Procedures to Characterize and Study $P_{2Z}/P2X_7$ Purinoceptor: Flow Cytometry as a Promising Practical, Reliable Tool

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The expression of $P_{2Z}/P2X_7$ purinoceptor in different cell types is well established. This receptor is a member of the ionotropic P2X receptor family, which is composed by seven cloned receptor subtypes ($P2X_1$ - $P2X_7$). Interestingly, the $P_{2Z}/P2X_7$ has a unique feature of being linked to a non-selective pore which allows the passage of molecules up to 900 Da depending on the cell type. Early studies of $P_{2Z}/P2X_7$ purinoceptor were exclusively based on classical pharmacological studies but the recent tools of molecular biology have enriched the analysis of the receptor expression. The majority of assays and techniques chosen so far to study the expression of $P_{2Z}/P2X_7$ receptor explore directly or indirectly the effects of the opening of $P_{2Z}/P2X_7$ linked pore. In this review we describe the main techniques used to study the expression and functionality of $P_{2Z}/P2X_7$ receptor. Additionally, the increasing need and importance of a multifunctional analysis of $P_{2Z}/P2X_7$ expression based on flow cytometry technology is discussed, as well as the adoption of a more complete analysis of $P_{2Z}/P2X_7$ expression involving different techniques.

Key words: flow cytometry - purinergic receptors - $P2X_7$ receptor - ethidium bromide - extracellular ATP

NUCLEOTIDES AND NUCLEOSIDES AS EXTRACELLULAR SIGNALING MOLECULES

Nucleotides and nucleosides comprise a recently established new family of extracellular messengers (see Ralevic & Burnstock 1998). The first evidence that such molecules can play a physiological role when applied extracellularly came from experiments of Drury and Szent-Györgyi (1929), where perfusion of adenosine and adenosine 5'-monophosphate promoted hipotension and bradicardia in the guinea pig cardiovascular system. Based on systematic investigations, Burnstock (1971, 1996) proposed the existence of a purinergic component in the vegetative nervous system, where the ATP is released by synaptic terminals as a neurotransmitter or co-transmitter in both sympathetic and parasympathetic systems.

In fact, the discovery of such purinergic component in the autonomic nervous system was a landmark that established the importance of nucleotides and their derivatives as extracellular messengers. The proposed new role of nucleotides and nucleosides as extracellular messengers was initially viewed with skepticism because of its importance in cell maintenance and survival. Yet, further studies, showing the direct physiological effect of such molecules in every system (respiratory, muscular, vascular, haemopoietic, immune and nervous system), stand for their relevance (Dubyak & El-Moatassim 1993, Alves et al. 1999). Additionally, ATP and derivatives have been found stored within vesicles of platelets, basophils and mast cells, being released with other known compounds when the appropriate stimulus is applied (Dubyak & El-Moatassim 1993).

Extracellular nucleotides and nucleosides, released from neural and non-neural sources, interact with a specific family of membrane associated-molecules named purinergic receptors (reviewed by Fredholm et al. 1994). The purinergic receptors have been classified into two types, P1 and P2, as originally proposed by Burnstock (1978); both types being ubiquitous (Ralevic & Burnstock 1998). P1 purinoceptors are specific for adenosine, being subclassified into four subtypes, namely A1, A2a, A2b and A3, according to pharmacological, functional and molecular criteria. Specific agonists as well as molecular biology techniques are available to distinguish each P1 receptor subtype (Olah & Stiles 1995, Ralevic & Burnstock 1998). P2 purinoceptors are specific for nucleotides and were classified in six subtypes: P_{2D}, P_{2T}, P_{2U}, P_{2X}, P_{2Y} and P_{2Z}. This classification was later sanctioned by the Interna-

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tional Union of Pharmacology (IUPHAR) Purinoceptor Classification Subcommittee. It was based on differences in terms of agonist potency rank order and selectivity, sensitivity to antagonist, functional response, signal transduction mechanism and desensitization features of the receptor after continuous agonist application (Fredholm et al. 1994). P_{2D}, P_{2T}, P_{2U} and P_{2Y} are G-protein coupled while P_{2Z} and P_{2X} are ligand-gated intrinsic ion channels. Interestingly, despite the differences in signal transduction all the receptor subtypes tested are able to induce an increase in the intracellular calcium concentration. Molecular cloning of P2 receptors led to the discovery and addition of new purinergic receptor subtypes that established the existence of two major families, P2Y and P2X (Abbracchio & Burnstock 1994). P2Y family is composed by metabotropic receptors, and is structurally related to G protein-coupled receptors, with seven putative α-helical transmembrane segments, extracellular amino-terminal, and intracellular carboxyl-terminal. The P2Y family comprises at least 5 functional cloned mammalian receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$) that have been described in several different cell types (Ralevic & Burnstock 1998, King et al. 1998). By contrast, the P2X family is composed by ionotropic receptors, i.e. ligand-gated ion channels, and so far seven receptor subtypes have been identified ($P2X_1$ – $P2X_7$) (Table I). The P2X receptors bear two putative transmembrane domains

TABLE I

Classification of P2X purinoceptors

Subtype	Species	Agonist rank order of potency	Amino acid number	GenBank accession number	References
$P2X_1$	Rat	2-MeSATP>ATP>α,βmeATP>>ADP	399	X80477	Varela et al. 1994
	Human	ATP>α,βmeATP	399	X83688[d]	Varela et al. 1995
	Human	Not determined	399	U45448[d]	Longhurst et al. 1996
	Human	α,βmeATP>ATP>ADP	399	AF020498[d]	Sun et al. 1998
$P2X_2$	Rat	Sensitive to ATP, 2-MeSATP and ATPγS	472	U14414	Brake et al. 1994
$P2X_{2\text{-}2}$[a]	Rat	ATP	403	Y09910	Brandle et al. 1997
$P2X_{2\text{-}3}$[a]	Rat	Not determined	212	AF013241	Salih et al. 1998
$P2X_{2\,(b)}$[a]	Rat	2-MeSATP=ATP>α,βmeATP	403	Y10473	Simon et al. 1997
$P2X_{2\,(c)}$[a]	Rat	Not sensitive to ATP	466	Y10474	Simon et al. 1997
$P2X_{2\,(d)}$[a]	Rat	Not sensitive to ATP	484	Y10475	Simon et al. 1997
$P2X_3$	Rat	ATP>2-MeSATP>ATPγS>ADP	397	X91167	Lewis et al. 1995
	Rat	2-MeSATP>ATP>α,βmeATP> ATPγS	397	X90651	Chen et al. 1995
	Human	2-MeSATP>ATP>α,βmeATP> ADP	397	Y07683	Garcia-Guzman et al. 1997a
$P2X_4$	Rat	ATP>ATPγS>2-MeSATP>α,βmeATP=ADP	388	X91200	Bo et al. 1995
	Rat	ATP>ADP>2-MeSATP>>>α,βmeATP	388	U47031	Wang et al. 1996
	Rat	ATP>2-MeSATP>α,βmeATP	388	U32497	Seguella et al. 1996
	Rat	ATP>2-MeSATP>α,βmeATP	388	X93565	Soto et al. 1996a
	Rat	ATP	388	X87763	Buell et al. 1996
	Human	ATP>2-MeSATP>α,βmeATP	388	Y07684	Garcia-Guzman et al. 1997b
	Human	Inactive channel	329[c]	-	Dhulipala et al. 1998
$P2X_5$[b]	Rat	ATP>2-MeSATP>ADP	455	X97328	Garcia-Guzman et al. 1996
	Rat	ATP>2-MeSATP>ATPγS >ADP	455	X92069	Collo et al. 1996
	Human	Not sensitive to ATP	422	AF016709	Lê et al. 1997
$P2X_6$	Rat	ATP>2-MeSATP>ADP>ATPγS	379	X92070 [e]	Collo et al. 1996
	Rat	Not sensitive to ATP	379	X97376 [e]	Soto et al. 1996b
$P2X_7$ (P_{2Z})	Rat	BzATP>>ATP>2-MeSATP>ATPγS>>ADP	595	X95882	Surprenant et al. 1996
	Human	BzATP>ATP	595	Y09561	Rassendren et al. 1997
	Mouse	BZATP>ATP	595	AJ009823	Chessell et al. 1998a

ADP: adenosine 5'-diphosphate; ATP: adenosine 5'-triphosphate; ATPγS: adenosine 5'-O-(3-thiotriphosphate); α,βmeATP: α,β-methylene ATP; BZATP: 3'-O-(4-benzoyl)benzoyl ATP; 2-MeSATP: 2-methylthio ATP; *a*: $rP2X_2$ receptor splice variant; *b*: unpublished data from Toyuyama et al. indicates the existence of $hP2X_5$ receptor splicing variants (GenBank accession: U49395, U49396; direct submission), not shown in the Table; *c*: non functional $hP2X_4$ receptor splice variant (obtained from clone HS-4); *d:* $hP2X_1$ receptors with the same amino acid sequence; *e:* $rP2X_6$ receptors with the same amino acid sequence; the homology among the receptors was analyzed comparing the respective amino acid sequence obtained from GenBank.

connected by a large extracellular ligand-binding loop and intracellular amino- and carboxyl- termini (Valera et al. 1994, Hansen et al. 1997). P2X receptors have been found expressed preferentially in neurons and muscle cells, except for $P2X_7$, as we shall discuss here. Thus, P2 receptors are similar to other known classical neurotransmitter receptors such as those that recognize acethylcholine, gamma-amino butyric acid (GABA), glutamate and serotonine, which present functionally and structurally distinct families of ionotropic and metabotropic receptors. These families mediate fast and slow responses, respectively, via different signaling mechanisms (Burnstock 1997). Probably during the evolution, each type of signaling pathway has accomplished a different advantage to the organism survival. In this context, ATP and other energetic nucleotides might have been chemotaxic molecules, indicating position of damage or dead cells. Thus, cells which had receptors for such molecules could have been positively selected, determining survival and reproductive success. In agreement with this hypothesis some unicellular organisms and invertebrate species express ATP and AMP nucleotide receptors, indicating that use of nucleotides as signaling molecules may be very ancient (Carr et al. 1986, Devreotes & Zigmond 1988).

THE P_{2Z}/$P2X_7$ RECEPTOR

The wide expression of P1 and P2 receptors in different systems has fostered the recent search for its physiological importance as well as its pharmacological applications. One P2 receptor subtype, the P_{2Z}, has interested mostly immunologists, hematologists and biophysicists. The P_{2Z} is the endogenous native counterpart of the cloned $P2X_7$ purinoceptor and for this reason it is also called P_{2Z}/$P2X_7$ by some authors. The existence of a distinct purinergic receptor named P_{2Z} was proposed by Gordon (1986) based on pharmacological analysis. Subsequent investigation established more precisely other P_{2Z} receptor properties. It has been proposed that P_{2Z} receptor is restrictively activated by the fully anionic ATP^{-4}, requiring higher concentrations of ATP to be activated (EC_{50}: 0.1 to 1 mM) when compared with other P2X purinoceptors (~ 1 - 10 µM). The P_{2Z} receptor is sensitive to few other agonists, particularly to BzATP, usually 10 to 100 times more potent than ATP (Dubyak & El-Moatassim 1993, Di Virgilio 1995). The agonist potency rank order of P_{2Z} was BzATP>ATP= ATPγS>>>ADP=AMP. Agonists such as adenosine and UTP, potent agonists for other P1 and P2 receptor subtypes, were ineffective for P_{2Z} (Steinberg et al. 1987, Dubyak & El-Moatassim 1993, Nuttle & Dubyak 1994). Additionally the P_{2Z} was specifically antagonized by oxidized ATP (in all cell types tested so far) and KN-62 (particularly in lymphocytes) (Murgia et al. 1993, Wiley et al. 1993, Gargett & Wiley 1997). Functionally, the hallmark for this receptor is the opening of a low selective pore permeable to molecules up to 900 Da (Steinberg et al. 1987). Interestingly, permeability differences have been found depending on cell type analyzed: thymocytes and peripheral lymphocytes present lower permeability, limiting the passage of molecules until 200-414 Da (Pizzo et al. 1991, Wiley et al. 1993, Nagy et al. 1995, Chused et al. 1996). This raises the possibility existing other P_{2Z} receptor subtypes or different pores, or the existence of pore subconductances.

The sensitivity to BzATP and the unique pore-forming capacity of the P_{2Z}/$P2X_7$ receptor make its characterization less uncertain than for the other purinoceptors.

The $P2X_7$ receptor was cloned from rat brain and expressed in HEK293 cells by Surprenant et al. (1996). This receptor presents 595 amino acids (a.a.) where the first 395 a.a. share 40% structural homology with other P2X receptors with the same putative structure: two transmembrane domains and a large extracellular loop. Conversely, the $P2X_7$ receptor presents a longer COOH-terminal compared to other P2X purinoceptors. It has been proposed that such extra intra-domain is responsible for, or participates in the formation of the pore. This hypothesis was based on an experiment where the expression of the $P2X_7$ protein truncated at 418 a.a. position in HEK293 cells did not induced pore formation as ascertained by dye uptake assay (Surprenant et al. 1996). This cloned rat $P2X_7$ receptor presented the following agonist potency rank order: BzATP>>ATP>2MeATP>ATPγS>> ADP. Additionally, it required high ATP concentrations to be activated (EC_{50}: 115 ± 9 µM), was antagonized by oxidized ATP (oATP), and promoted the non-selective pore formation. More recently, the human and mouse $P2X_7$ receptor was cloned and presented 80 to 85% of homology with the rat orthologue receptor (Rassendren et al. 1997, Chessell et al. 1998a).

Since these initial studies the native P_{2Z} purinoceptor and the cloned $P2X_7$ receptor matched many features, becoming possible the establishment of a consistent correlation between them. That was in opposition to some P2 receptors. The P_{2Z}/$P2X_7$ receptor has been expressed mostly in cells of haemopoietic origin, although it also may be found expressed in other cell types as well as in different cell lines (Table II). Such particularities distinguish the P_{2Z}/$P2X_7$ purinoceptor, making it quite different from all other known P2X receptors.

TABLE II

P_{2Z}/$P2X_7$ receptor expressing cells, properties and characterization approaches

Cell types expressing P_{2Z}/$P2X_7$ and/or its transcripts	Characterization approaches	EC_{50}[a]	Current reversal potential	Main agonists	P_{2Z}/$P2X_7$ mRNA	Dye uptake	Intracellular calcium increase	Antagonists	References
Astrocyte	CM, DUA	ND	ND	BzATP	ND	LY	+	oATP	Ballerini et al. 1996
CHO-K1 cell[b]	DUA, EF, LCE, NB, SF	1.2 µM	-2 mV	BzATP, ATP, ATPγS	+ (RT-PCR)	YO-PRO-1	+	ND	Michel et al. 1998
Dendritic cell[c]	CM, DUA, FC, PPP, RT-PCR, WB	721 µM	ND	BzATP, ATP, ATPγS	+ (RT-PCR)	EB, LY, YO-PRO-1	+	oATP	Coutinho-Silva et al. 1999, Mutini et al. 1999, Nihei et al. manus. submitted.
Fibroblast[c]	CM, EF, LSS, PPP, RT-PCR	2.2 µM	ND	BzATP, ATP	+ (RT-PCR)	EB, LY	+	oATP, PPADS	Gonzalez et al. 1989, Pizzo et al. 1992, Solini et al. 1999
Endothelial cell	RT-PCR	ND	ND	ATP	+ (RT-PCR)	ND	ND	ND	Von Albertini et al. 1998
Glanulocyte	NB	ND	ND	ND	+ (ISH)	ND	ND	ND	Collo et al. 1997
Macrophage[c]	EF, ISH, WB	>100 µM	-2 mV	BzATP, ATP, ATPγS	+ (ISH, WB)	CF, EB, EY, FU, LY, YO-PRO-1	+	oATP, KN-62	Steinberg et al. 1987, Greenberg et al. 1988, Picello et al. 1990, Alonso-Torres & Trautmann 1993, Murgia et al. 1993, Blanchard et al. 1995, Naumov et al. 1995, Chiozzi et al. 1997, Coutinho-Silva & Persechini 1997
Mast cell[c]	CM, DUA, EF	>100 µM	0 - 5 mV	ATP, BzATP	ND	EB, TPM-DPH	*	Brilliant blue G	Tatham & Lindau 1990, Sudo et al. 1996
Mesangial cell	DUA, NB, PPP	>100 µM	ND	BzATP, ATP, ATPγS	+ (NBH)	LY	ND	oATP	Schulze-Lohoff et al. 1998
Microglial cell[c]	CM, DUA, EF, SF	298 µM	+4.6 mV	BzATP, ATP,ATPγS, 2-MeSATP	ND	EB, LY	+	oATP, PPADS	Chessell et al. 1997, Ferrari et al. 1996, 1997
Monocyte[c]	DUA, FC, ISH	>100 µM	ND	ATP, BzATP	+ (ISH, RT-PCR)	EB, TO-PRO-3	ND	oATP, KN-62	Humphreys & Dubyak 1996, Collo et al. 1997, 1998, Persecchini et al. 1998
NG108-15[d]	CM, EF, RT-PCR	>300 µM	+9.3 mV	BzATP, ATP, 2-MeATP, ATPγS	+ (RT-PCR)	EB	+	ND	Kaiho et al. 1996, 1998, Song & Chueh 1996
NK	DUA, FC	ND	ND	ATP	ND	TO-PRO-3	ND	ND	Alves-Neto & Persecchini, pers. communi.

cont.

Cell types expressing P_{2Z}/$P2X_7$ and/ or its transcripts	Characterization approaches	EC_{50}[a]	Current reversal potential	Main agonists	P_{2Z}/$P2X_7$ mRNA	Dye uptake	Intracellular calcium increase	Antagonists	References
Lymphocyte[e]	DUA, FC, NB	189 µM	ND	BzATP, ATP, ATPγS, 2-MeATP	+ (ISH, RT-PCR)	EB, YO-PRO-1	+	oATP, HMA, KN-62	Wiley et al. 1993, 1994, 1998, Chused et al. 1996, Jamieson et al. 1996, Collo et al. 1997
Parotid acinar cell	CM, NB, SF	>100 µM	ND	BzATP, ATP	+ (RT-PCR)	ND	+	Brilliant blue G, DIDS, Reactive blue 2	Mcmillian et al. 1993, Fukushi et al. 1997, Tenneti et al. 1998
Phagocytic cell of thymic reticulum	DUA, EF	ND	+3 mV	ATP	ND	LY	ND	ND	Coutinho-Silva et al. 1996a
Smooth muscle cell	DUA, EF, NB	80 - 200 µM	+3 mV	ND	+ (RT-PCR, NBH)	EB	ND	oATP	Cario-Toumaniantz et al. 1998
Submandibular ductal cell	NB, PPP	220 µM	ND	BzATP, ATP	+ (RT-PCR)	ND	ND	Coomassie blue, oATP, suramin	Alzola et al. 1998
Thymocyte	CF, CM, DUA, SF	>100 µM	ND	BzATP, ATP	ND	EB, PI, TPM-DPH	+	ND	Lin et al. 1985, El-Moatassim et al. 1989, 1990, Nagy et al. 1995, Chused et al. 1996, Ross et al. 1997

CF: 6-carboxyfluorescein; DIDS: 4,4'-diisothiocyanatostilbene-2,2'-disulfonate; DUA: dye uptake assay; EB: ethidium bromide; EF: electrophysiology; EY: eosine yellowish; FU: FURA-2; HMA: 5-(N-hexamethyl)amiloride); ISH: *in situ* hybridization; KN-62: 1-[N,O-bis(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; LSS: liquid scintillation spectrometry; LY: lucifer yellow; NB: northern blotting; NBH: northern blotting reveled by hybridization; oATP: 2',3'-dialdehyde (oxidized ATP); PI: propidium iodide; PPADS: pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid; PPP: pharmacological studies based on physiological parameters; RT-PCR: reverse transcriptase-polimerase chain reaction; SF: spectrofluorometry; TPM-DPH: 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; WB: Western blotting; *a:* EC_{50}: >100 µM was chosen when the data were not precise but clearly evidenced high ATP concentration requirement to activate P_{2Z}/ $P2X_7$ receptor, in general its is a underestimated value; *b:* Variant of the Chinese Hamster Ovarian cell line (CHO); *c:* The analysis include primary cultured cells as well as cell lines; *d:* Hybrid cell line of the mouse neuroblastoma N18TG-2 and the rat glioma C6Bu-1 cells; *e:* the analysis was based on normal and leukemic lymphocytes; ND: not determined.d

$P_{2Z}/P2X_7$ PURINOCEPTOR CHARACTERIZATION APPROACHES

Methodologically different techniques have been used to characterize $P_{2Z}/P2X_7$ receptor (Table II). Most of them directly or indirectly investigate pores, ion channels and membrane alterations, comprising: (1) analysis of membrane biophysics (electrophysiological techniques); (2) analysis of transductional signaling that follows the $P_{2Z}/P2X_7$ activation (calcium microfluorometry); (3) indirect analysis of the receptor activation (dye uptake assay, membrane depolarization, different ion influx analysis); (4) analysis of physiological alterations that follow the $P_{2Z}/P2X_7$ receptor activation (for ex. cytotoxicity, cytokine secretion). More recently, with the availability of the $P2X_7$ receptor cDNA (Surprenant et al. 1996), molecular biology techniques have been gradually adopted to study the expression of the $P2X_7$ receptor at mRNA and protein level, making receptor study more accurate. Some of the most frequently used techniques to characterize and study $P_{2Z}/P2X_7$ receptor are presented below including the discoveries associated with each one.

Electrophysiology - One of the most common electrophysiological technique to study $P_{2Z}/P2X_7$ receptor is the patch clamp which was developed by Neher and Sakmann (1976) and has revolutionized the study of membrane biophysics. The patch clamp method isolates a tiny portion of the cell membrane and makes it possible to study single ion channels and pores individually or collectively (Sakmann & Neher 1995). Such electrophysiological technique has been widely used to study $P_{2Z}/P2X_7$ receptor and have been proved to be appropriate to elucidate important receptor properties such as its kinetics of activation, pore permeability, selectivity and conductance, current reversal potential and rectification, as well as the analysis of agonist and antagonist selectivity (Albuquerque et al. 1993, Surprenant et al. 1996, Coutinho-Silva & Persechini 1997, Rassendren et al. 1997, Virginio et al. 1997, Chessell et al. 1997, 1998a,b). Patch clamp studies in *whole cell* configuration have revealed that the receptor activation induces a fast inward current of Na^+ and Ca^{++} ions that appear in the first milliseconds. This current generally lacked inward rectification (or had it low) and showed a reversal potential near 0 mV, which is consistent with the non-selective ion channel. In addition, the current presented no or low desensitization under continuous agonist exposure. In murine macrophages and phagocytic cells of thymic reticulum, a secundary outward current due to the activation of Ca^{2+}-dependent K^+ channels was also recorded (Albuquerque et al. 1993, Coutinho Silva et al. 1996a). Nuttle and Dubyak (1994) showed a biphasic inward current response during the kinetics of activation of the $P_{2Z}/P2X_7$ receptor, consisting of an initial fast current due to the opening of a poorly selective cation channel, followed by a delayed large current due to the opening of an non-selective pore. This led to the notion that $P_{2Z}/P2X_7$ receptor has two transient forms (ion channel/pore). Patch clamp studies in *cell attached* and *out-side out* configurations have attributed low conductances and subconductances (2 to 17 pS) to $P_{2Z}/P2X_7$ receptor triggered single channels (Tatham & Lindau 1990, Naumov et al. 1995, Coutinho-Silva et al. 1996b, Markwardt et al. 1997, Persechini et al. 1998), what is consistent with the fast activating ion channel activity, but not with the low selectivity pore formation. More recently, the conductance compatible with the non-selective pore was reported by Coutinho-Silva and Persechini (1997). In this work two pores were described, showing conductances of 280 pS and 409 pS. Nevertheless, it is not yet clear if such findings actually represent two types of pores or two subconductance states of the pore.

In addition, there is an unsolved controversy whether the pore linked to $P_{2Z}/P2X_7$ receptor corresponds to the receptor itself or represents a distinct chemical entity. When the rat $P2X_7$ receptor was expressed in HEK293 cells, ATP application induced cell permeabilization to YO PRO-1 (Surprenant et al. 1996), but the same did not happen when the rat $P2X_7$ was expressed in Xenopus oocyte system (Petrou et al. 1997). Furthermore, studies of $P2X_7$ expression on chinese hamster ovarian variant cell line (CHO-K1) cells have demonstrated that at 22°C the permeabilization to YO-PRO-1 delayed up to 8 min when compared to that at 37°C. In contrast, calcium influx delayed just 10 seconds, suggesting two transient forms of the receptor (ion channel/pore) or two distinct entities (Michel et al. 1998). In keeping with this, electrophysiological studies in *cell attached* configuration conducted by Coutinho-Silva and Persechini (1997) reinforce the receptor/pore dissociation hypothesis. In such a configuration the $P2X_7$ receptor/pore structure is confined within the recording pipette tip isolated from extracellular bulk by a gigaseal. However, the authors observed that the external ATP application induced a 409 pS inward current, showing that the pore may be dissociated from the receptor, possibly been gated by an intracellular messenger. Thus, electrophysiological techniques have been critical to elucidate novel aspects of $P_{2Z}/P2X_7$ receptor activation that otherwise would be impossible.

Analysis of signal transduction: Calcium microfluorometry - The signal most commonly associated with the activation of all P2 receptors is the increase of intracellular calcium (Dubyak &

El-Moatassim 1993). In regard to the receptors of the P2Y family, the intracellular calcium increase is dependent on triphosphate inositol pathway, what induces the calcium release from intracellular stores. On the other hand in the P2X receptor family, calcium chiefly comes from the extracellular milieu and enters the cell through ligand-gated ion channels according to its electrochemical gradient (Harden et al. 1995). This response in particular has been explored to complement the characterization of $P_{2Z}/P2X_7$ receptor in different cell types. In keeping with this, calcium microfluorometry has been directed to pharmacological and functional studies since the calcium response amplitude is correlated with the agonist potency and concentration, when it is non-saturating. The calcium response induced by $P_{2Z}/P2X_7$ receptor activation has been described in the majority of cells analyzed (Table II). In general, the rise of intracellular calcium due to activation of $P_{2Z}/P2X_7$ receptor initiates milliseconds after the agonist application and presents a fast elevation, although its amplitude depends on the cell type. In thymocytes, intracellular calcium increases four times the baseline (0.1-1 μM) whereas in macrophages, it reaches at least ten times the baseline values under the same $P_{2Z}/P2X_7$ stimulation condition (Greenberg et al. 1988, Pizzo et al. 1991). Additionally, after a single agonist application, the $P_{2Z}/P2X_7$ receptor induces a sustained increase of intracellular calcium that is maintained for many seconds or even minutes (Greenberg et al. 1988, Ross et al. 1997). In this way, calcium microfluo-rometric evaluation allowed the distinction of the $P_{2Z}/P2X_7$ response from that of the other P2 receptors, as ascertained by distinct variables such as the required ATP concentration to trigger calcium response (high ATP concentrations), calcium response amplitude (high amplitude), duration of the response (sustained calcium response), agonist and antagonist selectivity (responsive to ATP and BzATP) as well as the calcium source (extracellular) (Greenberg et al. 1988, Macmillian et al. 1993).

Other transductional signaling pathways have been associated to $P_{2Z}/P2X_7$ receptor such as phospholipase A2 (Alzola et al. 1998) and phospholipase D (PLD) activation (El-Moatassim & Dubyak 1992,1993, Gargett et al. 1996, Humphrey & Dubyak 1996). Interestingly, in THP-1 monocytic cell line the PLD activation was explored as a marker of $P_{2Z}/P2X_7$ activation in the study of its modulation by pro-inflammatory factors such as interferon-γ (INFγ) and lipopolysaccharide (LPS) (Humphreys & Dubyak 1996).

Dye uptake assay - The $P_{2Z}/P2X_7$ purinoceptor differs from other known ligand-gated receptors due its link to a non-selective pore. The $P_{2Z}/P2X_7$ pore opening induces the exchange of ions and molecules up to 900 Da according to an electrochemical gradient. Electrophysiological, fluorometric and radiometric studies have revealed that the $P_{2Z}/P2X_7$ channel/pore is permeable to several ions such as Na^+, K^+, Li^+, Rb^+, Cl^-, Mn^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}, as well as larger molecules such as tris(hidroxymethyl)aminomethane (TRIS) (121.1 Da) and N-methyl-D-glucamine (NMDG+) (195.2 Da) (Steinberg et al. 1987, Naumov et al. 1992, Albuquerque et al. 1993, Wiley et al. 1993, Nuttle & Dubyak 1994).

Several studies have also demonstrated that the $P_{2Z}/P2X_7$ pore is permeable to different fluorescent markers such as 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) (290 Da), ethidium bromide (314 Da), YO PRO-1 (375.5 Da), 6-carboxyfluorescein (376 Da), propidium iodide (414 Da), TO-PRO-3 (417 Da), lucifer yellow (443 Da), eosine yellowish (646 Da) and FURA-2 (831 Da), but is not permeable to trypan blue (961 Da), evans blue (961 Da) and high molecular weight dextran conjugates (Steinberg et al. 1987, El-Moatassim et al. 1990, Picello et al. 1990, Wiley et al. 1993, Nuttle & Dubyak 1994, Nagy et al. 1995, Surprenant et al. 1996, Persechini et al. 1998). This permeability is a functional hallmark and has been explored in the majority of studies that characterize the $P_{2Z}/P2X_7$ receptor.

Additionally, this assay has been used to conduct pharmacological and functional studies. In macrophages, it was demonstrated that the pore opening is temperature and pH dependent; being inactive below 18°C and at pH 6.5 and optimally active at 37°C and pH 8.0-8.5 (Steinberg et al. 1987). Such phenomenon is also inhibited by Mg^{2+}, indicating that the active $P_{2Z}/P2X_7$ receptor ligand is the non-complexed ATP^{-4} (Steinberg et al. 1987). The dye uptake assay has also been useful to verify the functional expression of $P_{2Z}/P2X_7$ purinoceptor in studies that involve the generation of cell lines with altered expression of $P_{2Z}/P2X_7$ receptor (Chiozzi et al. 1996, 1997). $P_{2Z}/P2X_7$ receptor hiper (ATP-sensitive) and hipo (ATP-resistant) expressed in J774 cell lines can be clearly differentiated by the degree of permeabilization to fluorescent dyes (Chiozzi et al. 1996, 1997). Dye uptake assay may also demonstrate modulation of the $P_{2Z}/P2X_7$ receptor expression. Human THP-1 monocyte cell line presents increased expression of $P_{2Z}/P2X_7$ receptor when treated concomitantly with different pro-inflammatory and inflammatory factors such as INF-γ and LPS or INF-γ and tumor necrosis factor-α (TNFα), as ascertained by permeabilization assay (Humphreys & Dubyak 1996, 1998). The primary characterization of the $P_{2Z}/P2X_7$ receptor by dye

uptake assay can thus be used as a fast and practical method to ascertain its functionality.

However, additional techniques are required to ascertain the uncertain expression of $P_{2Z}/P2X_7$ purinoceptor when the cell analyzed is resistant to ATP-induced permeabilization. It has been the case with neutrophils and, in a lesser extent, monocytes and B lymphocytes (Walker et al. 1991, Hickman et al. 1994, Chused et al. 1996).

FLOW CYTOMETRIC ANALYSIS OF $P_{2Z}/P2X_7$ PURINOCEPTOR

The flow cytometry was developed by a collective effort that began in the 1950s. This invention allowed automatic counting and quantification of cell size for the first time, what significantly increased the reliability of such analysis (Melamed et al. 1991). Later a fluorescence detection system was coupled and evolved making it possible cell multiparametric studies. Additionally, the development of monoclonal antibody technology by Koehler and Milstein in the 70s increased the availability of reagents directed to research and clinical studies, and, as a consequence, the availability of fluorescent coupled ones (Koehler & Milstein 1975). Such technological evolvements made it real the use of flow cytometry as a powerful tool to improve research in immunobiology. Nowadays the flow cytometry usage is widespread in clinical and research laboratories, being applied to analyze multiple cell parameters such as cell cycle, cell membrane alterations, alterations of intracellular calcium and cell phenotype.

The $P_{2Z}/P2X_7$ receptor pore-formation capacity has been explored by several groups to permeabilize different cell types in order to introduce cell membrane impermeant molecules that could have clinical or research interest (Picello et al. 1990, Jaffar & Pearce 1993, Munerati et al. 1994, Gan et al. 1998). Additionally, using different techniques, these studies have analyzed the important parameters that could be affected by such ATP-induced membrane permeabilization such as the cell viability, morphology, intracellular calcium and pH, and apoptosis. Using flow cytometry all these analyses can be performed with accuracy.

Several studies have used the flow cytometry to study the $P_{2Z}/P2X_7$ receptor (Wiley et al. 1993, 1998, Hickman et al. 1994, Nagy et al. 1995, Chused et al. 1996, Persecchini et al. 1998). The main focus of such reports is the indirect $P_{2Z}/P2X_7$ receptor detection by means of dye uptake assays. A pioneer work that used this technology was that of Wiley et al. (1993), which demonstrated that lymphocytes obtained from B-cell chronic lymphocytic leukemia patients became permeable to ethidium bromide but not to propidium iodide after ATP-treatment. Later on, Hickman et al. (1994), demonstrated a possible augmented expression of $P_{2Z}/P2X_7$ receptor in monocytes, which varied with cultured time using the flow cytometry as an complementary technique and the YO-PRO-1 dye. In these studies, DNA binding dyes such as ethidium bromide, propidium iodide and YO-PRO-1 have preferentially been chosen to analyze dye uptake by flow cytometry. These fluorescent dyes presented two major advantages over other dyes such as lucifer yellow, that do not bind DNA: (1) these dyes are almost unaffected by diffusion; (2) they do not suffer the subtraction by organic transporters that could decrease its concentration in cytoplasm and, consequently, diminish the associated fluorescent signal.

Another important point before performing flow cytometry analyses of dye uptake assays is to ascertain by fluorescence microscopy if the phenomenon is simply due to $P_{2Z}/P2X_7$ activation, i.e., only pore opening, rather than endocytosis. In this regard, our group has identified the $P_{2Z}/P2X_7$ purinoceptor in primary cultured murine dendritic cells. In this study, permeabilization analyses were performed by flow cytometry using ethidium bromide as the standard dye. In this case the dye uptake analysis was also viewed by fluorescence microscopy in order to avoid any unwanted artifact. Dendritic cells treated with ATP concentrations compatible with that necessary to activate $P_{2Z}/P2X_7$ became permeabilized to ethidium bromide as shown in Fig. 1. Additionally, dendritic cells were sensitive to the agonist BzATP and the ATP-induced permeabilization was antagonized by oxidized ATP (oATP), thus showing that dendritic cells permeabilization is due to specific $P_{2Z}/P2X_7$ receptor activation (Fig. 1). In this regard, other P1 and P2 agonists such as adenosine, AMPc, ADP and UTP were ineffective (Fig. 2).

The time-resolved flow cytometry has also been explored to study the $P_{2Z}/P2X_7$ receptor properties. In this case, the mean fluorescence intensity of a pre-determined number of cells that pass in different time intervals is collected, what provides a continuous observation of the analyzed phenomenon. Wiley et al. (1998), using this method, confirmed that BzATP was a full agonist of the $P_{2Z}/P2X_7$ receptor-dependent permeabilization of human leukemic lymphocytes.

Flow cytometry has also been used to detect intracellular calcium alterations due to $P_{2Z}/P2X_7$ activation (Nagy et al. 1995, Chused et al. 1996). In theses studies indo-1 and fluo-3 dyes have been used. Chused et al. (1996) monitored the ATP dependent permeabilization and intracellular calcium alterations of different murine thymocyte and peripheral lymphocyte populations in a multiparametric analysis performed by flow cytometry. This

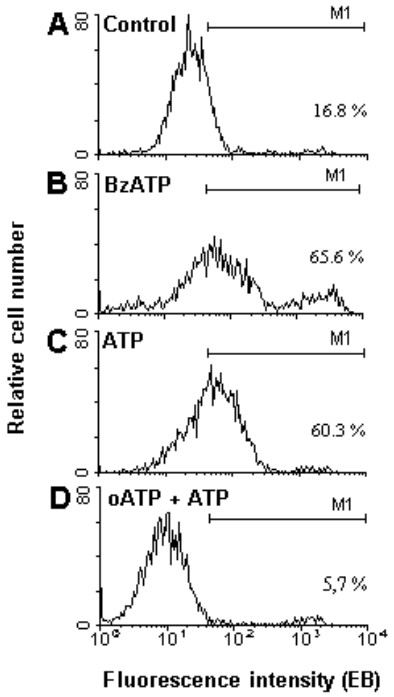

Fig. 1: dendritic cells are specifically permeabilized by P_{2Z}/$P2X_7$ purinoceptor activation. Dendritic cell suspension was treated with exogenous ligand ATP (5 mM) (C), or with the synthetic P_{2Z}/$P2X_7$ specific agonist BzATP (1 mM) (B), and incubated with ethidium bromide (10 µM) for 15 min at 37°C - 5% CO_2. Alternatively, the dendritic cell suspension was incubated with the P_{2Z}/$P2X_7$ antagonist oxidized ATP (oATP) (300 mM) (D) for 2 hr, being then treated with ATP (5 mM) and incubated with ethidium bromide in the same conditions. The fluorescence intensity was detected by flow cytometry (model EPICS ELITE - Coulter Eletronics/USA). The fluorescence intensity of the dendritic cells submitted to these different treatments was compared with that of control untreated dendritic cells (A). M1 marker delimits the positive cells, which percentage are also shown in each histogram. The debris were gated out based on low side scatter and forward scatter.

study demonstrated indirectly the presence of P_{2Z}/$P2X_7$ receptor in different cell types in the following decreasing expression sequence: spCD8+> thCD8+> spCD4+>thCD4+ >thCD4+CD8+ (sp:spleen; th:thymus). Persechini et al. (1998) and Alves-Neto and Persechini (pers. commun.) have used flow cytometry to determine the expression of the P_{2Z}/$P2X_7$ receptor in different peripheral blood mononuclear cell (PBMC) populations. The three color analysis of PBMC showed that T lymphocytes (CD3+) and monocytes (CD14+) became permeable to the TO-PRO-1 dye after ATP treatment. The same occurred with natural killer cells ($CD16^+$/$CD56^+$), thus evidencing the P_{2Z}/$P2X_7$ expression. Interestingly, among these PBMC populations monocytes presented the highest degree of permeabilization.

Recently, a monoclonal antibody (mAb) directed to the P_{2Z}/$P2X_7$ receptor was developed and tested (Chiozzi et al. 1997, Collo et al. 1997, Buell et al. 1998). Once largely adopted, it will facilitate the P_{2Z}/$P2X_7$ receptor expression analysis. Thus, the flow cytometry will consist in a more useful tool to study this purinergic receptor. Concomitantly with specific mAb labeling, P_{2Z}/$P2X_7$ pore functionality could be ascertained in the same experiment by dye uptake assay. Furthermore, multifunctional analyses of different cell populations can be performed through the use of other mAbs available.

CONCLUSIONS

Flow cytometry allows: (1) fast analysis of a large number of cells; (2) the sensitivity of a reliable fluorescence detection system; and (3) the possibility of distinguishing different cell populations due to the usage and availability of a variety of mAbs bearing distinct specificities. Such points are advantages that make flow cytometry distinctive from the all other technologies commonly used to detect cell fluorescence, such as fluorescence microscopy and fluorometry. Particularly regarding the study of purinergic receptors, this strategy has become gradually more used by different groups. Yet flow cytometry-based studies involving the analysis of P_{2Z}/$P2X_7$ receptor expression, its modulation and functionality are still underexplored. With the availability of the specific anti-P_{2Z}/$P2X_7$ mAb, the generation of new fluorescent dyes and the accessibility to flow cytometry apparatus coupled to two or more laser systems, different protocols could be envisioned for the investigation of more complex systemic parameters, such as the involvement of P_{2Z}/$P2X_7$ receptor in bone marrow cell differentiation and on thymocyte differentiation, including the intrathymic selection of the T cell repertoire.

It must be emphasized that the P2 receptor characterization based on classical pharmacological studies with the exclusive analysis of agonists and antagonist effects is nowadays considered incomplete, due to the cloning of many different new P2 receptors and the lack of specific pharmacological tools. The investigation of P_{2Z}/$P2X_7$ is not a exception, despite its unique hallmark properties. Its analysis must involve different techniques such as those described here as well as molecular biology approaches. Only the adoption of such procedures will clarify the precise characterization of the P_{2Z}/$P2X_7$ receptor in cells of different systems and distinguish the possible existence of different P_{2Z}/$P2X_7$ subtypes. This is the case of the P2 receptor characterized or just suggested in erythrocytes, gastric smooth muscle cells of toad, hepatocytes,

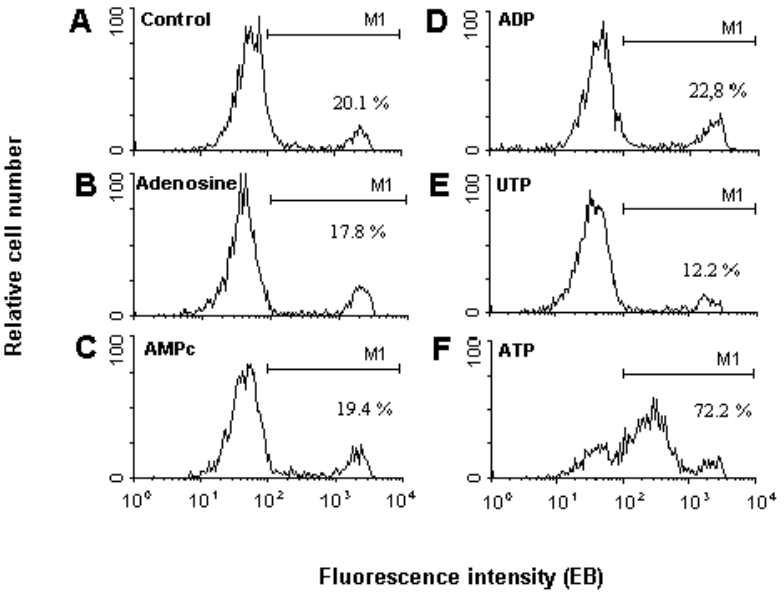

Fig. 2: dendritic cells do not become permeabilized when treated with other P1 or P2 agonists. Dendritic cells were treated with 5 mM of adenosine (B), AMPc (C), ADP (D), UTP (E), or ATP (F) and incubated with ethidium bromide (10 μM) for 15 min at 37ºC - 5% CO_2. The fluorescence intensity of the dendritic cells submitted to these different treatments was compared with that of control untreated dendritic cells (A). M1 marker delimits the positive cells, which percentage are also shown in each histogram. The debris were gated out based on low side scatter and forward scatter. The peak of positive cells with high labeling seen in all, untreated and treated cells, represents the basal dead cells.

rat pancreatic ducts, Leydig cells, supraoptic neurones and schwann cells (Parker & Snow 1972, Foresta et al. 1996, Zoeteweij et al. 1996, Ugur et al. 1997, Christoffersen et al. 1998, Grafe et al. 1999, Shibuya et al. 1999). In these cells the characterized P2 receptor shared some, almost all, or even all P_{2Z}/$P2X_7$ pharmacological properties, but it was not able to induce the formation of the large non-specific pore. In some cases such point was not investigated. This point also involves the controversy if the P_{2Z}/$P2X_7$ receptor and the linked pore are really the same or distinct entities. Further investigation involving different techniques will be necessary to clarify all these unsolved questions.

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