Ex vivo IP1 determination using Cisbio's HTRF® IP-One assay in rodent brain extracts after muscarinic M1 receptor activation

T. Smets[1], K. de Waepenaert[1], K. Hens[2], G. Vanhoof[1]

[1]C.R.E.A.Te Biomarkers & Translational Pharmacology and [2]Dept. Neuroscience, Janssen Research & Development, Janssen Pharmaceutical Companies of Johnson & Johnson, Beerse, Belgium

ABSTRACT

Muscarinic acetylcholine receptors are classified into 5 different subtypes, M1, M2, M3, M4 and M5. The M1 subtype is vital for processes involved in learning and memory and mediates its response to acetylcholine and pharmacological agonists via coupling to the Gq/11 class of G proteins[1]. The resulting activation of phospholipase C leads to a subsequent increase in phosphoinositide hydrolysis and causes the accumulation of inositol monophosphate (IP1) in the presence of LiCl[2].

In order to investigate the pharmacological effects of muscarinic activators, we developed an ex vivo functional assay measuring the increase of IP1 after treatment with test compounds.

The HTRF® IP-One assay is based on a competitive immunoassay using a monoclonal cryptate labeled anti-IP1 antibody and d2-labeled IP1.

We showed that apart from a non-selective muscarinic agonist also ectopic M1 agonists and M1 positive allosteric modulators increased IP1 levels in rodent brain extracts.

Abolishment of the increased IP1 levels by pretreatment with a M1-specific antagonist proved the specificity of the effect. The data support the use of the HTRF assay as a robust technology for IP1 measurement for target engagement of muscarinic activators.

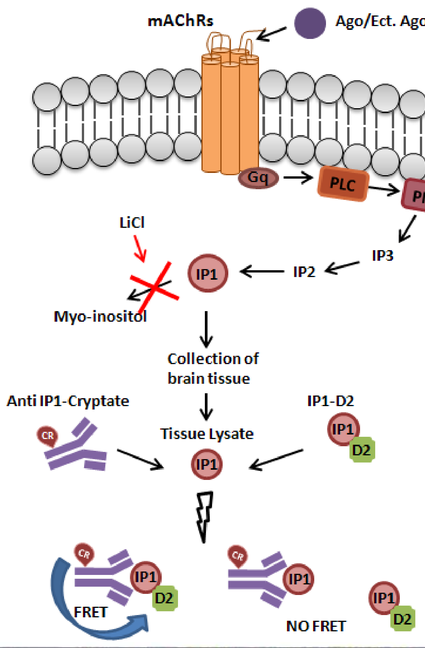
METHODS

Sample preparation

- The animals were predosed with 5 mmol/kg LiCl 60 min before administration of the compound. Sixty min after vehicle/drug treatment s.c., normal adult male C57BL/6 mice and Pgp-deficient mice or young (4-5 weeks) Wistar rats were sacrificed by decapitation. Hippocampus, striatum, cerebellum and cortex were removed and snap frozen.
- Brain tissue was homogenized in 10% (w/v) lysis buffer with 50 mM LiCl in TissueLyser (Qiagen). Lysates were clarified by centrifugation for 30 min at 4000 rpm. All lysates were stored at -80°C.

IP-one HTRF

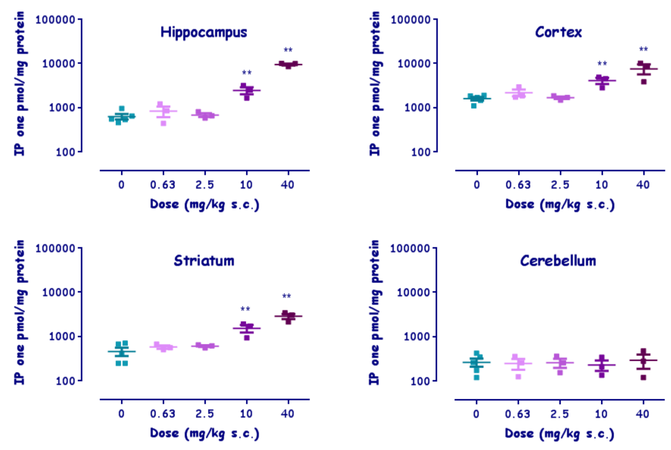
- The IP-One assay is a competitive immunoassay that uses a monoclonal cryptate-labeled anti-IP1 antibody and d2-labeled IP1. The resulting TRF (RFU @ 650 nM) signal is inversely proportional to the concentration of IP1 in the sample. A standard curve is constructed to convert raw data to IP1 concentration. Protein concentration of each sample is measured with the Pierce BCA Protein Assay (Thermo Scientific).



RESULTS

Effect of M1 PAM BQCA[3] on accumulation of IP1

Figure 1: Elevation of IP1 levels after muscarinic activation in different brain regions



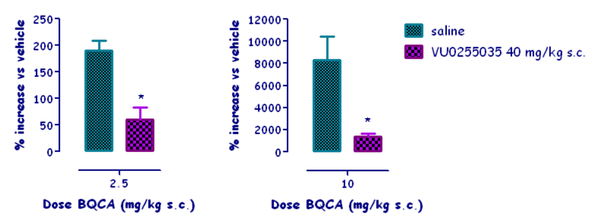
Dose-related increase of IP1 after drug treatment is seen in hippocampus, cortex & striatum. In cerebellum however there is no effect of BQCA on the accumulation of IP1.

Table 1. Elevation of IP1 levels after muscarinic activation in different species (hippocampus)

	BQCA dose (mg/kg) s.c.				
	2.5		10		40
	% increase vs vehicle	BQCA Brain conc (ng/g)	% increase vs vehicle	BQCA Brain conc (ng/g)	% increase vs vehicle
Mice					
C57BL/6JCrl			225±33	1055 ± 156	259±38
n			*6*	*2*	*6*
Pgp-deficient	190±19	789±71	8305±2105	5365±153	
n	*3*	*3*	*4*	*4*	
Rat Wistar					
			394±151	1080±60	3038±439
n			*5*	*2*	*4*
			564±191		1755±411
n			*5*		*5*
	8±11		289±70		1405±80
n	*3*		*3*		*3*
			432±72		
n			3		

Specificity of IP1 signal

Figure 2: Effect of VU0255035[4] a specific M1 antagonist on IP1 accumulation in Pgp-deficient mouse (hippocampus)



Approximately 75% of the effect of BQCA on IP1 increase can be abolished by pretreatment with VU0255035, a specific M1 antagonist, which proves that we are measuring a largely M1 dependent effect.

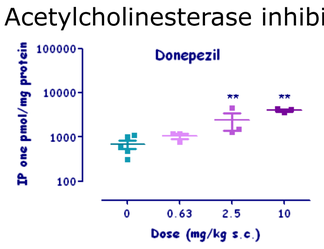
Pgp deficient mice are used to prevent VU0255035 being transported out of the brain through P-glycoprotein mediated efflux.

Values were depicted as mean ± SEM n=3-6. Data were analyzed by one-way ANOVA using the Student's t-test of unpaired data, $p<0.05$ was considered statistically significant. *: $p<0.05$ **:$p<0.01$

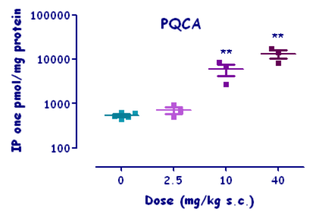
Muscarinic activators

Figure 3: Effect of different activators of the cholinergic system on IP1 levels in rat hippocampus

. Acetylcholinesterase inhibitor



. M1 positive allosteric modulator



Effect of PQCA[5] on IP1 levels is similar to the effect of BQCA (fig. 1)

. Ectopic M1 agonists (GSK1034702[7] and GSK 8k[8])

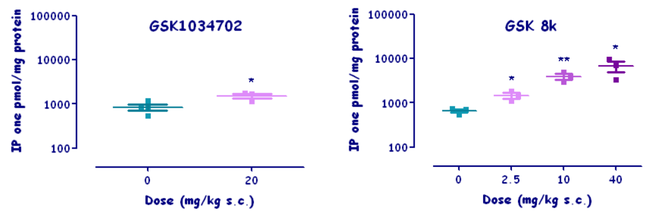


Table 2. Effect of different muscarinic activators on IP1 levels in mouse hippocampus

	Xanomeline M1/M4 Agonist	AC-260584[6] Ectopic M1 agonist	BQCA PAM
10 mg/kg	54±11	21±20	225±33
40 mg/kg	104±36	145±34	259±38

%increase compared to vehicle, n=6

A clear dose-related effect is seen with different modes of M1 activation in rat. In mice BQCA seems to reach a plateau in IP1 levels.

CONCLUSIONS

- The IP-one HTRF assay can be used for measuring IP1 accumulation in brain extracts.
- Muscarinic M1 receptor activation results in dose-related IP1 increase in hippocampus, striatum and cortex, but not in cerebellum.
- M1 activation results in IP1 increase in both mice and rats.
- The IP1 increase is exposure-related
- We proved that we measure M1-dependent signal, by largely abolishing the increase of IP1 after predosing with a M1 specific antagonist.
- Non-selective muscarinic agonists, ectopic M1 agonists and M1 positive allosteric modulators can increase IP1 levels after activation of the muscarinic receptor.
- Compounds differ in the extent of IP1 elevation.

REFERENCES

1. P.J. Conn et al. 2008 Trends in Pharmacological Sciences 30(3):148-155
2. J.R. Atack et al. 1992 J. Neurochem. 59(5):1946-1954
3. L. Ma et al. 2009 PNAS 106(37):15950-15955
4. D.J. Scheffler et al. 2009 Molecular Pharmacology 76(2):356-368
5. J.M. Uslaner et al. 2013 Psychopharmacology 225(1):21-30
6. S.R. Bradley et al. 2010 Neuropharmacology 58(2):365-373
7. P.J. Nathan et al. 2013 Int. J. Neuropsychopharmacology 16(4):721-31
8. D.J. Johnson et al. 2010 Bioorganic & Medicinal Chemistry Letters 20(18):5434-5438

Janssen Research & Development

Janssen PHARMACEUTICAL COMPANIES OF Johnson&Johnson