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THE ADENOSINE RECEPTORS AS A NEW TARGET FOR TREATMENT OF ATRIAL FIBRILLATION: BIOMARKERS, STRATIFICATION OF THE CARDIOVASCULAR RISK AND THERAPY

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1. Project summary

PURPOSE OF THE PROJECT

The general aim of the project La Marató 201520-30-31 was to test the potential of adenosine receptors as a new source of therapeutical targets and biomarkers for prevention, risk-stratification and treatment of atrial fibrillation. For this purpose, the project was designed to achieve the three primary goals outlined below.

1. To ascertain the spatiotemporal adenosine A_{2A} receptor functioning in cardiomyocytes from experimental models and humans
2. To explore druggability of adenosine receptors in atrial fibrillation
3. To evaluate the usefulness of adenosine receptors as biomarkers for risk-stratification

DESIGN AND METHODOLOGY

To achieve these goals, the project was structured in two subprojects with highly complementary skill sets employing state-of-the-art techniques such as optogenetics, photopharmacology, patch-clamp, and live cell imaging in order to develop new molecular tools for the discovery of new adenosine receptor-based biomarkers and targets for pharmacotherapy. Subsequently novel compounds were tested in isolated human atrial myocytes and *in vitro* models for the detection of arrhythmia in order to increase translation of the research to a clinical setting.

2. Results

Photopharmacological manipulation of adenosine A_1 and A_{2A} receptors

Photo-caged derivatives of adenosine A_1 activators and A_{2A} inhibitors were synthesized for the purpose of being able to induce controlled local photo-release of these compounds and hence prevent arrhythmic electrical activity caused by excessive adenosine A_{2A} receptor activation in patients with atrial fibrillation.

First, the compound MRS7145 was synthesized as a photo-caged derivative of SCH442416, a selective adenosine A_{2A} receptor ($A_{2A}R$) inhibitor. Subsequent testing revealed that photo-release of SCH442416 from MRS7145 effectively reduced $A_{2A}R$-mediated cAMP production in cardiomyocyte suspensions. Importantly, photoactivation

of MRS7145 also prevented arrhythmic responses in beating multicellular cardiomyocyte preparations and arrhythmogenic calcium release in isolated human atrial myocytes, pointing to MRS7145 as a useful tool for local pharmacological control of $A_{2A}R$ activity and spontaneous atrial electrical activity.

Subsequently, a new positive allosteric modulator of the adenosine A_1 receptor (A_1R) named T62 was developed and a photo-caged derivative (pc-T62) was synthesized. However, because initial testing of the efficacy of photo-released T62 revealed that it only produced an apparently modest A_1R activation (15%), another photo-caged derivative of the A_1R agonist CPA was also developed. Testing of the efficacy of photo-release of caged CPA revealed that it abolished forskolin induced cAMP production. The utility of T62 and CPA is currently being tested in human atrial myocytes (see below).

Assessing adenosine $A_{2A}R$ and A_1R expression and heteromerization

In order to optimize adenosine A_1 and A_{2A} receptor based therapy we first determined the A_1R and $A_{2A}R$ expression profiles in human atrial biopsies from patients with and without atrial fibrillation. This analysis revealed that atrial fibrillation is associated with opposite remodeling of A_1R and $A_{2A}R$ expression resulting in a 2.5 fold upregulation of $A_{2A}R$ levels and a 40% reduction the A_1R level in patients with atrial fibrillation. Consequently, the $A_{2A}R$:A_1R ratio is increased 7 fold in patients with atrial fibrillation pointing to a potential deregulation of adenosinergic signaling in atrial fibrillation and suggests that therapeutical strategies aiming to re-establish the balance between A_1 and A_{2A} receptors may be optimal.

Furthermore, to assess the $A_{2A}R/A_1R$ heteromer formation we also developed and implemented a new assay for this purpose. This assay uses specific $A_{2A}R$ and A_1R antibodies that engage into an energy transfer through specific secondary antibodies. Using this assay in atrial membranes from pigs with and without atrial fibrillation, we found a significant reduction in $A_{2A}R/A_1R$ heteromer formation in atrial fibrillation, pointing to a reduction of the negative allosteric modulation controlling $A_{2A}R$ function, and hence and increased intrinsic $A_{2A}R$ activity in atrial fibrillation.

Control of calcium homeostasis and electrical activity by manipulation of $A_{2A}R$ and A_1R activity

In order to determine the functional impact of the observed alterations in plasmatic adenosine levels, remodeling of A_1R and $A_{2A}R$ expression and heteromerization in atrial fibrillation, we investigated the impact of increasing adenosine levels as well as pharmacological manipulations of A_1R and/or $A_{2A}R$ activity on calcium homeostasis and electrical activity in isolated human atrial myocytes.

Thus, to determine if the above-mentioned deregulation of adenosinergic signaling in atrial fibrillation has a functional impact on intracellular calcium homeostasis, we performed patch clamp analysis of the effects of increasing adenosine concentrations on calcium release-induced transient inward (I_{TI}) currents and on the L-type calcium current in human atrial myocytes. These analyses showed that a low concentration of adenosine (6nM) is able to reduce the frequency of spontaneous I_{TI} currents induced by beta-adrenergic stress. However at higher adenosine concentrations (600 nM), this inhibitory effect disappears. For comparison, full activation of A_1R by the selective agonist R-PIA completely abolished the stimulatory effect of beta-adrenergic stimulation. These findings suggest that $A_{2A}R$ receptors are activated by higher adenosine concentrations, reversing the inhibitory effect of A_1R activation on I_{TI} currents. The corresponding analyses of the L-type calcium current revealed that while there is a rebound in the I_{TI} frequency at higher adenosine concentrations, the stimulatory effect of beta-adrenergic stimulation on L-type calcium current is progressively inhibited as the adenosine concentration increases, suggesting that the A_1 and A_{2A} receptors modulate L-type calcium channels and ryanodine receptors (that activate I_{TI} upon opening) differently.

Because arrhythmic electrical activity in myocytes from patients with atrial fibrillation can be triggered by increased phosphorylation of the RyR2 and adenosine A_{2A} receptors can mediate this phosphorylation, we investigated the relationship between adenosine $A_{2A}R$ activation, spatial heterogeneities in RyR2 phosphorylation and local sub-cellular alterations in intracellular calcium homeostasis and arrhythmic electrical activity in human atrial myocytes.

Our findings showed that stimulation of A_{2A} receptors with the selective agonist CGS21680 induced RyR2 phosphorylation at s2808, which was more pronounced at the cell membrane (where the A_{2A} receptors are located) than in the cell center. This in turn activated a larger number of spontaneous local calcium release events (sparks)

near the cell membrane. Consequently, larger amounts of calcium was extruded from the myocyte by electrogenic Na-Ca exchange, resulting in larger and more frequent arrhythmogenic membrane depolarizations. Interestingly, stimulation of A_{2A} receptors with CGS21680 in human atrial myocytes from patients without atrial fibrillation led to alterations in RyR2 phosphorylation and the incidence of spontaneous calcium release events and membrane depolarizations that resembled the alterations observed in patients with atrial fibrillation, demonstrating that excessive $A_{2A}R$ activation can transform healthy human atrial myocytes into myocytes with arrhythmic electrical activity similar to that observed in myocytes from patients with atrial fibrillation. These findings, combined with higher adenosine levels and $A_{2A}R$:A_1R ratio in patients with atrial fibrillation underscores the potential of adenosine A_1 and A_{2A} receptors as new pharmacological targets for the treatment of atrial fibrillation.

Evaluation of $A_{2A}R$ expression in human lymphocytes from patients with atrial fibrillation

To assess the utility of $A_{2A}R$ as a biomarker in atrial fibrillation, we first detected $A_{2A}R$ levels in peripheral lymphocytes, and demonstrated that $A_{2A}R$ levels are increased in lymphocytes from patients with atrial fibrillation. In addition, the $A_{2A}R$ mRNA expression in peripheral blood mononuclear cells from atrial fibrillation patients was also increased. Subsequently, we assessed the $A_{2A}R$ expression levels in different lymphocyte populations from subjects with and without atrial fibrillation. Thus, T-lymphocytes (i.e. CD3+ and CD8+) and B-lymphocytes (i.e. CD19+) were gated by flow cytometry and the $A_{2A}R$ density determined in each population. Interestingly, our results demonstrated that while $A_{2A}Rs$ are increased both in T and B lymphocytes from patients with atrial fibrillation, only B cells showed a significant increase in CD194, a heart homing marker. Importantly, a significant positive correlation between increased $A_{2A}R$ expression and presence of CD194 in B lymphocytes was observed. Overall, it seems that B lymphocytes from patients with atrial fibrillation were recruited to the heart and these showed an increased $A_{2A}R$ expression, thus becoming a specific hallmark for patients with this arrhythmia.

3. Impact, future research lines and clinical perspective

Adenosine receptors as mediators of arrhythmic atrial electrical activity

The discovery that patients with atrial fibrillation have lower plasma levels of adenosine deaminase and higher levels of adenosine than patients without the arrhythmia, combined with combined with findings demonstrating that $A_{2A}R$ activation induce alterations in the distribution and incidence of spontaneous calcium release events that promote arrhythmogenic electrical activity in myocytes from patients without atrial fibrillation and exacerbate these alterations in patients with the arrhythmia, suggest that there may be a correlation between plasma levels of adenosine and/or adenosine deaminase levels and ectopic atrial electrical activity, affording a pathophysiological motivation for investigating a possible correlation between adenosine and/or adenosine deaminase levels and the incidence of atrial ectopic activity in patients with paroxysmal atrial fibrillation or at risk of atrial fibrillation. The same is true for CD194 levels in B lymphocytes (see also adenosine A_{2A} receptors as biomarkers below). In collaboration with cardiologists, we are currently exploring the possibility of testing these hypotheses in an optimal clinical setting.

Adenosine receptors as therapeutical targets in atrial fibrillation

The fact that excessive $A_{2A}R$ activation promote arrhythmogenic electrical activity, combined with our finding that arrhythmogenic calcium release events induced by beta-adrenergic stress is sustained at an elevated adenosine level (600 nM) while the positive inotropic effect of beta-adrenergic stimulation is abolished by this adenosine level; suggest that the beneficial effect of reducing excessively high adenosine levels may be dual. In other words, our findings suggest that normalizing plasma adenosine (and/or adenosine deaminase) levels in patients with atrial fibrillation might at the same time reduce the incidence of ectopic electrical activity and increase the atrial inotropic reserve in patients with atrial fibrillation.

Our findings document that the selective $A_{2A}R$ antagonist SCH442416 can effectively be released by photo-activation (without damaging the myocytes) of the caged compound MRS7145 and fully reverse spontaneous calcium release events induced by $A_{2A}R$ activation in single and multicellular myocyte preparations. These findings open up the possibility of using photoactivation of MRS7145 as a means to deliver the $A_{2A}R$ antagonist SCH442416 locally minimizing potentially adverse systemic effects of the

antagonist. In this context new lines of research will be needed to explore the feasibility of delivering MRS7145 and releasing SCH442416 in intact heart preparations

Adenosine A_{2A} receptors as biomarkers for risk-stratification in atrial fibrillation

This study aimed to evaluate the usefulness of adenosine receptors as biomarkers for risk-stratification and the results of the project has revealed a significant positive correlation between increased $A_{2A}R$ expression and presence of CD194 (a homing marker for the heart) in B lymphocytes. Moreover, CD194 levels were significantly increased in patients with atrial fibrillation, pointing to increased CD194 levels as a specific hallmark for patients, suggesting that analysis of CD194 levels in B lymphocytes could be a useful new tool to predict risk and severity of atrial fibrillation. We are currently assessing the possibility of patenting these interesting results.

4. Publications

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