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Subunit-specific modulation of glycine receptors by cannabinoids and N-arachidonyl-glycine

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ABSTRACT

Glycine receptors (GlyRs) mediate inhibitory neurotransmission in spinal cord motor and pain sensory neurons. Recent studies demonstrated apparently contradictory (potentiating versus inhibitory) effects of the endocannabinoid anandamide on these receptors. The present study characterised the effects of cannabinoid agonists on α1, α1β, α2 and α3 GlyRs recombinantly expressed in HEK293 cells with the aims of reconciling effects of cannabinoids on these receptor subtypes and to establish the potential of different GlyR isoforms as novel physiological or analgesic targets for cannabinoids. The compounds investigated were anandamide, HU-210, HU-308, WIN55,212-2 and the endogenous non-cannabinoid, *N*-arachidonyl-glycine. The latter compound was chosen due to the structural similarity with anandamide and known analgesic actions in the spinal cord. Recombinant α1 and α1β GlyRs were potentiated by anandamide and HU-210 at submicromolar concentrations, whereas WIN55,212-2 had no effect and HU-308 produced only weak inhibition. By contrast, *N*-arachidonyl-glycine exerted complex effects including both potentiation and inhibition. Anandamide had no effect at α2 or α3 GlyRs although the other cannabinoids produced potent inhibition. On α2 GlyRs, the inhibitory potency sequence was HU-210 = WIN55,212-2 > HU-308 > *N*-arachidonyl-glycine but on α3 GlyRs it was HU-210 = WIN55212 = HU-308 > *N*-arachidonyl-glycine. These results suggest that α1, α2 and α3 containing GlyRs exhibit distinct pharmacological profiles for cannabinoids. We conclude that cannabinoid agonists may be useful as pharmacological tools for selectively inhibiting α2 and α3 GlyRs. Our results also establish GlyRs as potential novel targets for endogenous and exogenous cannabinoids.

1. Introduction

Pharmacological and gene knockout experiments have shown that the analgesic effects of cannabinoids are mediated primarily through CB_1 and CB_2 G protein-coupled receptors [1,2]. However, cannabinoids also produce actions that are not mediated by these receptors [3]. Because cannabinoid agonists exert effects on a wide variety of membrane proteins at

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Abbreviations: AEA, anandamide; $GABA_AR$, γ-aminobutyric acid type-A receptor chloride channel; GlyR, glycine receptor chloride channel; IPSC, inhibitory postsynaptic current; NA-Gly, *N*-arachidonyl-glycine; nAChR, nicotinic acetylcholine receptor cation channel; THC, Δ^9-tetrahydrocannabinol; WT, wild type.

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physiologically relevant concentrations, there are many potential candidate receptors for these actions [4].

Potent direct potentiating effects of the cannabinoids anandamide (AEA) and Δ^9-tetrahydrocannabinol (THC) have recently been identified at the glycine receptor (GlyR) chloride channel [5]. Because GlyRs mediate inhibitory neurotransmission onto nociceptive sensory neurons in peripheral laminae of the spinal cord dorsal horn [6–10], agents that specifically potentiate glycinergic inhibitory postsynaptic currents (IPSCs) should dampen the transmission of nociceptive impulses to the brain and thereby exert analgesic activity. It is thus feasible that GlyRs could mediate some of the analgesic effects of cannabinoids in the spinal cord.

AEA is closely related in structure to *N*-arachidonyl-glycine (NA-Gly), with the two molecules differing only by an OH group. NA-Gly is also found endogenously in the nervous system where it is distributed at highest levels in the spinal cord [11,12]. Spinal administration of NA-Gly also produces analgesia via an unknown mechanism [12–14]. Whereas AEA potently activates both CB_1 and CB_2 receptors, NA-Gly has no effects on either receptor [15].

GlyRs are members of the Cys-loop ligand-gated ion channel superfamily and can be formed either as pentameric homomers of α1, α2 or α3 subunits or as αβ subunit heteromers [16].

Several recent publications have investigated the effects of cannabinoids on GlyRs [5,17,18]. One study showed that the endocannabinoid agonists AEA and 2-arachidonylglycerol inhibited glycine-gated currents in isolated hippocampal and cerebellar neurons [17]. Since the effect was not abolished by CB_1 receptor antagonists or G protein inhibitors, a direct interaction between endocannabinoids and GlyRs was proposed. In apparent contradiction, AEA and THC increased the amplitude of subsaturating glycine-induced currents in *Xenopus* oocytes expressing homomeric α1 or heteromeric α1β GlyRs [5], with similar effects being observed in native GlyRs in rat ventral tegmental area neurons. This study also showed that GlyR modulation occurred independently of the CB_1 receptor. The third study identified no direct effect of the synthetic cannabinoid agonist, WIN55,212-2, on glycinergic IPSCs in hypoglossal motor neurons [18]. The inconsistency between these reports is possibly the result of the lower glycine concentrations used in the study reporting potentiation. Alternatively, it may have resulted from differences in GlyR subunit composition.

The present study systematically investigated the pharmacological profiles of several cannabinoids on recombinantly expressed α1, α1β, α2 and α3 GlyRs with the aims of reconciling the apparently contradictory results obtained to date, and to establish the potential of different GlyR isoforms as possible non-CB receptor analgesic targets for cannabinoids. At each of the four GlyR isoforms, we compared the actions of the following five compounds: AEA which is a non-specific agonist of CB_1 and CB_2 receptors [19], HU-308 which is highly specific for CB_2 over CB_1 receptors [20], HU-210 which is a highly potent CB_1 and CB_2 receptor agonist [19,21], WIN55,212-2 which is selective for CB_2 over CB_1 receptors [19,21] and NA-Gly which exhibits no activity at either CB_1 or CB_2 receptors, but is structurally related to AEA and exerts potent analgesic effects in the spinal cord. The synthetic cannabinoid compounds used in this study belong to three different structural classes: HU-210 is a classical cannabinoid, HU-308 is a non-classical bicyclic cannabinoid, and WIN55,212-2 belongs to the aminoalkylindoles which bear no obvious structural similarities to either classical or non-classical cannabinoids.

2. Materials and methods

2.1. Mutagenesis and expression of GlyR cDNAs

The human GlyR α1 subunit cDNA was cloned into the pCIS2 plasmid vector. The human α2 and rat α3L subunits were cloned into the pcDNA3.1 plasmid vector (Invitrogen, Carlsbad, CA). The human β subunit was cloned into the pIRES2-EGFP plasmid vector (Clontech, Mountain View, CA). HEK293 cells, cultured in Dulbecco's Modified Eagles Medium, were transfected using a calcium phosphate precipitation protocol. When co-transfecting plasmids encoding the GlyR α and β subunits, cDNAs were combined in a ratio of 1:20. After exposure to transfection solution for 24 h, cells were washed twice in calcium-free phosphate buffered saline and used for recording over the following 24–72 h.

2.2. Electrophysiology

Cells were visualised using an inverted fluorescent microscope and currents were measured by whole cell patch-clamp recording. Cells were perfused by a control solution that contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, with the pH adjusted to 7.4 with NaOH. Patch pipettes were fabricated from borosilicate hematocrit tubing (Vitrex, Modulohm, Denmark) and heat polished. Pipettes had a tip resistance of 1–2 MΩ when filled with the standard pipette solution which contained (in mM): 145 CsCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EGTA, with the pH adjusted to 7.4 with NaOH. After establishment of the whole cell configuration, cells were voltage-clamped at −40 mV and membrane currents were recorded using an Axon Instruments MultiClamp 700B amplifier and pClamp9 software (Molecular Devices, Union City, CA). The cells were perfused by a parallel array of microtubular barrels through which solutions were gravity-induced. All experiments were conducted at room temperature (19–22 °C).

Because α subunits can form functional GlyRs, it was necessary to confirm the incorporation of β subunits into functional αβ heteromers. As the GlyR β subunit cDNA was cloned into the pIRES2-EGFP plasmid vector, we used EGFP fluorescence to identify cells expressing the GlyR β subunit. The successful incorporation of β subunits into functional heteromeric GlyRs was inferred by their characteristic insensitivity to 5 μM picrotoxin [22]. Picrotoxin (PTX), from Sigma (St Louis, MO), was dissolved in DMSO at a concentration of 100 mM.

HU-210 and HU-308 (both gifts from Pharmos Ltd., Rehovot, Israel) were dissolved in ethanol at a concentration of 100 mM. WIN55,212-2 (Biomol, Plymouth Meeting, PA, USA) was dissolved in DMSO at a concentration of 50 mM. NA-Gly and AEA (both from Cayman Chemical, Ann Arbor, MI, USA) were