Molecular Pharmacology Fast Forward. Published on September 23, 2005 as doi:10.1124/mol.105.019141

MOLPHARM/2005/019141

Allosteric Binding Sites on Muscarinic Acetylcholine Receptors

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MOLPHARM/2005/019141

Running title: Allosteric muscarinic ligands

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Total number of text pages: 16

Number of figures**:** 1

Number of tables: 1

Number of references: 31

Number of words

abstract: 227

main body of text: 1534 (without references)

ABBREVIATIONS:

ACh, acetylcholine;

GPCR, G protein-coupled receptor;

mAChR, muscarinic acetylcholine receptor;

TM, transmembrane.

Molecular Pharmacology Fast Forward. Published on September 23, 2005 as DOI: 10.1124/mol.105.019141
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ABSTRACT

In this issue of *Molecular Pharmacology*, Tränkle et al. (**p. xx**) present new findings regarding the existence of a second allosteric site on the M_2 muscarinic acetylcholine receptor (M_2 mAChR). The M_2 mAChR is a prototypic class A G protein-coupled receptor (GPCR) that has proven a very useful model system to study the molecular mechanisms involved in the binding of allosteric GPCR ligands. Previous studies have identified several allosteric muscarinic ligands, including the acetylcholinesterase inhibitor tacrine and the bis-pyridinium derivative Duo3, which, in contrast to conventional allosteric muscarinic ligands, display concentration-effect curves with slope factors >1. By analyzing the interactions of tacrine and Duo3 with other allosteric muscarinic agents predicted to bind to the previously identified 'common' allosteric binding site, Tränkle et al. (**p. xx)** provide evidence suggesting that two allosteric agents and one orthosteric ligand may be able to bind to the M_2 mAChR simultaneously. Moreover, studies with mutant mAChRs indicated that the M_2 receptor epitopes involved in the binding of tacrine and Duo3 may not be identical. Molecular modeling and ligand docking studies suggested that the additional allosteric site probably represents a sub-domain of the receptor's allosteric binding cleft. Since allosteric binding sites have been found on many other GPCRs and drugs interacting with these sites are thought to have great therapeutic potential, the study by Tränkle et al. should be of considerable general interest.

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The human genome is predicted to code for ~1000 molecularly distinct G protein-coupled receptors (GPCRs) (Takeda et al., 2002; Fredriksson et al., 2003). GPCRs therefore represent one of the largest protein families found in nature. An extraordinarily large number of drugs in current clinical use exert their therapeutic effects via binding to specific GPCR subtypes, underscoring the great clinical importance of this class of receptors. The vast majority of these drugs stimulate or inhibit receptor activity by interacting with the binding site recognized by the endogenous ligand, the so-called 'orthosteric' binding site. Consequently, most clinically used GPCR ligands represent either orthosteric agonists (agents that mimic the action of the endogenous GPCR ligands), neutral antagonists, or inverse agonists. However, a considerable body of work suggests that GPCRs, like many other signaling molecules or enzymes, are endowed with one or more additional, so-called 'allosteric', sites that are recognized by allosteric ligands or modulators (Christopoulos 2002; Christopoulos and Kenakin, 2002; Birdsall and Lazareno, 2005; Gao and Jacobson, 2005). By definition, these allosteric binding sites are distinct from the sites involved in the binding of orthosteric ligands, allowing allosteric and orthosteric agents to bind to their target receptors simultaneously (Christopoulos 2002; Christopoulos and Kenakin, 2002; Birdsall and Lazareno, 2005). A variety of different radioligand binding and functional approaches have been used to characterize the binding and functional properties of allosteric GPCR ligands (for recent reviews, see Christopoulos 2002; Christopoulos and Kenakin, 2002; Mohr et al., 2003; Birdsall and Lazareno, 2005; Ehlert, 2005).

The binding of allosteric modulators is predicted to cause conformational changes in the receptor protein that can have different functional consequences. One important

outcome is that the binding of the allosteric agent increases or reduces the affinity of the endogenous ligand for the orthosteric site (such allosteric agents are also referred to as allosteric enhancers or inhibitors, respectively) and/or for exogenously applied orthosteric ligands.

During the past decade, considerable efforts have been focused on developing novel therapeutic agents that target allosteric GPCR binding sites. Research in this area has been driven primarily by considerations that such drugs are potentially more efficacious and less toxic than classic orthosteric GPCR ligands. Some of the major advantages associated with the potential therapeutic use of allosteric GPCR ligands are listed in Table 1 (for a detailed discussion, see Christopoulos 2002; Christopoulos and Kenakin, 2002; Birdsall and Lazareno, 2005).

Various members of each of the three major GPCR subfamilies (A, B, and C) have been shown to be subject to allosteric modulation by small molecule ligands (Christopoulos 2002; Christopoulos and Kenakin, 2002; Presland, 2005; Gao and Jacobson, 2005). During the past three decades, the muscarinic acetylcholine (ACh) receptors (mAChRs) have served as an excellent model system to study the molecular mechanisms by which GPCR activity can be modulated by allosteric drugs (Ellis, 1997; Christopoulos, 2002; Mohr et al., 2003; Birdsall and Lazareno, 2005). The five mAChRs (M_1-M_5), like most other GPCRs, are prototypic class A (rhodopsin-like) GPCRs (Wess, 1996; Caulfield and Birdsall, 1998; Hulme et al., 2003). All five mAChR subtypes have been shown to be subject to allosteric modulation. However, the molecular nature of this modulation differs among the individual receptor subtypes and depends on the choice of

orthosteric and allosteric ligands and their concentrations (Ellis, 1997; Christopoulos, 2002; Mohr et al., 2003; Birdsall and Lazareno, 2005).

The amino acids forming the binding site for orthosteric muscarinic ligands are located within the exofacial portions of various transmembrane (TM) helices, primarily TM domains III, V, VI, and VII (Wess, 1996; Hulme et al., 2003). The receptor binding site for allosteric muscarinic ligands is thought to be located close to the orthosteric site but at a more extracellular level, involving residues located in the extracellular loops and the outermost segments of different TM helices (Matsui et al., 1995; Ellis, 1997; Christopoulos, 2002; Mohr et al., 2003; Birdsall and Lazareno, 2005).

Drugs that can stimulate or inhibit the activity of individual mAChR subtypes with high selectivity may become therapeutically useful in the treatment of many pathophysiological conditions including Alzheimer's and Parkinson's disease, schizophrenia, and drug abuse (Felder et al., 2000; Wess, 2004; Eglen, 2005). However, the amino acids involved in ACh binding are highly conserved among the M_1-M_5 mAChRs (Wess, 1996; Caulfield and Birdsall, 1998; Hulme et al., 2003), most likely due to evolutionary pressures. For this reason, orthosteric muscarinic ligands that can interact with distinct mAChR subtypes with a very high degree of selectivity are not available at present. On the other hand, the extracellular surface of the M_1-M_5 mAChRs, including the three extracellular loops, is less well conserved than the TM receptor core containing the binding site for orthosteric ligands (Wess, 1996; Ellis, 1997; Caulfield and Birdsall, 1998). Since the allosteric muscarinic binding site is predicted to involve receptor epitopes located extracellular of the ACh binding site, it is likely that these sequence differences can be exploited to develop receptor subtype-selective allosteric muscarinic ligands.

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Consistent with this concept, it has been reported that thiochrome, a thiamine metabolite, selectively enhances the affinity of ACh for the M_4 receptor subtype without affecting ACh binding or function at the other subtypes (Lazareno et al., 2004). Moreover, several snake toxins have been identified that display an unprecedented degree of mAChR subtype selectivity (Karlsson et al., 2000; Potter, 2001). For example, MT7 (m1-toxin1) and MT3 (m4-toxin) are highly selective antagonists at M_1 and M_4 mAChRs, respectively (Karlsson et al., 2000; Potter, 2001). The binding of these polypeptide ligands appears to involve interactions with less well conserved amino acids present on the extracellular surface of the mAChRs (Potter, 2001).

The vast majority of known allosteric muscarinic ligands have no significant effect on receptor function in the absence of orthosteric ligands (agonists). However, two agents have been described recently, AC-42 (4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride) (Spalding et al., 2002) and the clozapine metabolite *N*-desmethylclozapine (Sur et al., 2003), which can activate M_1 mAChRs with a considerable degree of selectivity. Studies with mutant M_1 mAChRs and M_1/M_5 hybrid receptors suggested that the M_1 receptor residues involved in the binding of these agents may be different, at least partially, from those critical for the binding of ACh and other orthosteric muscarinic agonists (Spalding et al., 2002; Sur et al., 2003).

Interestingly, accumulating evidence suggests that mAChRs may be endowed with at least two allosteric binding sites (Christopoulos, 2002; Mohr et al., 2003; Birdsall and Lazareno, 2005). One key observation supporting this concept is that certain indolocarbazole (Lazareno et al., 2000) and androstane (Lazareno et al., 2002) derivatives are predicted to bind to a non-orthosteric site different from that recognized by other

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allosteric muscarinic ligands such as gallamine or strychnine. Moreover, several allosteric muscarinic ligands, including the acetylcholinesterase inhibitor tacrine (Potter et al., 1989) and the bis-pyridinium derivative Duo3 (Tränkle and Mohr, 1997; Schröter et al., 2000), have been identified which, in contrast to conventional allosteric muscarinic ligands, display concentration-effect curves with slope factors >1. The complex binding properties of Duo3 suggested that this ligand, similar to the above-mentioned indolocarbazole and androstane derivatives, may interact with a second allosteric binding site (Tränkle and Mohr, 1997; Schröter et al., 2000; Tränkle et al., 2003). In this issue, Tränkle et al. present new findings regarding the existence of a second allosteric site on the M_2 mAChR and provide data suggesting that two molecules of an allosteric agent and one orthosteric ligand may be able to bind to the M_2 mAChR simultaneously (Fig. 1). The authors based their conclusions on analyzing the interactions of tacrine and Duo3 with several other allosteric muscarinic agents predicted to bind to the 'common' allosteric binding site. Studies with M_2/M_5 hybrid receptors and mutant M_2 receptors indicated that the M_2 receptor epitopes involved in the binding of tacrine and Duo3 may not be identical, adding an extra layer of complexity. Molecular modeling and ligand docking studies suggested that two allosteric agents may be able to bind to neighboring areas of the allosteric binding cleft simultaneously. This cleft is predicted to be formed by the receptor's extracellular loops near the entrance of the orthosteric binding site (Voigtländer et al., 2003; Fig. 1). Thus, unless the analyzed allosteric agents exceed a certain critical size, cooperative interactions may occur between two allosteric molecules within the allosteric binding cleft.

The binding of muscarinic ligands to these allosteric sub-domains is predicted to be governed by different structure-function relationships (Mohr et al., 2003; Birdsall and Lazareno, 2005). This observation should guide medicinal chemists in designing novel allosteric drugs acting at distinct mAChR or other GPCR subtypes. Interestingly, the FDA has recently approved the first allosteric GPCR ligand, cinacalcet (an allosteric enhancer acting on the calcium-sensing receptor), for the treatment of certain forms of secondary hyperparathyroidism and hypercalcemia (Block et al., 2004; Lindberg et al., 2005).

Primarily due to the experimental designs used, past drug discovery efforts have yielded a large number of clinically important drugs acting on the orthosteric site of GPCRs. It is likely that the application of new methodologies including high-throughput functional screening techniques will lead to the identification of many novel allosteric GPCR ligands (Christopoulos 2002; Presland, 2005). Such agents are of considerable therapeutic potential since their use is predicted to be associated with increased efficacy and reduced side effects, as compared to classic orthosteric ligands (Table 1).

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Molecular Pharmacology Fast Forward. Published on September 23, 2005 as DOI: 10.1124/mol.105.019141
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Fig 1. Model of the M_2 mAChR (side view) showing the simultaneous binding of an orthosteric ligand (NMS) and two molecules of an allosteric muscarinic agent (tacrine). In this figure (kindly provided by Dr. Hans-Dieter Höltje), the muscarinic antagonist NMS (white structure) has been docked to the orthosteric binding site located within the TM receptor core. The study by Tränkle et al. **(p. xx)** suggests that two different allosteric agents (or two molecules of the same allosteric agent) can simultaneously bind to different sub-domains within the receptor's allosteric binding cleft. The amino acids predicted to be involved in the binding of allosteric muscarinic ligands are thought to be located within the extracellular loops and the outermost segments of different TM helices (Matsui et al., 1995; Ellis, 1997; Christopoulos, 2002; Mohr et al., 2003; Birdsall and Lazareno, 2005). In this model, two molecules of tacrine (yellow and green structures) are bound simultaneously within the allosteric binding cleft. Use of colors and abbreviations: TM helices (I-VII), red; N-terminal domain (N) and first (o1) and third (o3) extracellular loops, grey; second extracellular loop (o2), cyan; cysteine residues involved in disulfide bond formation, magenta.

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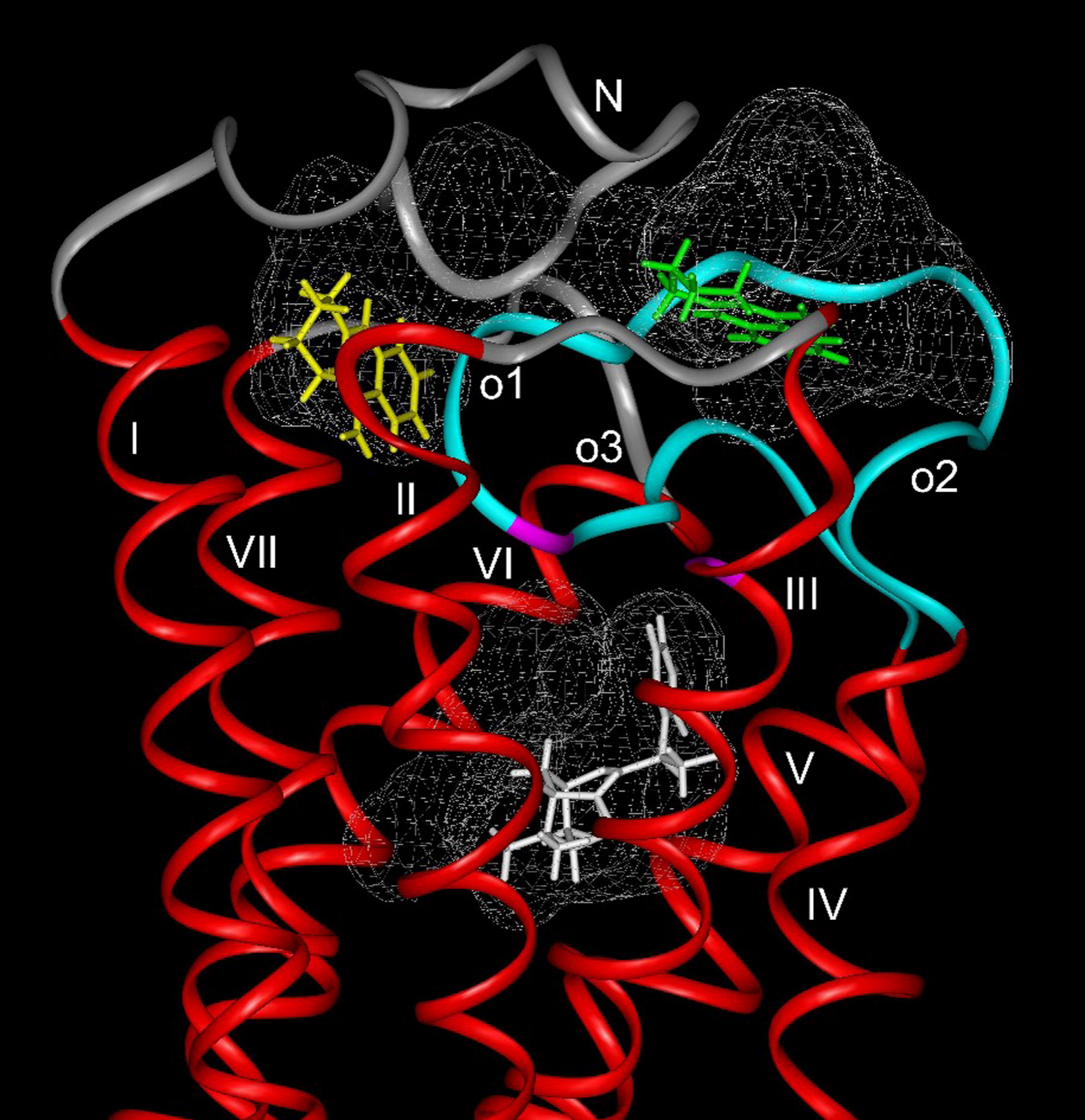
Table 1

Major advantages associated with the potential therapeutic use of GPCR ligands acting on allosteric sites

Properties of allosteric ligands or sites	Potential therapeutic benefit
The effect of allosteric modulators is saturable ('ceiling' effect)	Reduced risk of toxicity due to overdosage
The functional activity of allosteric enhancers is dependent on the presence/synaptic release of endogenous GPCR ligand	Maintenance of the physiological pattern of receptor activity
Allosteric binding sites on GPCRs are usually less well conserved among receptor subtypes as orthosteric binding sites	Potential for the development of ligands with increased receptor subtype selectivity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