functionally to metastasis. The Ca$^{2+}$-dependent metastatic cell behaviours (MCBs) would include apoptosis [39], which is also antagonized by blockade of the α7 receptor [40]. Other MCBs that may be modulated by intracellular Ca$^{2+}$ are motility, [41,42], although acetylcholine itself was found not to affect the migration of MDA-MB-231 cells [43], and gene expression [e.g., 44]. Finally, the Ca$^{2+}$ influx, in conjunction with AChE-peptide acting at an allosteric site [16], could induce further AChE expression [7] and with it release more (endogenous) AChE-peptide. Metastasis might be potentiated/sustained, therefore, by a positive feed-back process involving a receptor and an enzyme, traditionally linked to neurenmuscular transmission. Accordingly, the non-enzymatic function of AChE could be novel target for clinical management of metastatic breast cancer.

Acknowledgements

This work was supported by Synaptica Ltd. and the Pro Cancer Research Fund (PCRF).

References


