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Functional Cross-talk of Ca^{2+}-Mobilizing Endothelin Receptors in C6 Glioma Cells

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Summary

There are conflicting results concerning the receptor subtype(s) involved in calcium-mediated endothelin signaling in the glial cells. In order to elucidate the role of endothelin A and B receptors in these processes, we have studied the effect of a complex spectrum of endothelin receptor ligands on intracellular calcium concentration changes in proliferating and differentiated C6 rat glioma cells. Cell differentiation was induced by dibutyryl-cAMP and assessed by the glial fibrillar acidic protein content. Intracellular calcium changes were measured in cell suspensions using fluorescent probe Fura-2. The specific endothelin B receptor agonists sarafotoxin S6c and IRL-1620 did not influence the intracellular calcium concentration. However, calcium changes induced by endothelin-1 and especially by endothelin-3 after the pretreatment of cells with one of these endothelin B receptor specific agonists were significantly enhanced even above the values attained by the highest effective endothelin concentrations alone. Such endothelin B-receptor ligand-induced sensitization of calcium signaling was not observed in differentiated C6 cells. Moreover, endothelin-induced calcium oscillations in differentiated C6 cells were less inhibited by BQ-123 and BQ-788 than in their proliferating counterparts. In conclusion, the specific activation of endothelin B receptor in C6 rat glioma cells does not affect intracellular calcium *per se*, but probably does so through interaction with the endothelin A receptor. The pattern and/or functional parameters of endothelin receptors in C6 rat glioma cells are modified by cell differentiation.

Key words
Endothelin receptor • Glioma • Calcium signaling • Cell differentiation

Introduction

ETs were originally identified as a family of potent vasoactive peptides (Yanagisawa *et al.* 1988). Subsequent studies have demonstrated the presence of three isopeptides, named ET-1, ET-2 and ET-3, in vascular as well as non-vascular cells and tissues, where ETs, acting in a paracrine/autocrine manner, were found to have multiple biological activities (Goto *et al.* 1996). With respect to the central nervous system, ETs were shown to participate for example in the regulation of neovascularisation, brain perfusion and glial proliferation under both physiological and pathological conditions (Ehrenreich 1999).

Up to now, two subtypes of ET receptors (ET_A preferentially binding ET-1, and ET_B non-selective for ET isoforms) have been characterized, cloned and sequenced. However, further receptor complexity has arisen from pharmacological and biological studies (Sokolovski 1994). ET receptors have been found to be predominantly, but not exclusively, coupled to intracellular signaling pathways involving stimulation of Ca^{2+} fluxes (Gulati and Srimal 1992). There are conflicting results as regards the receptor subtype(s) expressed in the glial and glioma cells: while MacCumber *et al.* (1990) observed an expression of both ET_A and ET_B receptors mRNA, results of pharmacological studies (Martin *et al.* 1990) suggested only the ET_A subtype present.

Moreover, some authors proposed the existence of an atypical ET receptor in glial cells as well (Sakamoto *et al.* 1993, Šedo *et al.* 1993).

In order to interpret our previous preliminary observations suggesting the absence of ET_B receptor-mediated Ca^{2+}-signaling in glioma cells (Šedo *et al.* 1999), we studied the effect of a complex panel of ET receptor ligands on $[Ca^{2+}]_i$ changes in proliferating and differentiated C6 rat glioma cells.

Methods

Dulbecco´s modified Eagle's medium (DMEM) and fetal calf serum (FCS) were from GIBCO, Paisley, Scotland. Endothelin (ET)-1, ET-3 and BQ-123 were from Bachem, Bubendorf, Switzerland. BQ-788 and sarafotoxin S6c were from NovaBiochem, Lucerne, Switzerland. IRL-1620 was synthesized and kindly provided by Prof. P. Rovero, University of Salerno, Italy. Other chemicals used were purchased from Sigma-Aldrich, Prague, Czech Republic.

The C6 rat glioma cell line (Benda *et al.* 1968) from the European Collection of Cell Cultures (Wiltshire, United Kingdom) was obtained at passage number 103. In our laboratory, the cells were used up to additional 30 passages. To reach a comparable cell density at the time of harvesting, the cells were seeded at $1.2x10^4$ cells/cm^2 (proliferating cells) and at $2.4x10^4$ cells/cm^2 (differentiated cells) in plastic 6 well plates (Nunc, Roskilde, Denmark) and were cultured at 37 °C in DMEM supplemented with 10 % FCS under humidified (90 %) atmosphere of 5 % CO_2 / 95 % air for 48 hours. Then, the culture medium was replaced with the same fresh medium (proliferating cells) or with DMEM supplemented with 0.1 % FCS and 1 mM dibutyryl-cAMP (Db-cAMP; differentiated cells) and the cells were cultured for additional 72 hours to reach confluence of about 75 %. Cell viability evaluated by trypan blue exclusion was always greater than 90 %.

Cell differentiation was evaluated by glial fibrillar acidic protein determination and by morphological changes (Benda *et al.* 1968, Coyle 1995, Anciaux *et al.* 1997).

$[Ca^{2+}]_i$ was measured by the Fura-2 method (Garritsen and Cooper 1992). Cells were washed twice with phosphate-buffered saline (PBS; 12.1 mM Na2HPO4, 4 mM KH2PO4, 130 mM NaCl, pH 7.4) and then loaded with DMEM containing 4 μM Fura-2/AM and 0.02 % Pluronic F-127 for 30 min at 37 °C protected from the light. Then the cells were gently detached by up and down pipetting and resuspended in the Krebs buffer (120 mM NaCl, 4.75 mM KCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 1.44 mM $MgSO_4$, 1.1 mM $CaCl_2$, 0.1 mM EGTA, 11 mM glucose, 25 mM HEPES and 0.1 % bovine serum albumin fraction V) and kept at room temperature until use. Experiments were performed at 37 °C in the stirred cuvette, containing approximately $2x10^6$ cells in 2 ml of the measuring buffer. Dual excitation wavelengths were 340 nm and 380 nm, the emission was collected at 510 nm (Perkin-Elmer LS-50B). The calibration (Fmax, Fmin) was performed at the end of each measurement using 0.1 % Triton X-100 and 10 mM EGTA (Garritsen and Cooper 1992). The Ca^{2+} concentration changes are expressed as the fluorescence intensity ratio F/F0 (Šedo *et al.* 1999). Each experiment was repeated at least three times and results were statistically analyzed by Student's t-test.

Results and Discussion

Treatment of C6 cells with ET-1 as well as ET-3 induces a rapid transient rise in $[Ca^{2+}]_i$, followed by a lower level, sustained long-lasting elevation phase. The increase of $[Ca^{2+}]_i$ is concentration-dependent, starting from 10^{-10} M (ET-1) and 10^{-9} M (ET-3) and reaching plateau in both cases at agonist concentration of 10^{-6} M. ET-3 appears to be a less potent inducer than ET-1 on the equimolar basis.

Specific ET_B receptor agonists, sarafotoxin S6c (Williams *et al.* 1991) and IRL-1620 (Takai *et al.* 1992), are devoid of any effect on $[Ca^{2+}]_i$ (data not shown). However, the pretreatment of proliferating C6 cells with sarafotoxin S6c led to the 20 % and 90 % amplification of

$[Ca^{2+}]_i$ rise induced by ET-1 and ET-3, respectively (Tab. 1), above the values that could be induced even by the highest effective agonist concentration alone. The observed augmentation of ET-1 and ET-3 induced Ca^{2+}-signaling was sarafotoxin S6c dose-dependent (Tab. 1). Similar, but not significant, potentiation of ETs-induced response was observed after IRL-1620 pretreatment. It was impossible to induce fully developed "effect of the ligand-mediated potentiation" by simultaneous addition of both sarafotoxin S6c and ET (data not shown).

Table 1. Influence of ET_B-receptor ligands on ET-mediated Ca^{2+}-signaling in C6 cells

		ET-1				ET-3			
		Proliferating		Differentiated		Proliferating		Differentiated	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
STX S6c	*0 nM*	33.5	1.7	28.2	1.9	14.8	1.3	16.1	1.8
	1 nM	34.5	5.5	22.6	0.6	22.6	2.5	14.5	3.0
	10 nM	25.0	2.6	17.6	3.2	25.5*	2.0	12.0	2.0
	100 nM	43.3*	3.1	29.3	4.9	30.9*	1.1	15.6	2.0
BQ-788	*0 μM*	33.5	1.7	28.2	1.9	14.8	1.3	16.1	1.8
	0.1 μM	21.4	1.5	32.9	11.8	22.0	2.1	20.7	0.6
	1 μM	27.7	1.5	31.3	2.3	6.7*	0.8	16.6	1.6
	10 μM	25.5	3.3	24.5	2.5	0.0*	/	6.2*	2.9

*Values of F/F_{max} [%] are from at least three independent experiments done in triplicate. ETs were added 5 minutes after the BQ-788 or sarafotoxin S6c to reach the final concentration of 10^{-7} M. Probability of difference * < 0.01 compared to the ET effect without any pretreatment.*

Such potentiation could be explained by two hypotheses: (i) ET receptor ligand signaling is passed through one "atypical" receptor, preferring specific ligand combinations above all others. Indeed, on the basis of receptor-binding studies, Jensen *et al.* (1998) hypothesized the expression of such atypical ET receptor, with several binding sites for different ET ligands or ligand epitopes in primary rat astrocytes. However, the expression of both ET_A and ET_B receptor mRNA were observed in glial cells including those of the C6 rat glioma (MacCumber *et al.* 1990, Jensen *et al.* 1998). (ii) ET receptor ligands signaling is mediated and integrated by two receptors: an ET_A-like one, coupled with Ca^{2+}-signaling and an ET_B-like one, which, once activated by specific ligands, could modify the ET_A receptor availability or functional parameters thus influencing $[Ca^{2+}]_i$ only indirectly. Fig. 1 shows hypothetical functional ET receptor cross-talk. The observation of the dynamic relation between ET_A and ET_B, in the concrete ET_{B2}, receptor-mediated constriction of the rabbit basilar artery also speaks in support of the "two-receptors" hypothesis (Zuccarello *et al.* 1999). Previously described additional ET_B receptor heterogeneity could explain our observation that sarafotoxin S6c potentiates ET_A-mediated Ca^{2+}-signaling more efficiently than IRL-1620; sarafotoxin S6c interacts with both ET_{B1} and ET_{B2} receptors, whereas IRL-1620 prefers ET_{B1} subtype (Sudjarwo *et al.* 1995).

Surprisingly, slight but regular potentiation of ET-3, but not ET-1 effect was also induced by BQ-788, an ET_B receptor selective antagonist (Ishikawa *et al.* 1994), however only at concentrations ranging from 10^{-9} M up to 10^{-7} M. The higher BQ-788 concentrations inhibited the effect of ET-1 and ET-3, the latter more efficiently (Tab.1). The loss of BQ-788 effect at its higher concentrations could be caused by a non-specific interaction with ET_A receptor; the IC_{50} of BQ-788 on ET_A receptors in human neuroblastoma cells is 1300 nM (Ishikawa *et al.* 1994).

Ehrenreich *et al.* (1999), using the ET_B receptor-deficient rats, proposed ET_B receptor role in ET "clearance" and demonstrated the critical role of ET_B receptor activation for the rapid increase of $[Ca^{2+}]_i$ upon the ET-1 stimulation. Indeed, in the case of ET_B receptor

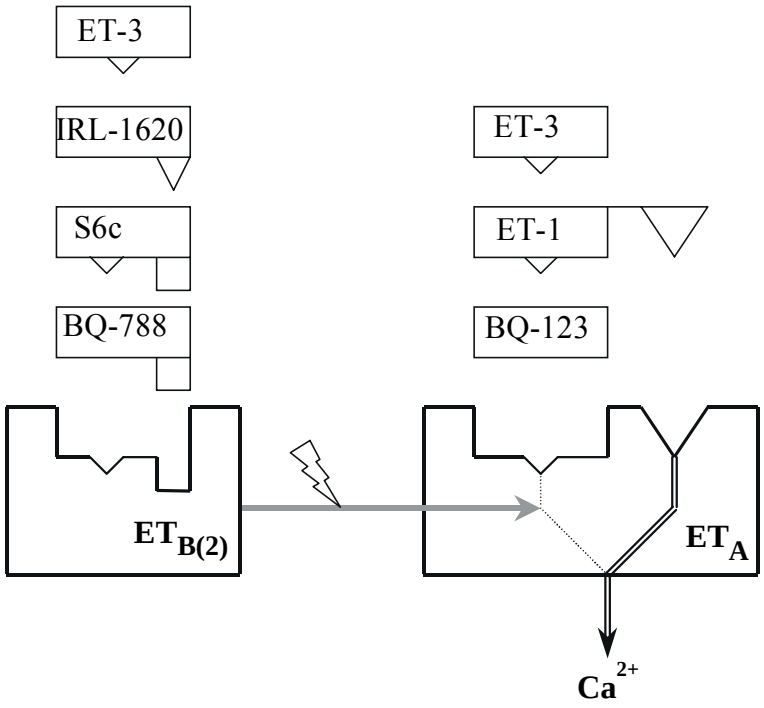

Fig. 1. *A putative model for functional ET_A and ET_B receptor cross-talk in Ca^{2+}-signaling in C6 rat glioma cells.*
ET-1 interaction with two ET_A receptor domains (Sakamoto et al. 1993) induces effective Ca^{2+}-signaling. Thus, ET_B receptor activation, even at high concentrations of its specific agonist, can only slightly increase such a signal. On the other hand, ET-3 binds only one of ET_A receptor domains (Sakamoto et al. 1993); to enable its full potential of Ca^{2+}-signaling, ET_{B2} receptor co-activation, either by ET-3 alone or by more specific ET_B receptor agonists, is required. To induce a full response, ET_B receptor has to be challenged first; ET-3 alone or simultaneous application of the mixture of ET-3 with ET_B specific agonists does not induce Ca^{2+}-signaling as efficiently as sequential treatment. IRL-1620, an ET_{B1}-specific agonist, was less efficient than sarafotoxin S6c, agonist of both ET_{B1} and ET_{B2} receptors. BQ-788, ET_B receptor specific antagonist, potentiates at low concentrations (up to 10^{-7} M) and blocks at higher concentrations ET-3-facilitated ET_A receptor-mediated Ca^{2+}-signaling. BQ-123 blocks signaling induced by both ETs; the ET-3 ones act even more efficiently. This observation is consistent with results obtained in other experimental systems (Gresser et al. 1996). It could be caused by the previously mentioned fact that ET-1 is interacting with two, whereas ET-3 only with one of the ET_A receptor domains.

saturation by its specific ligand, the relative increase of ET_A-agonist availability could be speculated. However, such a mechanism is not fully consistent with the fact that the sequential challenge of ET_A and ET_B receptors overshoots the highest Ca^{2+} signal attainable even by abundant concentration of a single ligand alone.

Finally, the hypothesis about the receptor cross-talk independent of the additional proteosynthetic steps, is supported by the fact that even short-time cell pretreatment with ET_B ligands was required (5 min, data not shown) for inducing the observed effect.

To assess the possible influence of chemically induced cell differentiation on ET signaling, we preformed additional experiments with Db-cAMP-treated C6 cells. The Db-cAMP-treated C6 cells exhibited an astrocyte-like shape, growth retardation and upregulated GFAP expression (data not shown), suggesting their morphological and biochemical differentiation (Sharma and Raj 1987). ET-induced Ca^{2+}-signaling was less inhibited by ET_A receptor antagonists BQ-123 (Šedo *et al.* 1999) as well as by ET_B-receptor antagonists BQ-788 (Tab. 1) in differentiated C6 cells compared to proliferating ones. Such a paradox could be explained, at least partially, by overall ET_A receptor pool down-regulation, known to be induced by Db-cAMP (Durieu-Trautman *et al.* 1991). In contrast to the proliferating C6 cells, significant ET_B-receptor ligand-induced sensitization of ET-1 and ET-3 induced Ca^{2+}-signaling was not observed in the differentiated cells (Tab. 1). This observation is in line with the recent results of Bhowmick *et al.* (1998), describing BQ-123-induced ET_A receptor internalization. Both phenomena could also be caused by the participation of more complex postreceptor

mechanisms, induced by the cell differentiation and still remain a matter for future studies.

In conclusion, C6 rat glioma cells probably express two types of ET receptors participating together in Ca^{2+}-mediated signaling. Their signaling patterns are most similar to ET_A and ET_{B2} receptor subtypes in proliferating C6 cells. However, ET-receptor expression patterns and/or functional parameters are modified by cell differentiation. A functional cross-talk seems to take place between the signal transduction pathways of ET_A and ET_B receptors in C6 rat glioma cells, the underlying molecular mechanism of which require further studies.

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