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Research Report

Functional endothelin receptors are selectively expressed in isolectin B4-negative sensory neurons and are upregulated in isolectin B4-positive neurons by neurturin and glia-derived neurotropic factor

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ABSTRACT

Activation of endothelin receptors expressed in DRG neurons is functionally coupled to translocation of PKCε from cytoplasm to the plasma membrane. Using immunocytochemistry we show that in DRG cultured neurons PKCε translocation induced by endothelin-1 was prominently seen in a peptidergic subpopulation of cultured DRG neurons largely negative for isolectin B4 staining, indicating that in basal conditions functional expression of endothelin receptors does not occur in non-peptidergic, RET-expressing nociceptors. Translocation was blocked by the specific ETA-R antagonist BQ-123 while it was unaffected by the ETB-R antagonist BQ-788. No calcium response in response to endothelin-1 was observed in sensory neurons, while large and long-lasting responses were observed in the majority of non-neuronal cells present in DRG cultures, which are ensheathing Schwann cells and satellite cells, identified with the glial marker S-100. Calcium responses in non-neuronal cells were abolished by BQ-788. The fraction of peptidergic PKCε-translocated neurons was significantly increased by nerve growth factor, while in the presence of neurturin or glia-derived neurotropic factor (GDNF), an IB4-positive subpopulation of small- and medium-sized neurons showed PKCε translocation induced by endothelin-1 which could be blocked by BQ-123 but not by BQ-788. Our in vitro results show that the level of expression of functional endothelin receptors coupled to PKCε is different in peptidergic and non-peptidergic nociceptors and is modulated with different mechanisms in distinct neuronal subpopulations.

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Abbreviations: DRG, dorsal root ganglia; ET-1,2,3, endothelin 1,2,3; ETA-R, endothelin receptor A; ETB-R, endothelin receptor B; ETFR, endothelin functional receptors; ETAFR, endothelin functional receptors A; GDNF, glia-derived neurotropic factor; IB4, isolectin B4; NFH, neurofilament H; NGF, nerve growth factor; NTN, neurturin; PKCε, protein kinase C epsilon; Prv, parvalbumin; PTX, pertussis toxin; SP, substance P; TTX, tetrodotoxin

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1. Introduction

The endogenous endothelins ET-1, ET-2, and ET-3 participate in a remarkable number of pain-related mechanisms. Pain caused by inflammation, by skin cuts, by cancer, by a sickle cell disease episode, and by neuropathy is decreased by local administration of antagonists of endothelin receptors (Nicol, 2004; Khodorova et al., 2009). ETs therefore are relevant members of a large number of local mediators important in pain initiation and in the modulation of nociceptor responsiveness to painful stimuli during inflammation and neuropathy (Khodorova et al., 2009). ETs were originally cloned from endothelial cells but they are also produced by other cell types, including neurons from dorsal root ganglia (DRG, Giaid et al., 1989). ETs act on ETA and ETB G-protein-coupled receptors (ETA-R and ETB-R) that can activate multiple G protein types and influence various signaling pathways (Nicol, 2004; Khodorova et al., 2009). ET-1 injection directly excites nociceptors and causes pain in animals and in humans. Activation of the ETA-R, which is the isoforms expressed in sensory neurons (Khodorova et al., 2009), produces little increases in calcium in DRG neurons (Pomonis et al., 2001; Yamamoto et al., 2006) and mediates the lowering of the threshold for activation of tetrodotoxin (TTX)-insensitive Na+channels which contributes to increased excitability of nociceptors (Zhou et al., 2002). Other significant effectors are involved, notably the nonselective cation channel TRPV1, a polymodal integrator of a number of noxious stimuli (heat above 42 °C, capsaicin, endocannabinoids, prostaglandins, arachidonic acid, and H^+ (Huang et al., 2006)), which is crucial for inflammatory thermal hyperalgesia (Caterina et al., 2000; Davis et al., 2000). TRPV1 increased activity is a chief mechanism of nociceptor sensitization, which occurs via several pathways (Cesare et al., 1999; Premkumar and Ahern, 2000; Vellani et al., 2001; Vellani et al., 2004; Vellani et al., 2006; Vellani et al., 2010). In particular, previous work showed that ET-1 produces translocation of the epsilon isoform of protein kinase C (PKCε) to the plasma membrane and subsequent phosphorylation of TRPV1 and potentiation of capsaicin-induced calcium responses (Yamamoto et al., 2006; Plant et al., 2007). Activation of several G-protein-coupled receptors results in PKCε activation, making this isoform particularly relevant for peripheral mechanisms of sensitization (Julius and Basbaum, 2001; Vellani and McNaughton, 2004; Huang et al., 2006). As its cellular localization can be easily visualized with immunocytochemistry, PKCε can be used as a useful tool to study the presence in cultured neurons of receptors functionally coupled to this enzyme, as illustrated in previous work (Vellani et al., 2004; Vellani et al., 2006; Vellani et al., 2010). In this study, we investigate in vitro the control of the expression of functional ET-1 receptors. We show, using translocation as a functional assay of receptor activation and specific antagonists, that ETA-R and not ETB-R are coupled to PKCε. ETA functional receptors (ETAFR) identified with this experimental approach were seen in a peptidergic subpopulations of DRG neurons and in myelinating neurons. The number of responding neurons was significantly upregulated by nerve growth factor (NGF) in an isolectin B4-negative (IB4–) subpopulation and by neurturin (NTN) and glia-derived neurotropic factor (GDNF) in a non-peptidergic, IB4-positive (IB4+) subpopulation. Calcium imaging experiments showed that, unlike in neurons, in satellite glial cells present in DRG cultures ET-1 causes a large and long-lasting calcium release which was blocked by the ETB-R antagonist BQ-788.

2. Results

2.1. *PKCε translocation in DRG neurons induced by ET-1*

Exposure of DRG neurons to ET-1 caused activation of PKCε, which was detected by observing the translocation from the cytoplasm to the cell membrane. Translocation occurred in a subpopulation of neurons not only of small and medium diameter but also in some large-diameter neurons—this caused the average size of translocated neurons to be 405 ± 28 μm^2 while the average size of the whole population was 352 ± 6 μm^2, as shown in Fig. 1A. Maximal translocation was obtained with 100 nM ET-1 applied for 30 s, and in these conditions the proportion of rat DRG neurons cultured in the presence of 50 ng/ml NGF exhibiting translocation was 10.2 ± 0.7% (41 cultures, 3 coverslips per culture, 200–500 neurons per coverslip, cells used at 2 days in vitro). The highest percentage of translocated neurons was consistently observed with exposure to this saturating concentration of ET-1 applied for 30 s, while the number decreased with a shorter time (15 s) and did not increase significantly with longer treatments (45–60 s), hence this time of exposure, similar to the one used before with other PKCε-activating mediators (see Vellani et al., 2004; Vellani et al., 2006; Vellani et al., 2010) was therefore adopted for all subsequent experiments. There was no evidence of a bell-shaped dose–response curve similar to the one seen with prokineticin receptors (Vellani et al., 2006) at higher concentrations of ET-1 (1 or 10 μM, not shown). Translocation was pertussis-toxin (PTX)-insensitive, as overnight pretreatment with 200 ng/ml PTX did not decrease translocation (10.7 ± 1.1%, n = 3).

2.2. *Upregulation by NGF and GDNF of PKCε translocation induced by ETA-R*

Nerve growth factor (NGF) upregulated the expression of ETAFRs, as culturing without NGF resulted in a smaller number of ET1-responsive neurons (6.4 ± 1.2%, n = 6, p < 0.05 compared to NGF-containing cultures) after 2 days in culture (Fig.1B, C). The percentage of neurons expressing ETAFRs was significantly upregulated also by neurturin (NTN, 50 ng/ml) and GDNF (50 ng/ml), both (as NGF) applied for 2 days in vitro. In the presence of NTN, 14.4 ± 1.2% of neurons responded to ET-1: NTN efficacy therefore was significantly stronger than that of NGF (p < 0.05, n = 6, see Fig.1C). A similar effect was seen in the presence of GDNF (14.1 ± 1.0, not shown, p < 0.05 compared to NGF). The upregulation caused by NGF and NTN was additive when the 2 factors were applied together in culture, suggesting that the effects of these growth factors were due to actions in distinct subpopulations of neurons, most likely the TRK-A and RET-expressing subpopulations. Upregulation by NGF occurred only in IB4– neurons, as both with and without NGF the fraction of ET-1-responding neurons positive to IB4 staining was only marginal (about 7%

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