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Characterization of novel store-operated calcium entry effectors ☆

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ABSTRACT

2-Aminoethyl diphenylborinate (2-APB) is a well-known effector of the store-operated Ca^{2+} entry of several cell types such as immune cells, platelets and smooth muscle cells. 2-APB has a dual effect: potentiation at 1–5 μM and inhibition at >30 μM. Unfortunately, it is also able to modify the activity of other Ca^{2+} transporters and, thus, cannot be used as a therapeutic tool to control the leukocyte activity in diseases like inflammation. Previously, we have shown that SOCE potentiation by 2-APB depends on the presence of the central boron-oxygen core (BOC) and that the phenyl groups determine the sensitivity of the molecule to inhibit and/or potentiate the SOCE.

We hypothesized that by modifying the two phenyl groups of 2-APB, we could identify more efficient and specific analogues. In fact, the addition of methoxyl groups to one phenyl group greatly decreased the potentiation ability without any significant effect on the inhibition. Surprisingly, when the free rotation of the two phenyl groups was blocked by a new hydrocarbon bridge, the BOC was no longer able to potentiate. Furthermore, larger aryl groups than phenyl also impaired the activity of the BOC. Thus, the potentiation site in the Ca^{2+} channel is not accessible by the BOC when the lateral groups are too large or unable to freely rotate. However, these molecules are potent inhibitors of store-operated calcium entry with affinities below 1 μM, and they can block the activation of the Jurkat T cells.

Thus, it is possible to characterize 2-APB analogues with different properties that could be the first step in the discovery of new immunomodulators. This article is part of a special issue entitled "Calcium Signaling in Health and Disease. Guest Editors: Geert Bultynck, Jacques Haiech, Claus W. Heizmann, Joachim Krebs, and Marc Moreau.

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1. Introduction

Cytosolic calcium concentration increase is a common feature in the initiation of biological processes such as cell activation, proliferation or apoptosis [1]. It is usually due to the release of Ca^{2+} ions from the internal stores, especially the endoplasmic reticulum (ER) and a massive entry of extracellular Ca^{2+} ions by a process known as "store-operated calcium entry" or SOCE. In a large number of non-excitable cells, this entry results from the opening of a class of channels known as "store-operated calcium channels" or SOCC. Although the SOCE of T lymphocytes was described in the 1980s, major progress has been made in the understanding of its activation in the last decade with the discovery and characterization of two proteins: the plasma membrane (PM) Orai1 protein, which forms the pore of the SOCC [2], and the ER membrane STIM1 protein, which induces the opening of the SOCC [3]. During the T cell activation, the T cell receptor (TCR) stimulation induces the synthesis of inositol 1,4,5-trisphosphate (IP_3), which allows the release of Ca^{2+} ions trapped in the ER lumen through IP_3 receptors. This release causes the oligomerization of ER membrane STIM1 proteins and their migration to ER-PM junctions where they directly interact with Orai1 proteins, the pore-forming unit of the Ca^{2+} release-activated Ca^{2+} (CRAC) channels. This physical interaction allows the opening of the channel and the massive entry of extracellular Ca^{2+} ions in a process known as the SOCE. The absence of functional Orai1 or STIM1 proteins impairs the SOCE and is responsible for severe combined immunodeficiencies [4]. In contrast under low agonist concentration stimulation, additional Ca^{2+} entry could be activated [5]. In non-immune cells, SOCE is less prominent and often activated together with other calcium entry pathways (example of neurons [6]).

In the immune system, pharmacological compounds acting on Orai1/STIM1 proteins are theoretically interesting to control T cell functions in order to be used as treatment for inflammation, auto-immune diseases and graft rejection for example. Noteworthy, during the last 30 years, several laboratories have developed SOCE inhibitors, but none of these molecules is used in therapy, mainly due to a lack of specificity. However, since the discovery of the Orai1/STIM1 couple and its

Abbreviations: 2-APB, 2-aminoethyl diphenylborinate; SOCE, store-operated calcium entry; BOC, boron-oxygen core; IL-2, interleukin 2

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involvement in T cell functions, research on SOCE effectors seem to has been boosted. Recently, Calcimedica, a company founded by some of the Orai1 and STIM1 inventors, has announced the development of an SOCE inhibitor already in phase 1 clinical trial to treat moderate to severe plaque psoriasis (CM2489, structure not published).

Among all the known effectors of SOCE, we have focused our work on 2-aminoethoxy diphenylborinate (2-APB), which has unique properties: at 1–5 μM, 2-APB potentiates the SOCE but inhibits it at >30 μM [7,8]. 2-APB was first described as an IP_3 receptor inhibitor (IC_{50} 42 μM [9]) and is known to block the SERCA (IC_{50} 91 μM [10]). Additionally, 2-APB was shown to inhibit some members of the TRP family: TRPC1 in neuronal cells (IC_{50} 80 μM [11]), TRPC3 with a partial block at 30 μM in DT40 cells [12] and TRPC5 expressed in HEK293 cells (IC_{50} 20 μM [13]). On the other hand, 2-APB activates TRPV1, TRPV2 and TRPV3 with an EC_{50} between 34 and 129 μM [14]. Likewise, it has been shown in another study that 2-APB has a dual activity on TRPM7 channels with a potentiation at concentrations around 100 μM and an inhibition for >1 μM [15].

Despite its lack of specificity, we previously showed that the basic molecule could be modified to give rise to analogues with distinct properties [8]. Structurally, 2-APB is made of three parts: a central boron-oxygen core, an ethanolamine and two phenyl groups. The absence of the central boron-oxygen core totally abolishes the potentiation capacity of the molecules, showing that this part of the 2-APB molecule is absolutely necessary for this ability [8]. On the other side, the ethanolamine and the two phenyl groups are implied in the inhibition capacity. To better understand the functioning of SOCE and to develop new regulators of the immune system, we need new molecules with better selectivity and efficacy. In this study, we based our work on the role of the two phenyls in 2-APB activities. For this purpose, we chose several 2-APB analogues with modifications such as the addition of methoxy groups on one phenyl group, or replacement of the two phenyls by thienyl or benzothienyl groups.

Previously, we have shown that even if the two aryl groups (any functional group with an aromatic ring) play a role in this inhibition process, they are also critical for the sensitivity of the SOCE for the 2-APB analogue; thus, the more there are cycles in the analogue, the more the SOCE is sensitive (Jurkat T cell SOCE is more sensitive to diphenylborinic anhydride with 4 rings than to 2-APB with 2 rings and to 2-aminoethoxy dibutylborinate with none [8]). In this work, we kept the central boron-oxygen core BOC and the ethanolamine parts intact and only changed the two aryl groups. All new compounds were tested on Jurkat cell SOCE and not with an Orai1/STIM1 overexpressing system. Indeed, the Jurkat cell SOCE has been intensively studied by numerous groups, and its pharmacological and kinetic properties are well established. Since the expression ratio between Orai1 and STIM1 modifies the pharmacological properties of the SOCE [16], we preferred to investigate endogenous SOCE. In Jurkat cells, SOCE is mostly due to Orai1 activation [17].

Results obtained with these new analogues showed that the 3D conformation of the two aryl groups is also important for the potentiation ability of the analogues. It also gives rise to a very potent SOCE inhibitor. As expected, some other changes in the parent molecule only modify the sensitivity for potentiation and inhibition. Unfortunately, these molecules, like 2-APB, lack specificity. However, even if they cannot be used outside the laboratories, they could represent another step in the discovery of new and more efficient molecules to treat diseases where the immune system is implied.

2. Materials and methods

2.1. Cell lines

The Jurkat E6.1 (acute T cell leukemia) cell line was maintained in RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum and 2 mM Glutamax® (Invitrogen, Cergy-pontoise, France) at 37 °C in a 5% CO_2 humidified atmosphere.

2.2. Measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$)

The cytosolic Ca^{2+} concentration was recorded by a fluorimetric ratio technique. Cells were centrifuged and resuspended in phosphate-buffered saline medium (PBS, Cambrex, Belgium) supplemented with 1 mg/ml bovine serum albumin (Sigma, Saint Quentin Fallavier, France) under gentle agitation. The fluorescent indicator indo-1 was loaded by incubating the cells at room temperature for 1 h with 4 μM indo-1-AM (Invitrogen/Molecular Probes, Eugene, USA). Cells were then spun down and resuspended in HEPES-buffered saline (HBS) medium without $CaCl_2$ (in mM): 135 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 11.6 HEPES, 11.5 glucose, pH 7.3, adjusted with NaOH.

One million cells were put in a 1-cm width to 3-ml quartz cuvette and inserted in a spectrofluorophotometer (RF-1501 Shimadzu Corporation, Japan), equipped with a thermostated (37 °C) cuvette holder. An ultraviolet light of 360 nm was used for excitation of indo-1, and emissions at 405 and 480 nm were recorded. Background and autofluorescence of the cells were subtracted from the values measured at 405 and 480 nm. The maximum indo-1 fluorescence ratio (R_{max}) was obtained by adding 1 μM ionomycin to the bath in the presence of 10 mM $CaCl_2$. Minimum fluorescence ratio (R_{min}) was determined from cells suspended in a calcium-free medium following the depletion of residual Ca^{2+} by 5 mM EGTA. All the measures were sent to a personal computer and analyzed. $[Ca^{2+}]_{cyt}$ was calculated according to the equation $[Ca^{2+}]_{cyt} = K_d\,(R - R_{min})\,/\,(R_{max} - R)$, where K_d is the apparent dissociation constant of indo-1 for Ca^{2+} (250 nM, [18]), and R is the ratio of fluorescence value at 405 nM over the fluorescence at 480 nm [18].

To induce SOCE, cells were treated with 1 μM TG in the absence of external Ca^{2+}. After 10 min, 1 mM $CaCl_2$ was added. For dose–response curves, the peak $[Ca^{2+}]_{cyt}$ was measured and expressed as a percentage of peak $[Ca^{2+}]_{cyt}$ measured in the absence of the 2-APB analogue.

To test the effects of 2-APB and its analogues on the Ca^{2+} release by the ER, the Jurkat T cells were stimulated by 10 μg/ml phytohemagglutinin (PHA) instead of TG, still in the absence of extracellular Ca^{2+}. PHA cross-links the T cell receptor (TCR), inducing the synthesis of IP_3 and Ca^{2+} release through IP_3 receptors. Cells were pretreated for 5 min with 2-APB or an analogue, and then stimulated by PHA.

2.3. IL-2 assay

One million Jurkat cells per well were incubated in 24-well plates for 24 h with 10 μg/ml phytohemagglutinin and 0 or 10 μM of certain 2-APB analogues. Then supernatants were collected and IL-2 amounts were quantified using a Quantikine Human IL-2 Immunoassay from R&D Systems (R&D Systems Europe, Lille, France).

2.4. Chemicals

2-Aminoethyl diphenylborinate (**1**, 2-APB), 2-aminoethoxy dibutylborate (**4**, 2-ABB) and other chemicals were from Sigma (Saint Quentin Fallavier, France), except 2-aminoethyl 3-methoxyphenyl(phenyl) borinate (**2**, "methoxy-APB")), 2-aminoethyl 3,4-dimethoxyphenyl (phenyl)borinate (**3**, "dimethoxy-APB"), 2-aminoethyl di(2-thienyl) borinate (**5**, "dithienyl-APB"), 2-aminoethyl 3-chloro-2thienyl (2-thienyl)borinate (6, "chloro-dithienyl-APB"), 2-aminoethyl di(-benzothien-2-yl)borinate (**7**, "dibenzothienyl-APB") and 2-(10,11-dihydro-5H-dibenzo[b,f]borepin-5yloxyl)ethylamine (**8** "cyclic-APB"), which were from Specs (Delft, The Netherlands). Structures of these molecules were drawn by Marvin software (http://www.chemaxon.com/products/marvin/) and are depicted in Fig. 1. Abbreviated trivial names (" ") were used for simplicity.

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