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Fluorophore assisted light inactivation (FALI) of recombinant 5-HT$_3$A receptor constitutive internalization and function

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ABSTRACT

Fluorescent proteins and molecules are now widely used to tag and visualize proteins resulting in an improved understanding of protein trafficking, localization, and function. In addition, fluorescent tags have also been used to inactivate protein function in a spatially and temporally-defined manner, using a technique known as fluorophore-assisted light inactivation (FALI) or chromophore-assisted light inactivation (CALI). In this study we tagged the serotonin$_3$ A subunit with the α-bungarotoxin binding sequence (BBS) and subsequently labeled 5-HT$_3$A/BBS receptors with fluorescently conjugated α-bungarotoxin in live cells. We show that 5-HT$_3$A/BBS receptors are constitutively internalized in the absence of an agonist and internalization as well as receptor function are inhibited by fluorescence. The fluorescence-induced disruption of function and internalization was reduced with oxygen radical scavengers suggesting the involvement of reactive oxygen species, implicating the FALI process. Furthermore, these data suggest that intense illumination during live-cell microscopy may result in inadvertent FALI and inhibition of protein trafficking.

Introduction

The 5-hydroxytryptamine type 3 (5-HT$_3$) receptor mediates the rapid excitatory currents evoked by serotonin both in the peripheral and central nervous systems (Maricq et al., 1991; Tecott et al., 1993). Currently 5-HT$_3$ antagonists are used clinically to treat irritable bowel syndrome and nausea and emesis during chemotherapy (Hesketh, 2008). Furthermore, 5-HT$_3$ receptor polymorphisms are associated with schizophrenia and bipolar disorder (Niesler et al., 2001). In addition, 5-HT$_3$A receptors may have roles in addiction to alcohol and other drugs of abuse as well as anxiety (Hodge et al., 1993, Olivier et al., 2000 reviewed in Grant, 1995 and McKinzie et al., 2000). Therefore, regulation of 5-HT$_3$A receptors at the cell membrane may play important roles in a number of neural functions, including nausea, anxiety and drug addiction.

The 5-HT$_3$ receptor is a member of the cysteine-loop pentameric ligand gated ion channel (pLGIC) family, which includes the nicotinic-acetylcholine receptor, γ-aminobutyric acid type A (GABA$_A$), and glycine receptors. Nicotinic-acetylcholine and 5-HT$_3$ receptors form cation channels, whereas GABA and glycine receptors form anion channels. Five 5-HT$_3$ isoforms have been described and have been termed 5-HT$_3$A–E (Maricq et al., 1991; Davies et al., 1999; Niesler et al., 2003; Karnovsky et al., 2003). The A subtype can combine to form functional homo-pentameric structures whereas B–E subunits must combine with A subunits to form functional hetero-pentameric receptors (Maricq et al., 1991; Davies et al., 1999; Niesler et al., 2007). Heteromeric 5-HT$_3$AB receptors have larger single channel conductance and are less permeable to Ca^{2+} compared to 5-HT$_3$A homomeric receptors (Davies et al., 1999). Despite our extensive knowledge of 5-HT$_3$ receptor structure and function, little is known about the cell surface stability and trafficking of this receptor.

The development of fluorescent proteins and strategies for tagging proteins with other fluorescent molecules has made it possible to visualize specific proteins and investigate their localization and trafficking. Furthermore, fluorescent proteins and molecules have been used to specifically inhibit proteins upon the excitation of the fluorophore by light and this technique is known as fluorophore assisted light inactivation (FALI) or chromophore assisted light inactivation (CALI) (Jay, 1988; reviewed in Jacobson et al., 2008). Fluorescent proteins such as enhanced Green Fluorescent Protein (eGFP) (McLean et al., 2009; Rajfur et al., 2002; Tanabe et al., 2005; Vitriol et al., 2007) and synthetic fluorophores like fluorescein or red biarsenical dye (ReAsH) (McLean et al., 2009; Tour et al., 2003; Yan et al., 2006; Guo et al., 2006; Marek and Davis, 2002; Lee et al., 2008) have been used to produce FALI.

FALI is mediated by the actions of singlet oxygen and is dependent on the irradiation energy (Horstkotte et al., 2005; McLean et al., 2009).

Abbreviations: 5-HT$_3$, 5-hydroxytryptamine (serotonin) type 3 receptor; BBS, bungarotoxin binding sequence; BTX, α-bungarotoxin; FALI, fluorophore assisted light inactivation; HA, hemagglutinin.

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Singlet oxygen is a reactive oxygen species (ROS) that can cause oxidation of tryptophan, tyrosine, methionine, histidine, and cysteine residues that may result in the cross-linking of residues (reviewed in Davies, 2003). The generation of singlet oxygen is a byproduct of all fluorescence in the presence of oxygen. Due to the close proximity of the fluorophore and the target protein, the singlet oxygen most likely reacts with the target protein (McLean et al., 2009). It has been suggested that FALI is specific to the protein labeled (Jay, 1988; Rajfur et al., 2002; Tanabe et al., 2005; Surrey et al., 1998; Yan et al., 2006; Marek and Davis, 2002; Tour et al., 2003). However, it has also been shown that FALI can lead to collateral damage to non-targeted proteins (Guo et al., 2006; Rahmanzadeh et al., 2007).

In this study, we identified robust constitutive internalization of recombinant 5-HT_3A receptors expressed in HEK-293 cells and N1E-115 neuroblastoma cells in the absence of an agonist. Furthermore, we show that receptor internalization and function were attenuated by fluorophore assisted light inactivation.

Results

α-bungarotoxin tagged 5-HT_3A receptor construct

5-HT_3A receptors were tagged with the binding sequence for α-bungarotoxin allowing us to label surface receptors with fluorescently conjugated α-bungarotoxin in live cells described previously (Sanders and Hawrot, 2004) and used by other groups (Wilkins et al., 2008; Sekine-Aizawa and Huganir, 2004; McCann et al., 2005; Guo et al., 2006). The bungarotoxin binding sequence (BBS) WRYYESSLEPYPD was added to the short extracellular c-terminus of the 5-HT_3A receptor by primer addition PCR. The addition of a single BBS yielded receptors that were unable to bind α-bungarotoxin conjugated to Alexa/555 (BTX/555) (data not shown). Repeats of glycine and asparagine were added to extend the BBS further from the membrane and the rest of the pentameric protein structure. Adding 3 or 8 gly-asp repeats before the BBS was not sufficient to allow for detectable BTX/555 binding. However, adding five gly-asp and a second BBS resulted in a construct that showed reliable labeling with BTX/555. A schematic representation of the construct is shown in Fig. 1a. We have previously shown that addition of the α-bungarotoxin pharmatope on the extracellular carboxyl terminus of the 5-HT_3A receptor does not alter general receptor function or the agonist concentration–response relationship (Sanghvi et al., 2009). Furthermore, currents mediated by 5-HT_3A/BBS receptors were not inhibited in the presence of varying concentrations of non-conjugated BTX (Fig. 1b). Therefore, the addition of the linker sequence and two BBSs did not significantly alter the function of 5-HT_3A receptors even in the presence of bound BTX.

Labeling of surface 5-HT_3A/BBS receptors

5-HT_3A/BBS receptors were expressed in HEK-293, or in N1E-115 neuroblastoma cells. Unlike HEK-293 cells, the N1E-115 cells endogenously express 5-HT_3A receptors (Peters and Lambert, 1989) and may express associated proteins that could influence cell surface stability and trafficking. Cells transiently transfected with 5-HT_3A/BBS receptors were incubated for 30 min at 4 °C in the presence of 1 μg/ml α-bungarotoxin conjugated to Alexa 488 (BTX/488). The cells were washed, fixed with 4% formaldehyde, and imaged using confocal microscopy. Transfected HEK-293 and N1E-115 cells were identified by the expression of mCherry that was co-transfected with the 5-HT_3A/BBS construct. Only transfected cells showed any detectable labeling with BTX/488 and, due to the impermeability of the BTX/488, only surface 5-HT_3A/BBS receptors were labeled with BTX/488 (Fig. 1c). The same staining patterns were seen when labeled 5-HT_3A/BBS receptors were imaged without fixation (discussed below). Binding of BTX/488 to 5-HT_3A/BBS receptors stably expressed in HEK-293 cells could be blocked by pre-incubation with non-conjugated BTX prior to BTX/488 application (Fig. 1d). Therefore, 5-HT_3A/BBS surface receptors can be labeled specifically with BTX conjugated to Alexa fluorophores with little to no background labeling.

Constitutive internalization of 5-HT_3A receptors

The ability to fluorescently label surface 5-HT_3A/BBS allows us to monitor the internalization and trafficking of surface receptors. 5-HT_3A/BBS receptors were expressed stably in HEK-293 and transiently in N1E cells and surface receptors were labeled with BTX/555 (1 μg/ml). Cells were fixed and imaged (Fig. 2a), or the cells were incubated at 37 °C for 30 min in the absence of an agonist then fixed and imaged. Following 30 min at 37 °C, confocal images show fluorescent puncta that appeared to be inside the cell, and there was very little evidence of cell surface fluorescence (Fig. 2b). Thus, the majority of labeled receptors appear to be internalized during the 30 min incubation period.

Following the internalization of BTX/555 labeled 5-HT_3A/BBS at 37 °C, cells were incubated at 4 °C in the presence of BTX/488 (1 μg/ml) to examine if unlabeled receptors were trafficked to the surface during the 30 min at 37 °C. Fig. 2c shows that the majority of BTX/555-labeled puncta appear to be inside the cell, while BTX/488 labeling is limited to the cell surface. These findings suggest that labeled receptors are internalized over the 30 min period and are replaced by unlabeled receptors. This apparent constitutive internalization of 5-HT_3A receptors is temperature sensitive, because surface receptors labeled with BTX/555 remain on the cell surface after 30 min at 4 °C (Fig. 2d top row). Furthermore, if cells are incubated with BTX/488 following the 30 min at 4 °C there is some labeling with the BTX/488 (Fig. 2d bottom row). Similar labeling was seen when cells were labeled with BTX/555, washed, and immediately labeled with BTX/488 (data not shown). Therefore, the labeling of the second fluorophore is most likely due to the unbinding of the first fluorophore that occurs even at low temperatures when internalization is prevented.

We next quantified the internalization rate when surface 5-HT_3A/BBS receptors were labeled with BTX/488 and the cells were incubated at 37 °C. Every 5 min for 30 min individual dishes were removed from the incubator and the cell membranes were labeled with WGA/555 (10 μg/ml) for 20 min at 4 °C. Following WGA labeling cells were fixed and imaged. To quantify receptor internalization we measured the BTX/488 fluorescence at the cell membrane and we observed a loss of fluorescence as receptors were internalized (Fig. 3a). To ensure that we were measuring the BTX/488 fluorescence at the cell surface a segmented line was drawn along the WGA/555 fluorescence and the BTX/488 fluorescence was averaged along that line. The majority of surface receptors are internalized in the first 10 min (Fig. 3a).

HEK-293 cells stably expressing the 5-HT_3A/BBS receptors were transiently transfected with 5-HT_3A subunits tagged with the hemagglutinin (HA) epitope to determine if the BTX/Alexa remained bound to 5-HT_3A/BBS receptors after internalization. Due to the pentameric structure of the 5-HT_3A receptors most if not all should contain at least one of each tagged subunit. Surface 5-HT_3A/BBS receptors were labeled with BTX/488 and then incubated at 37 °C for 30 min. Cells were fixed, permeabilized and incubated with HA-594 antibodies to label all 5-HT_3A/HA subunits. Cells that were transfected with the HA-tagged 5-HT_3A receptors showed that every internalized cluster of BTX/488 co-localized with an HA-594 cluster (Fig. 3b), suggesting that the BTX/488 remains bound to the 5-HT_3A/BBS receptors after internalization. As expected, there are many anti-HA labeled puncta that are not co-labeled with BTX/488, suggesting the majority of receptors are not on the cell surface at the time of BTX labeling (Fig. 3b).