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Adenine Nucleoside and Nucleotide Differentially Affect Heart Rhythm and Inotropy: Putative Therapeutic Implications

Bruno Miguel Martins Bragança

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

BRUNO MIGUEL MARTINS BRAGANÇA

ADENINE NUCLEOSIDES AND NUCLEOTIDES DIFFERENTIALLY AFFECT HEART RHYTHM AND INOTROPY: PUTATIVE THERAPEUTIC IMPLICATIONS

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Dissertation in fulfilment of the requirements for the PhD degree in Biomedical Sciences, submitted to Instituto de Ciências Biomédicas Abel Salazar of University of Porto.

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Bruno Miguel Martins Bragança - 2021

This research was partially supported by Fundação para a Ciência e a Tecnologia (FCT, FEDER funding, projects FCOMP-01-0124-FEDER-028726 - FEDER, COMPETE - FCT PTDC/DTP-FTO/0802/2012, PEst-OE/SAU/UI0215/2014, UID/BIM/4308/2016 and UIDB/04308/2020).

The author was in receipt of a PhD Studentship from FCT (POPH - QREN/FSE funding, SFRH/BD/104114/2014).

A journey of a thousand miles begins with a single step

Lao Tzu

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Data: 2022.01.27 23:11:17 +0000

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ACKNOWLEDGEMENTS

I want to express my deepest gratitude to my supervisor Professor Paulo Correia de Sá. His knowledge and vision were instrumental in guiding me to the best way of doing science. He is a true mentor, and I would be delighted to keep learning from him!

I am incredibly thankful to Professor Ana Patricia Fontes de Sousa for her guidance and friendship. Her perseverance, knowledge, and dedication were essential to help me in overcoming all the hurdles that I had during this long journey.

I am grateful to Professor Pedro Lima for his support and everything he taught me. I will never forget his hospitality and for having welcomed me into his family while I was venturing into the paths of electrophysiology in Lisbon.

I also had great pleasure of working with my colleagues and friends: Doctors Fátima Ferreirinha, Bernardo Matos, Cátia Vieira, Mariana Certal, Aurora Barros, Diogo Paramos, and Isabel Silva, as well as Drs. Carina Sousa, Nádia Monteiro, Mafalda Gonçalves, Rui Cardoso, Sílvia Marques, Rafael Neiva and Diogo Silva.

Many thanks to all professors from the Department of Pharmacology, in special to Professor Maria Adelina Costa, Professor Graça Lobo, Professor Laura Oliveira, Professor Margarida Araújo, Professor Miguel Faria, Dr. Teresa Magalhães Cardoso, and Dr. Alexandrina Timóteo. I would like to recognize the assistance and friendship from Mrs. Belmira and Mrs. Helena Costa e Silva.

A special thanks to family and friends, they were relentless in pushing me to success. I am blessed to have them. I cannot end without expressing my sincere thanks to Salomé for her endless support, love, and dedication throughout all these years.

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I. ABBREVIATIONS

$[Ca^{2+}]_i$, intracellular calcium
$[Na^{+}]_i$, intracellular sodium
ABC, ATP-binding cassette
AC, adenylyl cyclase
ACh, acetylcholine
ACS, acute coronary syndrome
ADA, adenosine deaminase
ADP, adenosine 5′-diphosphate
AK, adenosine kinase
AMP, adenosine 5’-monophosphate
AP, action potential
A_xR, adenosine receptors
ATP, adenosine 5′-triphosphate
ATPγS, adenosine 5′-[γ-thio]triphosphate
AVN, atrioventricular node
Ca^{2+}, calcium ion
cAMP, cyclic adenosine-3',5'- monophosphate
CD39, NTPDase 1
CD73, 5’-nucleotidase
cGMP, cyclic guanosine-3′,5′-monophosphate
CICR, calcium-induced calcium release (CICR)
CNT, concentrative nucleotide transporters
CTP, cytidine 5′-triphosphate
Cx43, connexin 43
DAD, delayed afterdepolarization
E-C, excitation-contraction coupling
E-NPP, ecto-nucleotide pyrophosphatase/phosphodiesterase
E-NTPDase, ecto-nucleoside triphosphate diphosphodyrolase
EAD, early afterdepolarization
ENT, equilibrative nucleotide transporters
EPAC, exchange protein directly activated by cAMP
GIRK, G-protein inwardly rectifying K^+ channel
GPCR, G-protein coupled receptors
HCN, hyperpolarization-activated cyclic nucleotide–gated channel
HF, heart failure
HIF-1, hypoxia-inducible factor-1
I_f, “funny current”
IP3, inositol 1,4,5-triphosphate
K^+, potassium ion
LCC, L-type Ca^{2+} channel
LCR, local Ca^{2+} release
M_xR, muscarinic ACh receptor
MAPK, mitogen-activated protein kinase
Na^+, sodium ion
Na_V, voltage-gated sodium channel
NBTI, nitrobenzylthioinosine
NCX, Na^+/Ca^{2+}-exchanger
NE, norepinephrine
NO, nitric oxide
NKA, Na^+/K^+-ATPase
PDE, phosphodiesterase
PI3K, phosphatidylinositol-3 kinase
PIP_2, phosphatidylinositol 4,5-bisphosphate
PKA, protein kinase A
PKC, phosphokinase C

PLC, phospholipase C
PLN, phospholamban protein
Pnx, pannexins
RyR, ryanodine receptors
SAH, S-adenosyl-L- homocysteine
SAN, sinoatrial node
SERCA, sarco/endoplasmic reticulum (SR) calcium-ATPase
SK, small-conductance calcium-activated K^+ channel
SSS, sick sinus syndrome
STEMI, ST-elevation myocardial infarction
UDP, uridine 5'-diphosphate
UTP, uridine 5'-triphosphate
VNUT, vesicular nucleotide transporter

II. RESUMO

As funções dos diversos sistemas do nosso organismo são reguladas, de forma precisa, pela atividade de purinas produzidas endogenamente, tanto em condições fisiológicas como patológicas. Desde há muito que se sabe que o ATP e a adenosina se comportam como metabolitos retaliatórios no meio extracelular; estas purinas são libertadas pelas células em situações de stresse na tentativa de restaurar o equilíbrio e a homeostasia do organismo. Os nucleótidos e nucleosídeos de adenina exercem as suas funções através da ativação dos recetores purinérgicos dos tipos P2 e P1, respetivamente. Estes recetores em conjunto com transportadores de purinas e ecto-nucleotidases presentes na membrana plasmática são os principais intervenientes da cascata da sinalização purinérgica. No sistema cardiovascular, as purinas são reconhecidas pelas suas ações cronotrópicas e inotrópicas, bem como pelos seus efeitos pró-trombóticos e na tonicidade vascular. Além destas respostas agudas, a sinalização purinérgica desencadeia respostas sustentadas e adaptativas em contexto de agressões prolongadas, como a inflamação, aterosclerose, trombose e remodelação miocárdica, levando em última instância à disfunção e/ou falência cardíaca.

A descoberta de novos alvos terapêuticos baseados na sinalização purinérgica tem constituído uma contribuição valiosa na cardiologia atual. Vários moduladores do sistema purinérgico têm sido utilizados tanto como o intuito terapêutico, nomeadamente no controlo de taquiarritmias ou no tratamento da doença cardíaca isquémica (onde se destacam, por exemplo, os antagonistas dos recetores $P2Y_{12}$), bem como para fins de diagnóstico da perfusão cardíaca. Para além dos potentes efeitos cronotrópicos e dromotrópicos negativos da adenosina, muito úteis no tratamento de diversas taquiarritmias em contexto de urgência, este nucleósido apresenta-se ainda como um potente vasodilatador coronário, restaurando a perfusão miocárdica e auxiliando no diagnóstico da doença arterial coronária.

Apesar da extensa investigação e do uso comum destes fármacos na prática clínica diária, a regulação aguda do sistema cardiovascular pelas purinas está ainda longe de estar completamente esclarecida. Algumas das lacunas existentes no nosso conhecimento incluem: 1) o significado fisiológico da cascata de sinalização purinérgica no controlo autonómico das funções cardíacas; 2) o envolvimento e a função dos diversos recetores P2 no tecido de condução cardíaco e no miocárdio contráctil em resposta ao ATP; 3) a compreensão dos mecanismos responsáveis pelos efeitos indesejados da adenosina e do ATP observados na prática clínica, e a forma para a sua minimização; 4) o esclarecimento da contribuição da sinalização purinérgica na fisiopatologia de diversas doenças cardiovasculares.

Atendendo às lacunas identificadas, esta tese teve como principal objetivo contribuir para o esclarecimento do papel do ATP e da adenosina na regulação da função

cardíaca através da estimulação de determinados subtipos de recetores P2 e P1, respetivamente. Os efeitos dos agonistas e antagonistas seletivos dos recetores purinérgicos sobre a frequência e força de contração cardíaca foram avaliados recorrendo a registos miográficos realizados tanto em preparações auriculares a contrair espontaneamente como em tiras de ventrículos estimuladas eletricamente provenientes de ratazanas da estirpe Wistar-Han. Foram, ainda, isolados cardiomiócitos auriculares para avaliação eletrofisiológica de correntes iónicas através da técnica de *patch-clamp* na configuração celular total (*whole-cell*). Para complementar os estudos funcionais foram realizadas experiências de imunolocalização de proteínas de interesse por microscopia confocal.

Os resultados mostram que a adenosina, atuando por via da estimulação do recetor do subtipo A_1 (A_1R), é mais potente a reduzir a frequência sinusal (cronotropismo negativo) do que deprimir a amplitude das contrações auriculares (inotropismo negativo), evidenciado, assim, uma atividade depressora "cronosseletiva". Esta propriedade não é partilhada por outros agentes cronotrópicos negativos conhecidos, como por exemplo o agonista do recetor muscarínico M_2 (M_2R) da acetilcolina - oxotremorina - que reduziu de forma equipotente a frequência e a força de contração auricular. O bloqueio seletivo dos canais GIRK pela tertiapina-Q preveniu os efeitos da adenosina e da oxotremorina de forma equivalente. Contrariamente, o bloqueio dos canais SK pela apamina e dos canais de cálcio Ca_v1 (tipo L, LCC) pela nifedipina ou verapamil favoreceram o efeito inotrópico negativo da adenosina, abolindo a "cronoseletividade" característica deste nucleósido. O envolvimento dos canais SK na resposta mediada pela ativação dos recetores A_1 da adenosina foi confirmada através da realização de experiências de eletrofisiologia em cardiomiócitos auriculares isolados. Utilizando a técnica de imunoflurescência aplicada à microscopia confocal foi possível demonstrar que as proteínas A_1R, SK e LCC estão colocalizadas no nó sinusal (SAN). Os resultados mostram pela primeira vez que a ativação do recetor A_1R limita a abertura dos canais SK nas aurículas de ratazana, prolongando assim o período de repolarização do potencial de ação auricular facilitando o influxo de Ca^{2+} através de canais de cálcio Ca_v1 (tipo L) e, consequente, uma maior atividade contráctil. Em suma, os resultados apresentados desvendaram o mecanismo pela qual a adenosina preserva parcialmente o inotropismo enquanto atua como potente agente cronotrópico negativo. Permitiram, ainda, explicar as diferenças a nível molecular entre a adenosina e outros agentes cardiodepressores com atividade sobre recetores acoplados à proteína $G\alpha_{i/o}$, como é o caso do M_2R.

Relativamente ao efeito cardíaco do ATP, demonstra-se neste trabalho que este nucleótido exerce um efeito cronotrópico negativo independente da sua conversão em adenosina. Esta conclusão foi retirada devido à persistência do efeito do ATP sobre a

frequência cardíaca na presença do antagonista seletivo dos A_1R, DPCPX, ou mediante a inibição da hidrólise enzimática do ATP após a adição do inibidor não-seletivo das ecto-nucleotidases, POM-1. O bloqueio do efeito inibitório do ATP pelo 5-BDBD sugere o envolvimento do recetor ionotrópico P2X4 (P2X4R). O mecanismo subjacente à ativação deste recetor pelo ATP no SAN parece envolver o trocador Na^+-Ca^{2+} (NCX), considerando que o mesmo foi abolido tanto pelo KB-R7943 com pelo ORM-10103, dois inibidores quimicamente distintos deste trocador. Curiosamente, a interação entre o P2X4R e o NCX associa-se a um efeito inotrópico positivo em preparações ventriculares estimuladas eletricamente. O P2X4R co-localiza com o NCX no SAN e no miocárdio ventricular direito, tal com foi possível provar através da técnica de imunoflurescência aplicada à microscopia confocal. Os dados sugerem que a ativação do P2X4R pelo ATP exerce um papel duplo muito interessante no coração, já que parece ser responsável por induzir a redução da frequência sinusal à qual se associa um aumento da força contráctil do miocárdio ventricular. Este efeito duplo parece dever-se à inibição do trocador NCX pelo P2X4R, cuja resultante é uma resposta funcional semelhante à dos digitálicos.

Em conclusão, os dados apresentados nesta tese desvendam novos mecanismos através dos quais a adenosina e o ATP controlam a função cardíaca. Este trabalho mostra, pela primeira vez, que a adenosina é um modulador cronosseletivo negativo da função auricular, afetando de forma diversa as correntes de potássio dos tipos GIRK e SK. Demonstra-se ainda que o ATP, através da estimulação do P2X4R, promove a atividade do trocador NCX em modo reverso, reduzindo a frequência cardíaca e estimulando a contração ventricular. Estas descobertas abrem novas perspetivas para a manipulação da cascata da sinalização purinérgica com potenciais implicações terapêuticas para o tratamento de diversas patologias cardiovasculares.

III. ABSTRACT

The functions of every system in the body are fine tuning controlled by endogenously produced purines, both in health and in disease conditions. Extracellular ATP and adenosine have long been considered retaliatory metabolites, which can be released by stressed cells in an attempt to restore the equilibrium when body homeostasis is compromised. Adenine nucleotides and nucleosides act via P2 and P1 purinoceptors, respectively, which together with plasma-membrane embedded transporters and ectoenzymes are the main players of the purinergic signaling cascade. In the cardiovascular system, purines are recognized by their cardiac chronotropic and inotropic actions, as well as by their potent vasodynamic and pro-thrombotic effects. Apart from these acute responses, signaling via purines undertake more sustained and adaptive responses to stressful conditions, such as inflammation, atherosclerosis, thrombosis and myocardial remodeling that may ultimately drive the heart to failure.

Targeting the purinergic system has been extremely valuable in modern cardiology. Several purinergic drugs have been used to manage tachyarrhythmias, myocardial infarction (e.g., $P2Y_{12}$ receptor antagonists), and for diagnostic purposes. Adenosine itself has potent negative chronotropic and dromotropic effects that are useful in medical emergencies to tackle with bothersome tachyarrhythmias; the nucleoside also acts as a potent coronary vasodilator restoring myocardial perfusion and helping in the diagnosis of coronary artery disease. Despite extensive research in this field and the compassionate clinical use of purinergic drugs, acute regulation of heart functions by purines is still far from being entirely resolved. Some existing gaps in our knowledge include: 1) the physiological significance of the purinergic signaling cascade in the autonomic control of heart functions; 2) information about the role of P2 receptor subtypes in the cardiac conduction system *vis a vis* the working myocardium responding to ATP administration; 3) knowledge about the mechanisms underlying the cardiac unwanted effects associated to adenosine and ATP and how these can be overcome; 4) more complete information about the pathophysiological implications of the purinergic signaling cascade in cardiovascular disease conditions.

With these questions in mind, this thesis aimed at providing further insights into the role of ATP and adenosine, via P2 and P1 receptors, respectively, in isolated hearts function. The myographic effects of selective purinoceptor agonists and antagonists on heart rate and cardiac inotropy was evaluated in spontaneously beating atria and electrically paced ventricular strips of Wistar-Han rats. Acutely dissociated atrial cardiomyocytes were also used for voltage-clamp electrophysiological recordings using the whole-cell patch configuration. Functional results were further strengthened by immunolocalization of interest proteins by immunofluorescence confocal microscopy.

Data demonstrate that adenosine, acting via the A_1 receptor (A_1R) subtype, is more

potent in reducing the sinus rhythm (negative chronotropism) than the amplitude of atrial contractions (negative inotropism), indicating that adenosine may be considered a negative "chronoselective" drug. This property contrasts with other known negative chronotropic agents, like the muscarinic M_2 (M_2R) acetylcholine receptor agonist, oxotremorine, which reduced atrial rate and contraction strength within the same concentration range. While the GIRK channel blocker, tertiapin-Q, prevented the effects of both adenosine and oxotremorine, only the negative chronotropic action of adenosine was sensitive to blockage of the SK channel blocker, apamin, as well as to inhibition of L-type calcium channel (LCC) with nifedipine and verapamil. Whole-cell voltage-clamp recordings confirmed that selective activation of the A_1R negatively modulates SK currents in isolated atrial cardiomyocytes. Co-localization of A_1R, SK and LCC proteins was demonstrated in the sinus region of rats atria by immunofluorescence confocal microscopy. Data show here for the first time that activation of atrial A_1R limits SK channels opening, thus prolonging plasma membrane repolarization, which allows more time for Ca^{2+} to enter the cell via L-type calcium channels. This feature finally explains why adenosine partially preserves inotropy while reducing the sinus rhythm (chronotropy), a situation that clearly contrasts with other cardiodepressants acting via Gαi/o-protein-coupled receptors, like the M_2R.

Regarding the effect of ATP, I show here that the nucleotide exerts a negative chronotropic effect that is independent of its enzymatic breakdown into adenosine. This was suggested because reduction of the spontaneous atrial rate by ATP was still evident in the presence of the A_1R antagonist, DPCPX, or upon preventing the enzymatic breakdown of extracellular ATP with the non-selective inhibitor of ecto-nucleotidases, POM-1. Instead, involvement of the P2X4 receptor subtype (P2X4R) has been confirmed because the negative chronotropic effect of ATP was abrogated by 5-BDBD, which is a potent and selective P2X4R blocker. The mechanism underlying the negative chronotropic effect of ATP seems to involve the Na^+-Ca^{2+} exchanger (NCX) located in the sinoatrial node (SAN), given that it was blocked by two chemically distinct inhibitors of this carrier, namely KB-R7943 and ORM-10103. Interestingly, the interplay between P2X4R and NCX increased inotropy in paced ventricular strips. Immunolocalization experiments confirmed that P2X4R and NCX co-localize in the SAN, as well as in the right ventricle. Data suggest that activation of P2X4R by ATP exerts a dual role in the heart, comprising a reduction in the sinus rhythm while increasing the amplitude of ventricular contractions. This dual effect is centered on the P2X4-mediated inhibition of NCX resulting in a unique digitalis-like phenomenon.

In conclusion, data presented in this thesis unravels novel mechanisms by which adenosine and ATP control cardiac function. It is shown here for the first time that adenosine is a chronoselective negative modulator of atrial function by differentially affecting GIRK and SK potassium currents, while the slow down effect of ATP on heart rhythm is accompanied

by a sustained ventricular inotropic action via inhibition of the NCX carrier in the reverse mode. These findings open new perspectives of manipulating the purinergic signaling cascade with putative therapeutic implications for heart disease conditions.

IV. INTRODUCTION

1. Purinergic signaling

The purinergic system remains one of the most ancestral cellular signaling systems found in living organisms. The first evidence of purinergic signaling date back to the beginning of the 20th century with the seminal work of Sir Alan Nigel Drury and Sir Albert Szent-Györgyi; they demonstrated for the first time that adenine-enriched extracts derived from bovine hearts were able to induce bradycardia, decrease blood pressure and promote dilation of coronary arteries (Drury and Szent-Gyorgyi, 1929). However, it was nearly five decades later when, in 1972, Professor Geoffrey Burnstock (who recently left us in 2 June 2020) proposed the groundbreaking theory of purinergic transmission, describing extracellular adenosine-5'-triphosphate (ATP) and its metabolite adenosine as important cellular messengers (Burnstock, 1972). Despite early resistance to this theory, soon it became gradually accepted by the scientific community, creating a fertile ground for what is currently an interesting and exciting research field that has been provided so much valuable knowledge in the interpretation of a wide variety of physiological and pathophysiological processes (Jacobson, 2020).

The purinergic transmission is based on purine (e.g., ATP, adenosine 5′-diphosphate (ADP), adenosine 5´-monophosphate (AMP)) and pyrimidine (e.g., uridine 5′-triphosphate (UTP), uridine 5′-diphosphate (UDP); cytidine 5′-triphosphate (CTP)) nucleotides and nucleosides (adenosine, inosine) and their extracellular activity over a wide family of transmembrane receptors globally designated by purinoceptors. The operation of this system involves several key processes, namely (1) the intracellular synthesis of purines and pyrimidines, (2) their release to the extracellular environment, followed by (3) activation of membrane-bound purinoceptors and, lastly, (4) removal of purines and pyrimidines from the extracellular space terminating the purinergic signaling cascade (Figure 1). In this introductory note, all processes that gear behind purinergic signaling will be detailed, in parallel with integrating their role in several physiological and pathophysiological mechanisms mainly focusing on the cardiovascular system.

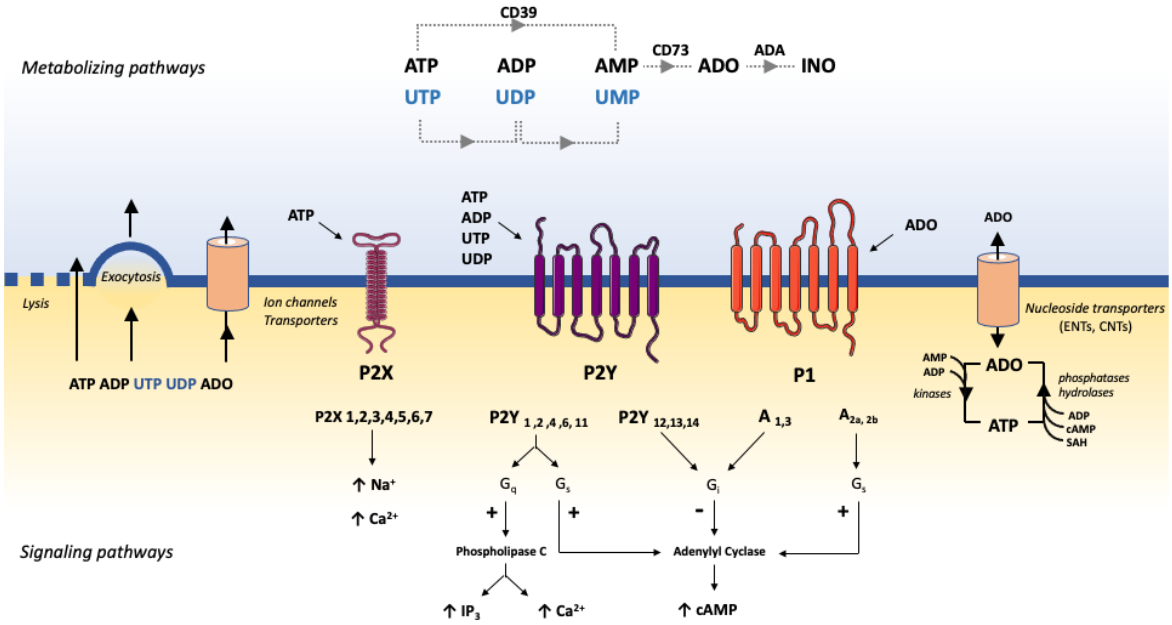

Figure 1. The purinergic signaling. The source of purines is either provided by both anabolic (*de novo* purine biosynthesis) and catabolic pathways from hydrolysis of compounds containing purine moieties (cAMP, S-adenosyl-L-homocysteine (SAH)). Intracellular purines are released to the extracellular space either by lytic or non-lytic pathways. Extracellular nucleotides are short-lived due to ecto-nucleotidases (CD39 (ecto-nucleoside triphosphate diphosphohydrolase); CD73 (ecto-5'-nucleotidase)) that sequentially remove phosphate groups, generating adenosine (ADO). Adenosine deaminase (ADA) then metabolizes ADO to inosine (INO). During this metabolizing pathway, adenine and uridine nucleotides are able to bind to P2 receptors (ionotropic P2X and metabotropic P2Y), whereas adenosine binds to metabotropic P1 receptors. Stimulation of P1 and P2 receptors trigger different signaling pathways and physiological responses. Illustration used elements from Servier Medical Art.

2. Purinergic Receptors

2.1.1. P1 receptors

Adenosine receptors (ARs) are collectively denominated as P1 receptors. Four subtypes of ARs have been cloned and include A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R (Fredholm et al., 2011) (Table 1). ARs belong to the class A of membrane-bound G-protein coupled receptors (GPCRs). ARs are highly conserved in mammals, with a reported sequence homology that exceeds 84% among all receptors with the exception for A_3R, which exhibits about 72% sequence variation between humans and rats (Jacobson, 1995). Each AR has its own "fingerprint" according to its sensitivity to adenosine and other synthetic ligands, downstream signaling pathways, as well as tissue/cell and development stage specific distribution throughout the body. Therefore, it is possible to resolve the receptor involvement in a particular adenosine-mediated response based on these characteristics. The rank affinity order of ARs for adenosine is as follows: $A_1R > A_{2A}R > A_3R > A_{2B}R$ (Fredholm et al., 2011). Different affinities imply that when the concentration of a certain ligand changes, one receptor could be preferentially activated over another (Table 1). Under resting conditions, extracellular levels of adenosine are set to be low. Tiny physiological fluctuations of

extracellular adenosine are primarily sensed by the high-affinity A_1R, which has a reported affinity within the nanomolar range, followed by $A_{2A}R$, A_3R, and lastly by $A_{2B}R$, with the latter exhibiting an affinity for the nucleoside within the micromolar concentration range. Low-affinity receptors are thought to play a meaningful role only when extracellular adenosine is high, which may occur following stressful insults, such as ischemia or tissue injury. Interestingly, endogenous ligands of ARs are not limited to adenosine. Adenosine deamination by adenosine deaminase (ADA) generates inosine, which can bind and activate ARs (Ciruela et al., 2010). Inosine is a low-affinity agonist of the A_3R (Jin et al., 1997; Herman-de-Sousa et al., 2020); evidence for its binding over other ARs is questionable, at least within reasonable physiological levels (Welihinda et al., 2016). Interestingly, extracellular ADA may function as an allosteric modulator of ARs, increasing cooperativity for ligand binding, and limiting receptor desensitization (Gracia et al., 2008; Ciruela et al., 2010).

Table 1. Selected pharmacology of P1 receptors

	Signaling pathway	**Main location**	**Agonists** (Ki μM for endogenous agonist)	**Antagonists** (Ki μM)
P1 receptors			CADO; NECA	Caffeine,Theophylline
A_1R	$G\alpha_i$-protein ↓cAMP; K^+ channels; ↑IP_3	Conduction system, myocardium	R-PIA > CPA >> Adenosine (0.1)	LUF5891; Rolophylline; DPCPX
$A_{2A}R$	$G\alpha_s$-protein ↑cAMP	Endothelium; VSMC; fibroblasts; immune cells	CGS-21680C > Adenosine (0.3)	SCH-420814; ZM-241385; SCH-5826
$A_{2B}R$	$G\alpha_s$-protein ↑cAMP	Endothelium; VSMC; fibroblasts; immune cells	BAY 60-6583 >> Adenosine (15)	PSB-603; PSB-1115
A_3R	$G\alpha_i$-protein ↓cAMP; ↑IP_3	Myocardium	Cl-IB-MECA ≥ MRS-5151 >> Adenosine (0.3) >> Inosine*	PSB-10; MRS1191; MRS 1523

Adapted from (Fredholm et al., 2011; Headrick et al., 2013). Receptor affinities of agonists and antagonists are reported in micromolar (μM) for humans. *Inosine has been described as an adenosine receptor agonist (Jin et al., 1997). Abbreviations: 2-chloro-adenosine (CADO); adenosine 3',5'-cyclic monophosphate (cAMP); inositol 1,4,5-trisphosphate (IP_3); (R)-N6 -phenylisopropyladenosine (R-PIA); vascular smooth muscle cells (VSMCs)

Regarding downstream targets of ARs, the odd-numbered receptors (A_1R and A_3R) are preferentially coupled to pertussis-sensitive $G\alpha_{i/o}$-protein, thereby inhibiting adenylyl cyclase (AC) and, hence, formation of 3',5'-cyclic adenosine monophosphate (cAMP) and downstream activation of protein kinase A (PKA). On the other hand, stimulation of even-numbered receptors ($A_{2A}R$ and $A_{2B}R$) are preferentially coupled to $G\alpha_s$-protein and have an opposite effect on AC (Fredholm et al., 2011). While regulation of AC-cAMP-PKA axis assumes a central position in AR signaling, other "non-canonical" signaling pathways have also been reported. One example of such pathway is related with stimulation of phospholipase C (PLC), a key enzyme involved in regulation of intracellular calcium dynamics via formation of inositol 1,4,5-triphosphate (IP_3) and phosphorylation of target proteins via protein kinase C (PKC). While $A_{2B}R$ and A_3R are both coupled to $G\alpha_q$-proteins and stimulate PLC, A_1R also activates this enzyme through other "non-canonical" pathways involving the release of Gβγ subunits and stimulation of PLC (Biber et al., 1997; Fenton et al., 2010). Examples of other signaling pathways include the mitogen-activated protein

kinase (MAPK), phosphatidylinositol-3 kinase (PI3K), and exchange protein directly activated by cAMP (EPAC) (Mustafa et al., 2009; Villarreal et al., 2009; Liu and Xia, 2015; Borea et al., 2018). Nevertheless, caution should be taken regarding the physiological role of these “non-canonical” pathways, as most of them were detailed in non-native systems.

ARs also regulate signaling proteins other than intracellular enzymes, namely ion channels. Stimulation of A_1Rs and other $G\alpha_{i/o}$-protein-coupled receptors, like muscarinic acetylcholine (ACh) receptor M_2 (M_2R) receptors, promote an increase of K^+ conductance through Gβγ subunits-mediated opening of G-protein inwardly rectifying K^+ (GIRK) channels (Leaney et al., 2000), decreasing cellular excitability and complementing the inhibitory action over AC (Kobayashi and Ikeda, 2006). Interestingly, stimulation of PLC that occurs with A_1R stimulation functions as a negative feedback mechanism for membrane hyperpolarization, given that the substrate of this enzyme, phosphatidylinositol 4,5-bisphosphate (PIP_2), is required for a proper function of GIRK channels (Kobayashi and Ikeda, 2006; Cho, 2010). Another example comes from stimulation of A_2Rs and opening of K_{ATP} channels, an important family of ion channels involved in vasodilation and ischemic preconditioning, as well as in insulin secretion and glucose homeostasis (Kleppisch and Nelson, 1995).

Intracellular ends of ARs have in their amino-acid sequence several consensus regions to dock multiple accessory proteins, which regulate their signaling, internalization, and desensitization (Ciruela et al., 2010). In addition, ARs associate to form homo-oligomers (e.g., A_1R-A_1R) or hetero-oligomers, not only between ARs (e.g., A_1R-A_{2A}R) but also with other GPCRs (e.g., A_1R-$P2Y_1$R, A_{2A}R-D_2R) (Fredholm et al., 2011). Altogether, these findings support portentous signaling plasticity that raises additional complexities in detailing responses mediated by adenosine.

2.1.2. P2 receptors

Following the discovery of P1 receptors, a large family of ATP--sensitive receptors, collectively denominated as P2 receptors, soon emerged. Mammalian P2 receptors are subdivided into seven ionotropic P2X receptors (P2X1-7R) and eight metabotropic P2Y receptors ($P2Y_{1,2,4,6,11,12,13,14}$R). They are a target for several endogenous purine and pyrimidine nucleotides (e.g., ATP, ADP, UTP, UDP, CTP) (Jacobson et al., 2020) (Table 2).

Table 2. Selected pharmacology of P2 receptors

Receptors	Signaling pathway	Main location	Agonists (pEC_{50} for endogenous agonists)	Antagonists
P2X receptors				Suramin; PPADS; RB-2; TNP-ATP
P2X1	Ion influx	VSMC, platelets, myocardium, immune cells	BzATP > ATP (6.2) ≥ α,β-MeATP ≥ 2-MeSATP >ATPγS	PSB-2001; PSB-2013; NF023
P2X2	Ion Influx	Perivascular nerves	BzATP > 2-MeSATP = ATP (5.7) = ATPγS >> α,β-MeATP	NF770; PSB-1011
P2X3	Ion influx	Perivascular nerves	BzATP > 2-MeSATP ≥ α,β-MeATP ≥ ATP (6.3) ≥ ATPγS	AF-906; AF-353; BLU-5937
P2X4	Ion influx Pore formation	Endothelium, immune cells, myocardium	2-MeSATP > ATP (6) > BzATP ≥ α,β-MeATP ≥ ATPγS > CTP; Ivermectin (PAM)	PSB-15417; NP-1815-PX; 5-BDBD
P2X5	Ion influx		ATP (6.4) > ATPγS ≥ 2-MeSATP ≥ BzATP >> α,β-MeATP	
P2X6	Ion Influx		ATPγS > 2-MeSATP ≥ ATP (4.9) ≥ BzATP >> α,β-MeAT	
P2X7	Ion influx Pore formation	Immune cells, erythrocytes, VSMC	BzATP > ATP (4) ≥ ATPγS ≥ 2-MeSATP > α,β-MeATP	AZ10606120; A-804598; A-438079
P2Y receptors				Suramin; PPADS; RB-2
$P2Y_1$	$G\alpha_q$-protein ↑IP_3	Endothelium, platelets, perivascular nerves	MRS2365 >2-meSADP > ADPβS > ADP (5.1)	MRS2500; MRS2279; MRS2298; MRS2179
$P2Y_2$	$G\alpha_q$-protein ↑IP_3	Endothelium, VSMC, platelets, myocardium, immune cells, perivascular nerves	MRS2698 ≥ UTP (8.1) > ATP (7.1) > PSB-1114	AR-C118925; PSB-716
$P2Y_4$	$G\alpha_q$-protein ↑IP_3	VSMC	MRS406> MRS2927 > UTP (6.3 = ATP in rat, mouse)	PSB-16133; PSB-1699
$P2Y_6$	$G\alpha_q$-protein ↑IP_3	Myocardium, endothelium, VSMC	MRS2957 ≥ MRS2693> PSB-0474 >UDP (6.5)	MRS2578
$P2Y_{11}$	$G\alpha_s/_q$-protein ↑cAMP; ↑IP_3	Myocardium, endothelium	ARC67085 > NF546 > ATP (4.8) ≥ ATPγS	NF340; NF157
$P2Y_{12}$	$G\alpha_i$-protein ↓cAMP	Platelets, VSMC, immune cells	2-meS-ADP > ADPβS ≥ ADP (7.2)	PSB-0739; Cangrelor; Ticagrelor; Clopidogrel; Prasugrel
$P2Y_{13}$	$G\alpha_i$-protein ↓cAMP	Erythrocytes; immune cells	ADP (7.9) ≥ 2-meS-ADP	Cangrelor; MRS2603; MRS2211
$P2Y_{14}$	$G\alpha_i$-protein ↑IP3		MRS2905 > MRS2690 > UDP (6.8) > UDP-glucose (6.4)	MRS4147; PPTN; MRS4608; MRS4458

Adapted from (Erlinge and Burnstock, 2008; Jacobson et al., 2012; Jacobson et al., 2020; Illes et al., 2021; von Kugelgen, 2021)
Receptor affinities of agonists and antagonists are reported for humans
Abbreviations: adenosine 3',5'-cyclic monophosphate (cAMP); inositol 1,4,5-trisphosphate (IP_3); positive allosteric modulator (PAM); vascular smooth muscle cells (VSMC)

P2XRs are ATP-gated ion channels formed by three subunits assembled in a dolphin-like structure. Each subunit comprises two transmembrane (TM) α-helices, with two intracellular N- and C-terminal domains and a large extracellular loop where ATP binds. Stoichiometrically, three ATP molecules putatively bind to the three orthosteric sites available in each receptor. P2XRs are pharmacologically differentiated between them by characterizing relative affinities for ATP and other ligands, as well as by activation and deactivation kinetics, selective permeability and conductance to ions, and response to allosteric modulators. P2X1R and P2X3R present the highest affinities for ATP and are rapidly desensitized with continuous stimulation; P2X2R and P2X5R have intermediate affinities and slow desensitization kinetics; P2X4R has both intermediate affinity and desensitization kinetics; P2X7R has the lowest affinity for ATP and shows little or no desensitization to repetitive or continuous stimulation (Schmid and Evans, 2019).

Interestingly, some of these ionotropic receptors, namely P2X4 and P2X7, have conformational states that allow passage of large molecules, including nucleotides (Pellegatti et al., 2005; Bernier et al., 2012).

Like ARs, P2YRs are also members of the membrane GPCRs family. They are much more diverse in terms of structural and functional properties than their P2XR counterparts. Although formally called ATP-sensitive receptors, some receptors exhibit either low or null sensitivity for this ligand, while others are sensitive to other adenine and uracil nucleotides. Indeed, each receptor has its preferential endogenous ligand(s). ATP is the native ligand of $P2Y_2R$, $P2Y_6R$, $P2Y_{11}R$ and $P2Y_{12}R$, while ADP preferentially binds to $P2Y_1R$, $P2Y_{12}R$, and $P2Y_{13}R$; UTP binds to $P2Y_4R$ and $P2Y_6R$, UDP to $P2Y_6R$ and $P2Y_{14}R$; and UDP-glucose/galactose to $P2Y_{14}R$. One caveat to this endogenous agonist profile is that it significantly varies among mammal species. In addition, some P2YRs have more than one endogenous ligand, which raises significant difficulties in their identification and characterization. Fortunately, the past decades of research were fruitful in developing new selective agonists and antagonists, and some are listed in table 2 (Jacobson et al., 2020). Much of the current understanding about these receptors' physiology has been resolved by detailing their downstream transduction pathways. P2YRs are phylogenetically clustered in their signaling and functional properties as $P2Y_1$-like receptor, which includes the first five receptors of the P2YR nomenclature ($P2Y_1R$, $P2Y_2R$, $P2Y_4R$, $P2Y_6R$, and $P2Y_{11}R$), and $P2Y_{12}$-like receptors, corresponding to the remaining three receptors ($P2Y_{12}R$, $P2Y_{13}R$, and $P2Y_{14}R$). Stimulation of $P2Y_1$-like receptors has an overall excitatory effect due to their preferential coupling to $G\alpha_q$-proteins, with a small deviation for $P2Y_{11}R$ as it also couples to stimulatory $G\alpha_s$-proteins. $P2Y_{12}$-like receptors, for instance, are coupled to $G\alpha_i$-proteins and other inhibitory signaling pathways (Jacobson et al., 2020). P2YR signaling also includes G-protein independent pathways, such as modulation of ion channels, β-arrestin pathway, and regulation of MAPK and other mitogenic kinases (Abbracchio et al., 2006; Miras-Portugal et al., 2019).

3. Purine-releasing pathways

Purines and pyrimidines are virtually released from every cell either by lytic (e.g., cellular damage and death) or non-lytic mechanisms (Figure 1). Non-lytic mechanisms include exocytosis and channel-mediated release and can be further classified as either passive (e.g., connexins (Cx), pannexins (Pnx), equilibrative nucleotide transporters (ENT) or ionotropic receptors) or active transport (e.g., exocytosis, facilitated transport by nucleoside-specific ATP-binding cassette (ABC) transporters, concentrative nucleotide transporters (CNTs)), according to the energetic expenditure necessary for the transport of purines (Yegutkin, 2008). There is now plenty evidence that these mechanisms operate

uninterruptedly in the background of several physiological processes (Lazarowski et al., 2000; Lazarowski, 2012; Noronha-Matos et al., 2012). Certain conditions, such as inflammation, hypoxia, shear-stress, second messengers, and action potentials, amplify these mechanisms of releasing purines (Lazarowski et al., 2000; Lazarowski, 2012; Lohman and Isakson, 2014; Makarenkova et al., 2018; Taruno, 2018).

Vesicular storage of ATP and its co-release with other neurotransmitters during exocytosis represents the pioneering concept behind Burnstock's theory (Burnstock, 1972). The heart is densely innervated by afferent and efferent sympathetic and parasympathetic fibers, particularly in atria, where numerous neuronal cell bodies can be found (Burnstock and Pelleg, 2015). In the sympathetic autonomic nervous system, norepinephrine (NE) is actively stored in vesicles using vesicular monoamine transporters that utilize transmembrane H^+ gradient generated by vesicular H^+-ATPase. Although H^+-ATPase consumes ATP to acidify vesicles, ATP is also incorporated during this process by vesicular nucleotide transporters (VNUT). ATP and other nucleotides, but not adenosine, are substrates of VNUT (Moriyama et al., 2017). With the arrival of an action potential (AP) to the synapse, intracellular vesicles fuse with the plasma membrane, releasing ATP together with NE to the extracellular space. Of note, purinoceptors control presynaptically this exocytotic process (Burnstock and Pelleg, 2015; Burnstock, 2017b). Interestingly, the vesicular release of ATP is not limited to neurons, as other cells, such as platelets, urothelial and endothelial cells, also release vesicles containing ATP (Burnstock and Pelleg, 2015). A recent study performed in rat tail arteries demonstrated that ATP and NE are stored in different vesicles and utilize different channels to release them from sympathetic varicosities, challenging the hypothesis of co-transmission (Kalkhoran et al., 2019). The release of purines through membrane proteins is particularly relevant for cells that lack proper machinery for exocytosis, such as non-neuronal cells. Multiple membrane proteins are involved in either electrodiffusional or facilitated release of purines, namely: connexin/pannexin hemichannels, chloride channels (maxi anion channels, volume regulated channels, CFTR), ABC transporters, CNT and ENT, and P2XRs as well (Pellegatti et al., 2005; Bernier et al., 2012; Taruno, 2018; Matsuura et al., 2021).

Hemichannels are of particular importance for cardiac function not only because they form gap junctions, allowing the heart to function as a syncytium, but also due to their role in releasing purines to the extracellular space (Lazarowski, 2012). Connexins are the building blocks of gap junctions and participate in cell-to-cell communication, and their dysfunction increases the likelihood of arrhythmias (Gonzalez et al., 2015). Cx43 is one of the most abundant connexins in the heart, being also permeable to ATP (Kang et al., 2008). The pannexin family include 3 isoforms that share some structural and functional properties with connexins. Pannexin 1 is ubiquitously expressed, being the predominant isoform found

in the heart (Makarenkova et al., 2018). Despite some conflicting evidence, pannexins are not usually present in gap-junctions but rather present as isolated membrane ion channels (Sosinsky et al., 2011) and, hence, considered to have better secretagogue properties (at physiological conditions) than connexins (Bao et al., 2004; Lazarowski, 2012; Lohman and Isakson, 2014). In cardiac myocytes, a large conductance current, with properties resembling the opening of pannexin 1, was demonstrated in response to raised intracellular calcium from the sarcoplasmic reticulum, stretch, and P2R activation (Kienitz et al., 2011).

In the heart, endothelial cells and cardiomyocytes are the primary source of ATP and adenosine. Other cells also release purines, namely neurons, fibroblasts, smooth muscle cells, and blood circulating cells (e.g., immune cells, platelets, erythrocytes). The relative contribution of each cell to the extracellular pool of purines changes in health and disease; for example, erythrocytes represent a major source of purines in hypoxic conditions (Burnstock and Pelleg, 2015). In the normal functioning heart, intracellular adenosine is synthesized by two main pathways: metabolization of S-adenosylhomocysteine (SAH) or dephosphorylation of AMP by 5'-nucleotidase (CD73) (Figure 1). While the former prevails in normoxic conditions, adenosine generating source shifts to AMP pathway in ischemic or hypoxic conditions (Hori and Kitakaze, 1991). Other putative substrates for adenosine formation include the breakdown of cAMP via phosphodiesterase (Sassi et al., 2014). Under resting conditions, roughly 92% of adenosine is localized in the intracellular compartment (Deussen et al., 1999). This transmembrane gradient is mainly maintained by adenosine kinase (AK) driving continuously adenosine to phosphorylation for AMP or other high-energy nucleotides, thereby preventing its extrusion or deamination to inosine by ADA (Figure 1).

Hypoxic, ischemic, cardiotonic agents and mechanical stimulation are potent stimuli for adenosine and ATP release (Vassort, 2001). For instance, Borst's study demonstrated that resting levels of extracellular adenosine and ATP, measured in coronary venous effluent were below 1 nM. However, with myocardial hypoxia, they increased up to 40-fold and 3-fold, respectively (Borst and Schrader, 1991). Interstitial levels of ATP and adenosine are highly variable, but it was estimated to be within the low nanomolar range, with about 11:1 ratio of adenosine to ATP (Kuzmin et al., 1998; Lorbar et al., 1999). UTP is also released during myocardial ischemia (Erlinge et al., 2005), with a proportion of UTP estimated to be 10% of ATP release (Wihlborg et al., 2006).

4. Purine-inactivating mechanisms

The purinergic signaling is terminated by extracellular catabolism, reuptake and diffusion of purines (and pyrimidines) away from the receptor sites (Figure 1). A specialized collection of membrane-bound and soluble enzymes sequentially hydrolyze extracellular

nucleotides. This group of enzymes includes: (1) ecto-nucleoside triphosphate diphosphodyrolases (E-NTPDase1-8); (2) ecto-nucleotide pyrophosphatase / phosphodiesterases (E-NPP 1-7); (3) ecto-5'-nucleotidase (isoforms 1 to 7) and (4) alkaline phosphatases (Yegutkin, 2008). Despite the existence of 8 subtypes of NTPDases, only NTPDases 1,2,3 and 8 ecto-enzymes have a meaningful role in the purinergic transmission; other enzyme subtypes are located intracellularly, thus not amenable for the catabolism of extracellular nucleotides. NTPDases hydrolyze extracellular tri- and/or di-phosphates (e.g., ATP, ADP) with different preferences, but do not hydrolyze monophosphates (e.g., AMP). E-NTPDase1 hydrolyzes ATP and ADP equally well, E-NTPDase2 preferentially hydrolyzes triphosphonucleosides, and E-NTPDase3 and E-NTPDase8 have an intermediate hydrolysis profile (Yegutkin, 2008). NTPDase 1 (CD39 or apyrase) and NTPDase 2 are present in the cardiac vasculature, being NTPDase 1 predominantly located in endoluminal surfaces (endocardium and endothelium). In contrast, NTPDase 2 is primarily located in the subendocardium and in muscular layers of blood vessels (Sevigny et al., 2002). NTPDase2 is the most abundant subtype observed in the left ventricle from rats (Rucker et al., 2008). Regarding E-NPPs, NPP1 is the most expressed in human tissues, particularly in bone and cartilaginous tissues where it controls mineralization. NPP1 is also expressed in the heart and blood vessels where it seems to mediate vascular calcification (Yegutkin, 2008). Apparently, alkaline phosphatase lacks any relevant role in the heart.

The extracellular hydrolysis of tri- and di-phosphonucleosides yields AMP as the final product, which can be fully dephosphorylated to adenosine by E-5′-nucleotidase/CD73. Adenosine resulting from the extracellular catabolism of adenine nucleotides plays paramount effects in the heart, which could be confirmed by the deficits exhibited in transgenic mice lacking CD73 (Eckle et al., 2007). Both ATP and adenosine have a short (<2 s) half-life in the plasma (Moser et al., 1989). However, this scenario may change under pathological conditions, such as myocardial infarction where the hydrolysis of adenine nucleotides is impaired, thus favoring the extracellular accumulation of adenine nucleotides in detriment of its end product, adenosine (Kuzmin et al., 1998).

Adenosine effects on membrane-bound P1R receptors have been claimed to be terminated by the deamination of the nucleoside by adenosine deaminase (ADA 1 and 2). Nonetheless, recent evidence indicates that this might not always be the case, given that the adenosine deamination product, inosine, may also activate A_3R (Ciruela et al., 2010). ADA exists both in intracellular and extracellular compartments, but its extracellular location assumes a particular interest in cellular signaling taking into consideration that it is present in multiple cell types, including immune cells, where it acts as a pro-inflammatory enzyme through the hydrolysis of adenosine. In the heart, ADA present in the vascular endothelium and erythrocytes removes most of the circulating adenosine. Besides deamination, the

nucleosides resulting from the ecto-nucleotidase cascade may also follow two distinct pathways: 1) they may be eliminated via the hypoxanthine-urate pathway, or 2) they might enter the salvage pathway being sequentially re-phosphorylated by adenylate kinase (AK 1-9; AK 1 is the most abundant) and nucleoside diphosphate (NDP 1 to 8) kinase. These pathways are mostly intracellular, but they can also occur outside cells due to secreted soluble forms of these enzymes (Yegutkin, 2008).

Adenosine may also be rapidly taken up by cardiac and other circulating cells (e.g., erythrocytes) via equilibrative and concentrative nucleoside transports (Reiss et al., 2019). Nucleoside transporters control the extracellular levels of adenosine generated from the extracellular catabolism of ATP during hypoxia and inflammation conditions (Li et al., 2012). Special attention has been given to these transporters in recent years. They represent the principal pathway for the cellular uptake of nucleoside analogs used in anti-cancer/anti-viral therapies. Nucleotide transporters are permeant to a wide range of purine and pyrimidine nucleosides but hardly transport nucleotides. Nowadays, nucleoside transporters are divided into two not-evolutionary related family groups: ENT (SLC29) and CNT (SLC28). Each family has three members: ENT1 to ENT3 and CNT1 to CNT3. A putative fourth ENT (ENT4) subtype can be found in the literature, which seems to be abundantly expressed in the heart (Barnes et al., 2006). However, the ENT4 lower affinity for adenosine and its resemblance to monoamine transporters have challenged its role as a functional purinergic transporter (Boswell-Casteel and Hays, 2017).

ENTs are ubiquitously expressed; they passively and bi-directionally transport nucleosides down to an electrochemical gradient. ENTs can be further classified as sensitive (ENT1) or insensitive (ENT2-3) to nitrobenzylthioinosine (NBTI). ENT1 and ENT2 are located in the cellular membrane, while native ENT3 is predominantly located in endosomal/lysosomal membranes (Boswell-Casteel and Hays, 2017). ENT1 is the main adenosine transporter in blood vessels (Li et al., 2012). Interestingly, hypoxia down-modulates the ENT1 expression via a mechanism associated to the hypoxia-inducible factor-1 (HIF-1), thus favoring the accumulation of cardioprotective adenosine (Chaudary et al., 2004b; Eltzschig et al., 2005; Rose et al., 2011; Li et al., 2012). As passive carriers, ENTs may also promote the efflux of nucleosides, which is favored under hypoxic and other stressful conditions when the intracellular adenosine/ATP ratio increases (Chaudary et al., 2004a). ENTs, particularly ENT1, are also permeable to several cardioactive drugs that interfere with adenosine uptake, namely dilazep, dipyridamole, nifedipine, which might explain their potency to induce vasodilation and inhibition of platelet activation (Li et al., 2012; Boswell-Casteel and Hays, 2017).

CNTs are active sodium-dependent nucleoside transporters; CNT2 is the major cardiac isoform (Barnes et al., 2006; Loffler et al., 2007). CNTs function as symporters by

carrying either Na^+ or H^+ together with nucleosides. CNT shows different selectivity for nucleosides, namely: CNT1 is transport preferentially pyrimidine nucleosides, CNT2 prefers purines nucleosides, while CNT3 has a broader selectivity transporting equally well both pyrimidine and purine nucleosides. Despite their presence, CNTs have minimal contribution to the uptake of nucleosides compared to ENTs (Archer et al., 2004; Loffler et al., 2007).

5. Purinergic regulation of cardiovascular functions

Purinergic receptors are important signaling transducers in the heart. They are expressed in the working myocardium and in other cardiac cells, including those of the conduction system, neurons, vessels, and valves of the heart (Table 1 and 2). ATP and other purines are deeply involved in acute and chronic physiological processes of the cardiovascular system (Burnstock and Pelleg, 2015; Burnstock, 2017b; Burnstock, 2018). The retaliatory action of adenosine has been the most notorious effect of purines in the heart. Adenosine, via inhibitory A_1R, reduces cardiac work by decreasing heart rate and the force of muscle contraction, while increasing the myocardium oxygen supply through coronary vasodilation operated by A_2R. These cardioprotective effects and its short half-life granted adenosine an important role in modern clinical practice. Adenosine is indicated to treat supraventricular tachyarrhythmias and to assess coronary artery disease in myocardial perfusion imaging studies. Acute regulation of the cardiovascular system is particularly centered on cardiac output and hemodynamics. The following sections of this Introduction are aimed to provide an overview of the purinergic signaling roles on acute regulation of the cardiovascular system, with a special focus on chronotropy and inotropy, which will be further detailed in the Results chapter. Chronic effects of purines will also be detailed to provide some background for interpretation of cardiovascular effects that are expected with prolonged administration of drugs targeting this signaling system.

5.1. Short-term regulation of the cardiovascular function by purines

5.1.1. Heart rate and rhythm

The sinoatrial node (SAN) is the heart's natural pacemaker that sets the rate of the sinus rhythm. It is a delicate structure discovered by Keith and Flack in the early years of the twentieth century. In their landmark paper, the SAN was described as a "*remarkable remnant of primitive fibers at the sino-auricular junction"* with both neuronal and arterial supply (Keith and Flack, 1907). The SAN is conserved between mammals (Solc, 2007), and usually assumes an epicardial location in the right atria, near the insertion of superior vena cava. A distinctive property of SAN cells is their ability to self-generate action potentials (APs). Auto-excitability is not exclusive of SAN cells, as other cells of the conduction system also have this property. SAN cells are specialized in pacemaking as they have more

intricate and robust mechanisms that allow a reliable generation of APs. Although several theories have emerged to explain the spontaneous activity of SAN cells, nowadays, the concept of a coupled-clock system represents the most unifying theory (Yaniv et al., 2015) (Figure 2).

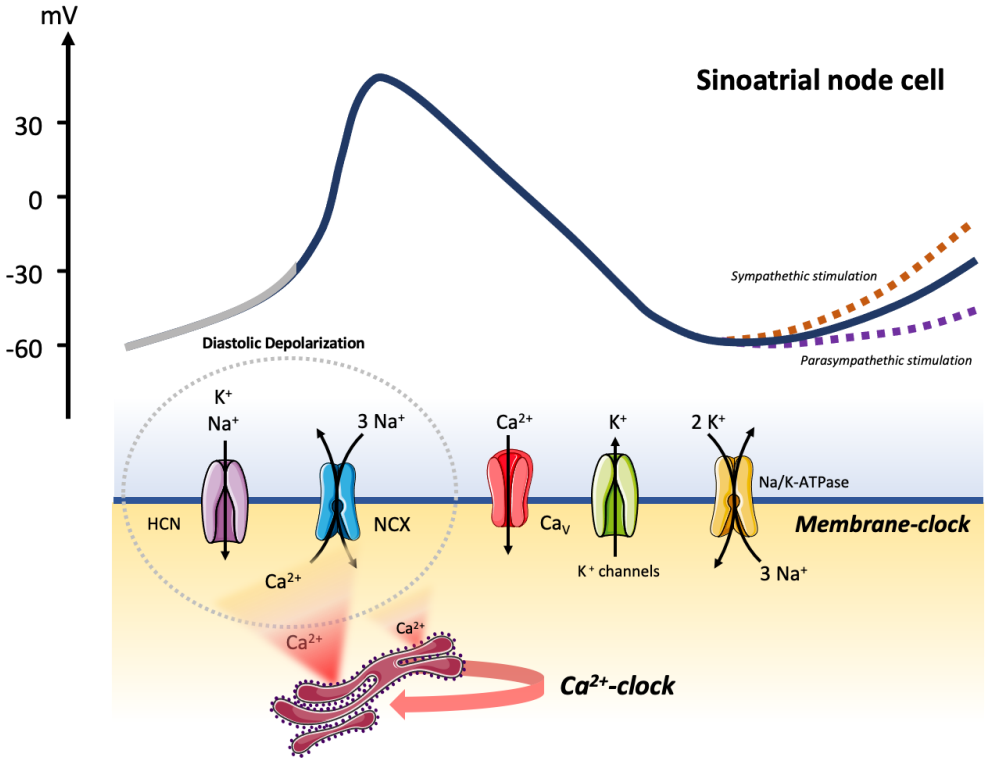

Figure 2. Spontaneous generation of action potentials in the sinoatrial node. Timely generation of APs in the sinoatrial node (SAN) results from an intricate interplay between oscillations of both intracellular Ca^{2+} (Ca^{2+}-clock) and membrane potential ("membrane-clock"). The sarcoplasmic reticulum (SR) of SAN is leaky and constantly releases Ca^{2+} to the intracellular space. During the diastolic depolarization phase, Ca^{2+} release from SR increases in frequency and magnitude, stimulating forward or electrogenic mode of Na^+/Ca^{2+} exchanger (NCX). Negative membrane potentials during the diastolic depolarization phase also stimulate the opening of HCN channels, further pushing membrane potential to the threshold of calcium voltage-dependent channels (Ca_V) activation. The opening of Ca_V is accompanied by a sharp rise in membrane potential that depolarizes SAN cells. Repolarization then succeeds with the opening of K^+ channels and inactivation of depolarization currents. The Na^+/K^+-ATPase operates in the late period of the repolarization phase to restore resting ionic gradients necessary for forthcoming APs. The illustration used elements from Servier Medical Art. Adapted from (Tsutsui et al., 2018).

The coupled-clock system integrates both experimental and computational data and essentially describes the existence of mutual entrainment between two "clocks", the M-clock, or membrane-clock, and the Ca^{2+}-clock. The M-clock represents a temporal assembly of ion channels, exchangers, and other membrane proteins that set the membrane potential. On the other hand, the Ca^{2+}-clock is the representation of a time-based assembly of intracellular Ca^{2+} dynamics. As each clock has voltage and Ca^{2+}-sensitive proteins, the two clocks mutually regulate themselves (Yaniv et al., 2015). The generation

of APs by the SAN shares many fundamental processes of the excitation-contraction (E-C) coupling (described below), but with two main striking differences: while in the myocardium contraction stimulus arise from an electrical impulse conveyed by the conduction system, in the SAN electrical stimuli are generated by the SAN cells themselves. Another striking difference is that SAN cells lack Na_V channels and, therefore, do not have the typical "phase 0" or fast upstroke of AP observed in the working myocardium (Chandler et al., 2009).

Beginning in the diastole, the coupled-clock system is ignited during the diastolic depolarization phase. In the Ca^{2+}-clock, generation of an AP begins with spontaneous and periodically release of Ca^{2+} from sarcoplasmic reticulum (SR) through ryanodine receptors (RyRs) (Vinogradova et al., 2004), and perhaps through L-type calcium channels (Torrente et al., 2016), a process called diastolic local Ca^{2+} releases (LCRs). The SR can be viewed as a "dam" that stores "water" for energy production, whereas "water" represents Ca^{2+} ions. The sarcoendoplasmic reticulum calcium transport ATPase (SERCA) pumps Ca^{2+} upstream the "dam" or, in other words, it refills SR for another Ca^{2+} release through RyRs. SR is periodically alternating between pump and release of Ca^{2+}. This SR Ca^{2+} kinetics generates Ca^{2+} fluctuations that are responsible for the spontaneous and periodical generation of APs in SAN cells (Yaniv and Maltsev, 2014; Yaniv et al., 2015). As RyRs are just beneath the membrane surface and very close to Na^+/Ca^{2+} exchangers (NCX), LCR will drive NCX to function in the Ca^{2+}-extrusion mode. As three Na^+ ions enter for each Ca^{2+} ion that leaves the cell, the Ca^{2+}-extrusion mode is electrogenic and slowly depolarizes the SAN. Indeed, deletion of NCX1 from SAN cells impairs pacemaker activity (Groenke et al., 2013; Torrente et al., 2015). As pointed by Maltsev and co-workers in their numerical simulations, NCX has a bell-shaped behavior in pacemaking that needs to be balanced; otherwise will barely respond to β-AR stimulation (Maltsev et al., 2013).

In parallel to the Ca^{2+}-clock, another important depolarizing process occurs at the M-clock with activation of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and related "funny" current (I_f) (DiFrancesco, 2019). The HCN channel family comprises four members (HCN 1-4), providing a functional differentiation between SAN cells and other cardiac cells lacking pacemaking capabilities (DiFrancesco, 2019). Among the four members of the HCN channel family, HCN4 is the most expressed in the SAN. HCN channels are the target of ivabradine, a selective heart frequency-lowering drug used to treat patients with heart failure (HF) and coronary artery disease (Ponikowski et al., 2016; Knuuti et al., 2020). Of note, some HCN polymorphisms are associated with inherited arrhythmias (DiFrancesco, 2013). Contrary to most voltage-gated channels, HCN channels are activated by negative or hyperpolarizing potentials (DiFrancesco et al., 1986; Vaccari et al., 1999), which means that they "switch-on" when SAN cells reach the maximum diastolic potential. Once activated, HCN channels carry a mixed inward Na^+/K^+ depolarizing

current (DiFrancesco, 2019). Operation of both NCX and HCN gradually push the membrane potential to the threshold of T-type and L-type calcium channels (LCC); once reached this level, a sudden increase in membrane permeability occurs along with a massive influx of Ca^{2+} ions that ultimately depolarize SAN cells and generate an AP (Yaniv et al., 2015). Then, a period of membrane repolarization follows, where the closing of LCCs and the opening of both voltage and Ca^{2+}-dependent K^+ channels push the membrane potential to its resting level (Chandler et al., 2009; Monfredi et al., 2010; MacDonald et al., 2020).

The SAN is under control of the autonomic nervous system being highly innervated by autonomic nerve fibers (Pauza et al., 2014; Burnstock and Pelleg, 2015). Several autacoids also regulate its function (MacDonald et al., 2020). The adrenergic system boosts the SAN in its capabilities of generating APs by increasing the coupling between membrane- and Ca^{2+}-clocks, while ACh released from the vagus nerve does the opposite (Figure 2). Stimulation of β_1-adrenoceptors, a $G\alpha_S$-protein coupled receptor, stimulates cAMP production and increases SAN spontaneous activity, with every LCR occurring earlier in the diastolic depolarization period (Tsutsui et al., 2018). Likewise, the α_1-adrenoceptor, a $G\alpha_q$-protein coupled receptor whose stimulation generates IP_3 and, consequently, RyR-dependent Ca^{2+}-release, also increases the SAN cells firing rate (Kapoor et al., 2015). Hence, these two types of receptors, or others with similar signal transduction pathways, are able to increase heart rate. Noteworthy, basal cAMP levels are already elevated in quiescent SAN cells (Vinogradova et al., 2006), which means a raised cAMP-PKA-dependent excitatory tonus in these cells. The cAMP-PKA axis phosphorylates and stimulates several Ca^{2+} cycling proteins, namely SERCA, RyR, LCCs (Yaniv and Maltsev, 2014; Yaniv et al., 2015). With increased activity of these Ca^{2+} cycling proteins, periodic Ca^{2+} oscillation and APs generation are also more probable (Yaniv et al., 2015). The balance between phosphorylation and dephosphorylation is achieved by phosphodiesterases (PDEs) that spatially and temporally compartmentalize cGMP/cAMP-mediated effects (Vinogradova et al., 2018a; MacDonald et al., 2020). cAMP also stimulates HCN channels upon its binding to an intracellular cAMP-sensitive domain (DiFrancesco and Tortora, 1991). Both, $G\alpha_s$-protein and $G\alpha_q$-protein coupled receptors increase intracellular calcium $[Ca^{2+}]_i$ at some branching point of their signaling pathway. Ca^{2+} itself and indirectly by stimulating CaMKII, a Ca^{2+}-activated kinase of several Ca^{2+}-handling proteins, propels the automaticity of SAN cells in a feed-forward manner (Yaniv and Maltsev, 2014; Yaniv et al., 2015). Regarding the parasympathetic control of SAN, ACh acting on inhibitory $G\alpha_i$-protein coupled M_2 receptor decreases sinoatrial rate by 1) inhibiting AC and decreasing stimulatory effect of cAMP, and 2) increasing transmembrane efflux of K^+ by opening GIRK/$K_{IR}3$ channels (Kurachi et al., 1986a; DiFrancesco et al., 1989).

Another important issue of pacemaking comes with the repolarization of SAN cells. The repolarization phase also needs to be accelerated; otherwise, the firing rate of SAN cells would not be increased. The rationale for shorter repolarization periods during adrenergic stimuli is probably related to the existence of several voltage-, time- and Ca^{2+}-dependent proteins. Current evidence suggests an involvement of Ca^{2+}-dependent K^+ channels in repolarization, including small-conductance calcium-activated (SK) channels. Interestingly, the role of these channels is not limited to repolarization as they seem to be also important to pacemaking, and, when dysfunctional, they may precipitate bradycardia or rhythm disturbances (Weisbrod et al., 2013; Lai et al., 2014; Zhang et al., 2015; Torrente et al., 2017).

5.1.1.1. Purinergic modulation of heart rate and rhythm

The bradycardic effect of adenosine has been recognized for a long time. Adenosine has an inhibitory activity on pacemaking structures of the cardiac conduction system, including the SAN, atrioventricular node (AVN) and the His-Purkinje system (Mustafa et al., 2009). A_1R is the main receptor involved in this inhibitory effect; its stimulation closely resembles the M_2R, another cardiac inhibitory GPCR. The A_1R decreases cellular excitability by two mechanisms: 1) inhibition of excitatory mechanisms that are dependent of cAMP levels (e.g. HCN, calcium channels), also known as the anti-adrenergic effect, and 2) opening of GIRK channels and subsequently membrane hyperpolarization and shortening of action potential duration (Belardinelli et al., 1995; Mustafa et al., 2009). The A_1R is one of the most expressed purinergic receptors found in the heart, which is highly expressed in the SAN (Musa et al., 2009). Notably, cardiac responses to adenosine differ between atrial and ventricular tissues, with the AVN representing a functional boundary between these two territories. Below the AVN, adenosine effects are mostly limited to its anti-adrenergic effect, while above that, both direct and indirect mechanisms operate (Belardinelli et al., 1995; Lerman, 2015). This functional variation matches regional GIRK channel expression (Dobrzynski et al., 2001; Anderson et al., 2018). In SAN cells, the opening of GIRK channels and inhibition of cAMP formation counteract the activity of both NCX and hyperpolarization-activated HCN, two key elements involved in the spontaneous generation of APs (chronotropy) (Yaniv et al., 2015). In contrast, adenosine has a minor impact on the His bundle and ventricular myocardium during resting conditions, in agreement with the low expression of GIRK channels in these territories, but the nucleoside turns into a potent inhibitory agent upon increasing the sympathetic drive (Belardinelli et al., 1995; Dobrzynski et al., 2001; Calloe et al., 2013; Lerman, 2015; Anderson et al., 2018). In the AVN, adenosine decreases the upstroke velocity of AP and, consequently, the AV conduction velocity (negative dromotropy). High

levels of circulating adenosine may cause complete AVN block, usually seen during myocardial ischemia, exogenous administration of adenosine or of drugs that increase its bioavailability (e.g. dipyridamole) (Belardinelli et al., 1995; Shenasa, 1995; Brignole et al., 1997).

Negative chronotropy and dromotropy operated by adenosine, along with its short half-life, have granted it an important role in the treatment of supraventricular arrhythmias. It is also important in unmasking supraventricular rhythms, as most atrioventricular accessory pathways are relatively insensitive to adenosine (Shenasa, 1995; Pelleg et al., 2002; Matthews and Grace, 2020; Gupta et al., 2021). ATP and other adenine nucleotides, by their breakdown to adenosine, have inhibitory roles on the conduction system, thus explaining why ATP and adenosine are both effective in terminating paroxysmal supraventricular tachyarrhythmias (Shenasa, 1995; Burnstock and Pelleg, 2015; Burnstock, 2017b). Notwithstanding this, evidence has been provided suggesting that ATP is more effective than adenosine in terminating supraventricular tachyarrhythmias, and that the nucleotide may account for an additional bradycardic component carried out by vagal activation triggered via a P2X2/3-mediated pulmonary-cardiac central reflex (Pelleg and Belhassen, 2010). Adenosine and ATP have little effects on ventricles, but once again due to their anti-adrenergic effects these purines are highly effective in terminating catecholamine-induced ventricular tachycardia (Lerman, 2015).

Curiously, adenosine-based therapies may also be pro-arrhythmogenic. The A_1R-mediated opening of K^+ channels shortens AP and decreases the effective refractory period, increasing the likelihood of reentry mechanisms and occurrence arrhythmias, namely atrial flutter and atrial fibrillation (Shenasa, 1995; Soattin et al., 2020). Moreover, the occurrence of several malignant tachyarrhythmias have also been described, particularly in patients with accessory conduction pathways (Pelleg et al., 2002). The pro-arrhythmogenic effects of adenosine depend on multiple issues, including the levels of the nucleoside, the AR amounts, and co-morbidities. For instance, HF evolves with increased susceptibility to arrhythmias. Indeed, Long and co-workers found that in a canine model of HF induced by chronic high pacing rhythm the levels of A_1R and GIRK channels were upregulated, which was accompanied by a higher sensitivity to adenosine and likelihood to bradycardia occurrence (Long et al., 2020b). The vasodilation effect of adenosine $A_{2A}R$ also favors reflex tachycardia (Dhalla et al., 2006), an effect that is most often overridden by the A_1R activation. ATP can also be arrhythmogenic, particularly when concurrent adrenergic stimulus is verified, thus increasing the occurrence of both early and delayed afterdepolarizations (Vassort, 2001).

5.1.2. Excitation-contraction coupling and inotropy

Excitation-contraction (E-C) coupling is the concept behind the relationship between AP and the contraction of cardiac muscle cells. Given the pivotal role of $[Ca^{2+}]_i$ for contraction and relaxation of myocardial fibers, diastolic and systolic $[Ca^{2+}]_i$ must be balanced to ensure proper heart pumping. Indeed, abnormal Ca^{2+} dynamics and uncoupling of E-C are associated with arrhythmias and progression to HF (Bers, 2006; Gilbert et al., 2020). E-C coupling continues to be extensively studied, but it remains far from being fully resolved (see reviews (Bers, 2002; 2006; Eisner et al., 2017); Figure 3).

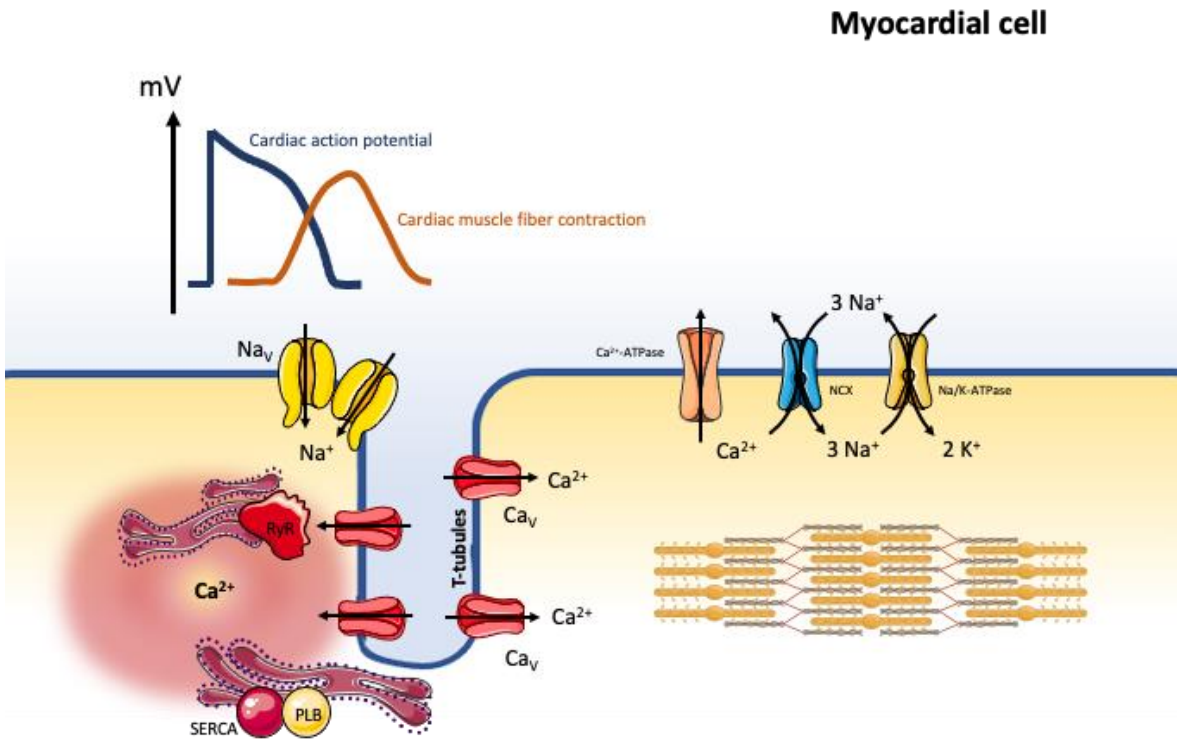

Figure 3. Excitation-contraction coupling in the heart. The arrival of an action potential to a myocardial cell from the cardiac conduction system stimulates opening of voltage-gated sodium (Na_V) channels and its depolarization. The depolarization wave propels throughout the entire myocardial cell reaching highly branched invaginations of cardiomyocyte sarcolemma (T-tubules), where a high density of calcium voltage-dependent (Ca_V) channels are found to be in close proximity with ryanodine receptors (RyRs) located in the sarcoplasmic reticulum (SR). Opening of Ca_V channels stimulates RyRs and triggers a massive release of intracellular Ca^{2+}, also known as Ca^{2+}-induced Ca^{2+} release. An increase of intracellular Ca^{2+} stimulates sliding of actin and myosin myofilaments and shortening of the cardiac muscle fiber. Following contraction, Ca^{2+} is pumped out the cytoplasm to the SR via SERCA protein and to the extracellular space through Ca^{2+} pumps (e.g. Ca^{2+} -ATPase) and exchangers (NCX) located in the membrane. The illustration used elements from *Servier Medical Art*. Adapted from (Bers, 2002; Eisner et al., 2017).

Briefly, depolarizing waves originated at the SAN spreads throughout the myocardium stimulating its contraction. Voltage-gated sodium channels (Na_V) are the first group of ion channels that sense an AP from neighboring cells, generating an inward current of Na^+ and a fast upstroke of membrane potential responsible by excitation of other ion

channels. A huge variety of ion channels are located in the sarcolemma of these cells, many of them with voltage-dependent properties that allow them to sense every change in membrane excitability (Grant, 2009). Among the variety of ion channels involved in this process, LCCs have a detrimental role in E-C coupling. LCCs channels are particularly abundant in t-tubules of cardiomyocytes. T-tubules are deep invaginations of the sarcolemma where LCCs are in close juxtaposition with RyRs of the SR, forming functional units know as dyads. In the dyadic space, Ca^{2+} influx through LCC ignites a massive Ca^{2+} release from SR, in a process called calcium-induced calcium release (CICR) (Bers, 2002; Eisner et al., 2017). Upon rising $[Ca^{2+}]_i$, thin and thick myofilaments begin to slide, shortening contractile fibers – the systole. Inotropy derives from the magnitude and duration of $[Ca^{2+}]_i$ rise. Noteworthy, myofilaments and other Ca^{2+}-binding proteins function as Ca^{2+} buffers; post-translational modifications of these proteins, such as (de)phosphorylation by phosphatases/kinases, also have a detrimental role in inotropy regulation (Eisner et al., 2017), being of particular interest in the pursue of new sarcomeric modulators to treat HF (Hwang and Sykes, 2015; Teerlink et al., 2021).

Following the rise of $[Ca^{2+}]_i$ and contraction, a diastolic phase must succeed. Diastole, rather than being a passive process, it involves energy expenditure through active pumping of Ca^{2+} from sarcoplasm back to internal stores, such as SR by SERCA (SR Ca-ATPase), and to the extracellular milieu, mostly via forward or Ca^{2+}-extrusion mode of the NCX exchanger (Bers, 2002; Eisner et al., 2017). At the myofilament level, relaxation occurs with the replacement of ADP by ATP in the actin-myosin complex, terminating the contraction cycle (Hwang and Sykes, 2015). Dysfunction of Ca^{2+} removal concurs with fibrosis and stiffness of myocardium for the development of diastolic HF (Franssen and Gonzalez Miqueo, 2016; Eisner et al., 2017). Indeed, HF progression is accompanied by an increase in diastolic Ca^{2+} concentration, along with an increase in intracellular Na^+ (Eisner et al., 2020). In some mammals, including humans, there is a positive force-frequency relationship in the working myocardium, defined by an increase of inotropy with elevations of heart rate. Increases in diastolic Ca^{2+} is the basis for this phenomenon, which rationale implicates a lesser time to Ca^{2+} extrusion at higher stimulation frequencies (Dibb et al., 2007). With increases in diastolic calcium, the Ca^{2+}-buffers become saturated and the amount of $[Ca^{2+}]_i$ available for contraction also increases (Franssen and Gonzalez Miqueo, 2016; Eisner et al., 2017). Some studies have explored new drugs to restore Ca^{2+} homeostasis and E-C coupling in diseased hearts (reviewed by (Roe et al., 2015).

The NCX is a major route for Ca^{2+} removal following depolarization and contraction of cardiomyocytes. Na^+ and Ca^{2+} handling capabilities of NCX have been implicated in arrhythmogenesis (delayed (DAD) and early afterdepolarization (EAD)), hypertrophy and myocardial remodeling (Roe et al., 2015). Although NCX activity for Ca^{2+} removal varies

among different species, it is expected to be responsible for 28% of the total Ca^{2+} removal in humans. In coordination with SERCA, which removes another 70% of total intracellular Ca^{2+}, these two proteins, SERCA and NCX, leave only about 1% of Ca^{2+} at the end of diastole (Bers, 2002). In E-C coupling, NCX and SERCA keep low levels of $[Ca^{2+}]_i$. The exchange of Ca^{2+} by Na^+ via NCX is bidirectional, which means that it can be either electrogenic, replacing 3 intracellular Na^+ ions by each extracellular Ca^{2+} ion, or hyperpolarizing when Na^+ and Ca^{2+} ions flow in the opposite direction. These two modes of NCX operation are primarily controlled by the electrochemical potentials of Na^+ and Ca^{2+} across the plasma membrane (Kang and Hilgemann, 2004). When intracellular $[Na^+]_i$ increases and the membrane potential becomes more positive as occurring during membrane depolarization, NCX functions predominantly in the reverse-mode (Bers, 2002; Kang and Hilgemann, 2004; Roe et al., 2015; Eisner et al., 2017). In addition, Na^+/K^+-ATPase (NKA), the main Na^+ extrusion pathway of cardiomyocytes, is also functionally coupled to NCX (Swift et al., 2010; Roe et al., 2015). Thus, changes in $[Na^+]_i$ may also interfere with $[Ca^{2+}]_i$ and, ultimately, with the myocytes contraction performance. Indeed, digitalis, used for decades in patients in HF, and, more recently, istaroxime, increase $[Na^+]_i$ by inhibiting the Na^+/K^+-ATPase, which by limiting the Ca^{2+}-extrusion mode of NCX facilitate the intracellular accumulation of Ca^{2+}making it more available for contraction (Swift et al., 2010; Peana and Domeier, 2017).

Another level of regulation of NCX is located on phospholemman, an intracellular protein that that is substrate of PKA and PKC during adrenergic stimulation (Zhang et al., 2006; Despa et al., 2008). When phosphorylated, this cardiac stress protein inhibits NCX and stimulates cardiac contractility (Wang et al., 2011). Another important feature of phosphorylated phospholemman is its ability to stimulate NKA, thus limiting intracellular Na^+ overload during $[Ca^{2+}]_i$ rise and decreasing the likelihood of arrhythmias (Despa et al., 2008). Interestingly, phospholemman is also a phosphorylation target of P2R activation. P2R agonists increase NKA activity in rat skeletal muscle (Walas and Juel, 2012; Juel et al., 2014).

All these mechanisms that modulate E-C coupling overlap with those presented for autonomic regulation of the SAN. Norepinephrine (NE) stimulates $G\alpha_s$-protein coupled receptors (e.g., β_1-AR), increasing inotropy, while the opposite occurs when ACh binds to inhibitory $G\alpha_i$-protein coupled receptors (e.g., M_2R). Activation of β_1-AR stimulates AC and increases cAMP levels; then cAMP binds to PKA that phosphorylates several key proteins of the E-C coupling (e.g., SERCA inhibitory protein phospholamban (PLN), LCCs, RyRs, troponin I). The net effect of PKA phosphorylation is an increase of Ca^{2+} kinetics, which means an increase of systolic Ca^{2+} in each contraction in parallel with an increased re-

uptake, explaining why β_1-AR stimulation has both positive inotropic and lusitropic effects (Bers, 2002; Peana and Domeier, 2017).

5.1.2.1. Purinergic modulation of excitation-contraction coupling

Purines and pyrimidines regulate the contractile performance of the heart. Their cardiotonic properties originate from their activity over several key steps of E-C coupling, including intracellular calcium handling, as well as (de)phosphorylation of enzymes and other contractile proteins (Neumann et al., 2019). Despite the importance of ventricular inotropy for cardiac output, it is worth mentioning that atrial inotropy also has an important role in keeping cardiac output, particularly in exercise or when the ventricular filling is compromised.

ATP and other nucleotides modulate the inotropy of both atria and ventricles. The inotropic effect of ATP and adenosine depends on several factors, including their concentration near receptors, as well as the region of the heart being targeted (e.g., ventricle vs atria). For research purposes, it also important to note that the inotropic properties of purines change significantly between different experimental preparations (e.g., isolated myocytes vs whole tissue) and different animal species. Almost every purinergic receptor can be found in the myocardium, and despite regional and species variation, some expression patterns can be identified. In atria and ventricles of rats, P2X5, P2X7, P2X4, and P2X1 receptors are the most expressed P2X receptors, while for the P2Y receptors the relative abundance is dominated by $P2Y_1$, $P2Y_2$, $P2Y_{14}$. Contrariwise, the human heart expresses higher levels of $P2Y_2$ followed by $P2Y_1$>>$P2Y_{11}$>$P2Y_6$>$P2Y_4$ in all four cardiac chambers (Wihlborg et al., 2006). Regarding P1 receptors, the A_1R is undoubtedly the most expressed receptor in cardiomyocytes throughout the entire heart (Musa et al., 2009). $A_{2A}R$ and $A_{2B}R$ are also found in cardiomyocytes, but it is on cardiac fibroblasts where they have a meaningful function in controlling fibrosis (Epperson et al., 2009).

Adenosine has a general inhibitory effect on E-C coupling and contraction through stimulation of A_1R. A_1R controls inotropy through different mechanisms depending on whether it operates in atria or ventricles. In atria, stimulation of A_1R increases hyperpolarization currents through stimulation of GIRK channels and inhibits cAMP-stimulated pathways. In ventricles, due to low expression levels of GIRK channels, negative inotropic effects of adenosine are mostly restricted to inhibition of AC, explaining why most ventricular effects of adenosine were only observed when adrenergic tonus is raised. Other adenosine receptors are also involved in the regulation of inotropy, but their role compared to A_1R is smaller, at least under physiological conditions. Even so, positive inotropic effects have been described for stimulation of $A_{2A}R$ and $A_{2B}R$ by stimulating cAMP-dependent pathways (Dobson et al., 2008; Chandrasekera et al., 2010; Boknik et al., 2020).

In mammalian atria, ATP has a biphasic inotropic effect that is characterized by a transient decrease of inotropy followed by a sustained positive inotropic effect (Froldi et al., 1994; Headrick et al., 2013). This dual response suggests the involvement of multiple receptors acting with different time-courses, but which identity remains to be fully resolved. Current evidence supports that the negative inotropic effect of ATP may be a consequence of A_1R activation after the nucleotide breakdown to adenosine (Froldi et al., 1994; Vassort, 2001). However, data showing that ATP-mediated effects were relatively insensitive to A_1R blockade in human atria questioned this theory (Gergs et al., 2008a). Aside from species differences, other P2 receptors are likely to be involved in this inhibitory effect, since enzymatically stable ATP analogues, such as ATPyS, as well as UTP or UDP, which breakdown do not directly generates adenosine, also caused transient negative inotropic effects (Froldi et al., 1994; Gergs et al., 2008a).

In ventricles, ATP is consistently associated with positive inotropic effects. This positive inotropy has been regarded as beneficial for contractile recovery following ischemia (Matsuura et al., 2021). The mechanism underlying the positive inotropic action of ATP entails increases of intracellular calcium. Although calcium influx was initially attributed to the opening of calcium channels, current evidence points towards Ca^{2+} permeation through the ionotropic P2X4R. It has been shown that the P2X4R has a positive inotropic effect both in atria and ventricles (Hirano et al., 1991; Hu et al., 2001; Vassort, 2001; Gergs et al., 2008a). UTP also stimulates myocardial contraction but, contrary to what was initially expected, $P2Y_{2,4,6}R$ receptors might not be involved in this effect, as either selective antagonists or knockout models for these receptors were unable to change the positive inotropic responses of UTP (Gergs et al., 2018; Neumann et al., 2019). Prostanoids are possibly involved in this UTP-mediated effect (Froldi et al., 2005). This diverges from previous studies where $P2Y_4$ and $P2Y_6$ receptors were found to be involved in the positive inotropism caused by UTP and UDP (Wihlborg et al., 2006).

5.1.3. Vascular control

The heart pumps blood into a large and complex network of vessels, nourishing all body territories. Control of vascular tonus, either by contracting or dilating vessels, is essential to ensure hemodynamic homeostasis and guarantee adequate tissue perfusion. Vascular tonus is determined by different mechanisms, including metabolic, myogenic, endothelial, and neuronal in nature. Autonomic innervation provides the bulk control in most vascular beds; here, sympathetic nerves that project into blood vessel walls co-release NA and ATP resulting in contraction of smooth muscle fibers. In small arteries, perivascular ATP induces fast and short-lived vasoconstriction, which precedes the long-lasting contraction held by NA (Burnstock, 2017b). ATP-mediated vasoconstriction is mostly

mediated via P2X1 receptors located on smooth muscle cells, but P2X2 and P2X4 have also been implicated. On the other end, endothelial cells sense increases in shear stress caused by vasoconstriction and increased resistance to blood flow, releasing paracrine and autocrine vasorelaxant mediators, such as ATP and adenosine. On the luminal side, ATP and adenosine counteract vasoconstriction promoted by perivascular nerves by means of stimulating $P2Y_1R$-like, P2XR, and A_2R located on endothelial cells. Besides the role played both P1 and P2 purinoceptors, NTPDase 1, the main vascular ecto-nucleotidase isoform, controls vascular reactivity by hydrolyzing adenine and uracil nucleotides (Kauffenstein et al., 2010), evidence exist showing that impairment of NTPDase1 activity is associated with arterial hypertension (Roy et al., 2018) and vasculogenic erectile dysfunction (Faria et al., 2008). The vasorelaxant activity of luminal ATP is largely blunted by endothelium damage; yet, under such conditions, adenosine is still able to induce vasodilation (Gordon and Martin, 1983; Faria et al., 2006; Arsyad and Dobson, 2016).

As the main metabolite of cellular activity, adenosine has a central role in the metabolic control of vascular tonus, Berne (1963) proposed that in highly demanding circumstances, like extraneous exercise or tachyarrhythmias, or even during hypoxic and ischemic conditions following myocardial infarction or respiratory failure, the circulating levels of adenosine resulting from the breakdown of adenine nucleotides dramatically increase in coronary arteries (Berne, 1963). The myocardial perfusion is highly dependent on coronary perfusion under resting conditions, with the normal myocardium consumption situated near the limit of oxygen supply and up to 75% of total oxygen content being extracted from the coronary arterial blood (Feigl, 2004). To match oxygen needs during highly demanding conditions, coronary arteries dilate and increase coronary blood flow. Under such conditions, adenosine acts as a potent vasodilatory mediator via endothelial $A_{2A}R$ and $A_{2B}R$, adding their actions to the activity of β-adrenoceptors in releasing vasoactive mediators, such as NO, prostacyclin, while opening K^+ channels (Sato et al., 2005; Sanjani et al., 2011). This vasodilatory effect supports the rationale for using either adenosine or drugs that increase its bioavailability, such as dipyridamole (an inhibitor of extracellular adenosine uptake), for the treatment and diagnosis of coronary artery diseases (Brink et al., 2015; Rai et al., 2017; Garcia-Baizan et al., 2019). Even in the presence of damaged endothelium, arteries keep adenosine's vasorelaxant activity due to stimulation of $A_{2A}R$ and $A_{2B}R$ directly on smooth muscle cells (Sato et al., 2005; Arsyad and Dobson, 2016; Burnstock, 2017b). The role of adenosine on the metabolic regulation of vascular tone is currently expanding from small to large vascular beds, with growing evidence supporting a role for this nucleoside in controlling compliance and stiffness of the aorta, which is important to maintain ventricular-arterial coupling and myocardial perfusion during acute and chronic hemodynamic changes (Dobson et al., 2017).

Hemostasis and thrombosis are other critical issues associated to purinergic signaling in blood vessels. Myocardial infarction, which is the leading cause of death in developing countries, most often results from rupture of cholesterol-rich atherosclerotic plaques (Collet et al., 2020). Plaque disruption causes vascular injury and exposure of subendothelial structures to circulating cells and coagulation factors, namely platelets, ADP, and ATP. ADP binds to $P2Y_{12}$ receptors and functions as a potent platelet activator leading to secretion of ADP-containing granules and release of other prothrombotic factors. ATP binding to platelet $P2Y_1$ and P2X1 receptors enhance platelet activation and aggregation (Hechler and Gachet, 2015). Interestingly, vascular injury also promotes ADP accumulation by means of increased activity of subendothelial ATPases, namely NTPDase 2 (Sevigny et al., 2002). Interruption of this deleterious vicious cycle was made possible with the introduction of $P2Y_{12}R$ antagonists in clinical practice. Clinical trials have successfully demonstrated that $P2Y_{12}R$ antagonists, such as clopidogrel or ticagrelor, are effective in improving the clinical outcomes of acute coronary syndromes (ACS) and stroke (Collet et al., 2020). This therapeutic alternative has gained more and more adepts since it was demonstrated that platelets from patients with myocardial infarction exhibit increased resistance to acetylsalicylic acid by a mechanism that possibly involves higher release levels of ADP (Borna et al., 2005). High levels of $A_{2A}R$ and $A_{2B}R$ are also found in human platelets (Amisten et al., 2008), which may partially counteract the effects of ADP after its conversion to adenosine by ecto-nucleotidases.

5.2. Long-term regulation of the cardiovascular function by purines

The purinergic signaling has a retaliatory nature by promptly reacting to perturbations of body homeostasis, as aforementioned. Nevertheless, this knowledge may be only the tip of a vast iceberg comprising intricate signaling pathways. It also runs in the background of several physiological processes, from the early stages of embryonic development to adulthood.

Diabetes, obesity, dyslipidemia, and hypertension are all relentless inflammatory states that promote atherosclerosis and heart disease development. Emerging evidence shows that modulation of purinergic signaling represents an exciting approach for better control of these risk factors. For instance, hypertensive phenotypes have been observed with loss-of-function of endothelial P2X4R (Ganbaatar et al., 2018), activation of vascular P2X1R (Inscho et al., 2011), stimulation of P2X3R in the carotid body (Pijacka et al., 2016), and activation of renal P2X7R (Menzies et al., 2013). Moreover, the development of atherosclerotic plaques can be restrained upon activation of $A_{2A}R$ (Reiss et al., 2019), or inhibition of A_3R (Park et al., 2018), $P2Y_1R$ (Zerr et al., 2011), $P2Y_6R$ (Stachon et al., 2014; Sunggip et al., 2017) and $PY_{12}R$ (Ganbaatar et al., 2018). The pleiotropic actions of

purinergic receptors may also extend to the control of adipogenesis and low-grade inflammation associated with the metabolic syndrome (de Oliveira et al., 2020).

Heart failure is within the spectrum of most forms of cardiovascular risk factors and associated diseases. Despite the progress made in the past few years regarding diagnosis and treatment, hospitalization and deaths from HF still have pandemic proportions. Targeting purinergic signaling has attracted increasing interest in cardiovascular medicine, particularly after the development of $P2Y_{12}R$ antagonists and their meaningful impact on ACS. As we already noticed in the previous sections, pathophysiological processes behind every cardiovascular disease eventually converge to purinergic signaling implications. Development of HF is accompanied by a disorganization of the structure and function of the heart, also commonly referred as pathological cardiac remodeling, which includes hypertrophy and death of cardiomyocytes, fibroblast proliferation and fibrotic debris deposition, metabolic reprogramming and insulin resistance, and vasculopathy ranging from endothelial dysfunction to abnormal smooth muscle cells function (Burchfield et al., 2013). All these processes that run silently in the setting of HF are all amenable to purinergic regulation (Headrick et al., 2013; Burnstock, 2017b).

Taking ischemic heart disease as an example, as it is the commonest etiology of HF, numerous preclinical studies demonstrate that all adenosine receptors and some P2 receptors are cardioprotective in ischemia-reperfusion models, with some redundancy among their actions (Headrick et al., 2013; Procopio et al., 2021). In the acute phase of myocardial infarction, adenosine acting via A_1R and A_3R limits damage before and after an ischemic insult, while activation of $A_{2A}R$ and $A_{2B}R$ contribute to increase the coronary blood flow, to inhibit platelet aggregation, and to control inflammation subsequent to the ischemic insult (Headrick et al., 2013; Burnstock and Pelleg, 2015). This led some researchers to hypothesize that adenosine or ATP might be helpful as adjuvant therapy in ischemic disease conditions (Ross et al., 2005; Sakuma et al., 2010). Specifically, the randomized placebo-controlled trial AMISTAD-II, which enrolled over than 2000 patients, showed that adenosine administrated to patients with ST-elevation MI (STEMI) was able to reduce the infarct size, yet (unfortunately) without any major impact in their clinical outcome (Ross et al., 2005). In contrast, the PROMISE trial, which addressed STEMI patients treated with primary coronary angioplasty and intracoronary adenosine as adjuvant therapy, failed to demonstrate any improvement in ventricular remodeling (Garcia-Dorado et al., 2014). These conflicting results were further explored in a subsequent meta-analysis, where adenosine, used as adjuvant therapy to reperfusion, improved clinical outcomes in STEMI patients with less incidence of HF but, again, no impact in major clinical endpoints (e.g., death) (Bulluck et al., 2016). Noteworthy, in PLATO trial, ticagrelor performed better than clopidogrel in improving major cardiovascular events in ACS (Wallentin et al., 2009).

Although speculative, it appears that ticagrelor, but not clopidogrel, may act as an inhibitor of ENT-1. It was also suggested that the protective effect of ticagrelor was probably related with its ability to raise the circulating levels of adenosine, thus favoring vasodilation, anti-platelet activity, and anti-inflammatory effects. This off-target effect of ticagrelor probably also explains the documented increase of ventricular pauses and dyspnea (Cattaneo et al., 2014). A protective role for UTP was also claimed in the MI acute phase (Yitzhaki et al., 2006).

The plasmatic levels of adenosine are increased in HF (Funaya et al., 1997; Burnstock, 2017b). Likewise, expression of myocardial purinergic receptors (e.g., A_1R, P2X1R and $P2Y_2R$) also change in HF (Hou et al., 1999; Burnstock, 2017b). Enhancing circulating levels of adenosine with chronic administration of the nucleoside uptake blocker, dipyridamole, was shown to be protective in HF patients (Sanada et al., 2007). Moreover, adenosine formation from the extracellular breakdown of ATP seems to be relevant to protect the heart from ischemia, as low levels of this nucleoside were found in mice lacking CD73 giving a rationale for the loss of the cardioprotective effects operated by $A_{2B}R$ (Eckle et al., 2007). NTPDase1/CD39 is cardioprotective by facilitating termination of the pro-inflammatory and thrombogenic actions of ATP and ADP, respectively (Kohler et al., 2007; Yegutkin, 2008). Genetic or pharmacological interference with NTPDase1/CD39, aimed at impairing adenosine formation, is shown to be deleterious in myocardial ischemic injury (Kohler et al., 2007; Smith et al., 2017). Consistent with this theory, it was observed an increase of myocardial injury following infarction with prior disruption of ecto-nucleotidases activity (Takahashi-Sato et al., 2013). Notwithstanding this, the cardioprotection afforded by NTPDase1/CD39 has been recently challenged (Sutton et al., 2017).

In this context, clinical trials are ongoing with several adenosine receptor agonists for HF. These include capadenoson, a partial $A_1R/A_{2B}R$ agonist, and neladenoson, a partial A_1R agonist, which conclusions are expected to be released soon (Borah et al., 2019). While adenosine seems to be protective in the acute phase of ischemic insults, chronic elevation of the nucleoside levels may activate some mitogenic pathways involved in the expression of hypertrophic genes and maladaptive remodeling, such as the PI3K/AKT and Wnt/beta-catenin pathways (Procopio et al., 2021). Therefore, it has been claimed that activation of the $A_{2B}R$ may have beneficial effects if restricted to the acute phase, while its antagonist may be desirable in chronic phases of this disease condition (Lasley, 2018).

Regarding P2 receptors, it has also been shown that P2X7R activity exacerbates inflammatory damage and fibrosis (Zhou et al., 2020), while stimulation of the P2X4R subtype had an opposite effect by protecting the heart from failure (Yang et al., 2015). Myocardial fibrosis has a negative impact in HF, being associated with arrhythmias and impairment of ventricular filling caused by myocardial stiffness. Noteworthy, ATP may have

both pro and anti-fibrotic roles; UTP has also been characterized as a pro-hypertrophic factor (Pham et al., 2003). While $P2Y_2R$ and $P2Y_6R$ increase the production of pro-fibrotic gene expression and deposition of extracellular matrix, $A_{2A}R$, and $P2Y_{11}R$ counteract this effect (Lu and Insel, 2014; Certal et al., 2015; Shimoda et al., 2020).

V. AIMS

In general terms, this project aimed at providing further insights into the role of P1 and P2 purinoceptors in acute hemodynamic regulation of cardiac function operated by adenosine and ATP, respectively.

The specific aims of this project were as follows:

1- Considering that adenosine, unlike other cardiodepressant drugs, reduces more potently heart rate compared to the contraction force, we thought it would be interesting to explore the molecular mechanisms underlying adenosine's chronoselectivity by directly implicating changes in ion currents and intracellular second messenger systems in the spontaneously beating rat atria model.

2- To identify the most relevant P2 purinoceptor among the various subtypes existing in the SAN, as well as to characterize the molecular mechanisms responsible for the dual effect of ATP in the regulation of the sinus rhythm and inotropy using spontaneously beating rat atria and paced right ventricular strips.

VI. RESEARCH PAPERS

The results obtained in this thesis were published as original research papers in peer-reviewed JCR international journals, as follows:.

Paper 1: **Bragança B***, Oliveira-Monteiro N, Ferreirinha F, Lima PA, Faria M, Fontes-Sousa AP, Correia-de-Sá P (2016). Ion Fluxes through $K_{Ca}2$ (SK) and Ca_v1 (L-type) Channels Contribute to Chronoselectivity of Adenosine A_1 Receptor-Mediated Actions in Spontaneously Beating Rat Atria. Front Pharmacol 2016 Mar 7;7:45. doi: 10.3389/fphar.2016.00045.

Paper 2: **Bragança B***, Nogueira-Marques S, Ferreirinha F, Fontes-Sousa AP, Correia-de-Sá P. (2019). The Ionotropic P2X4 Receptor has Unique Properties in the Heart by Mediating the Negative Chronotropic Effect of ATP While Increasing the Ventricular Inotropy. Front Pharmacol 2019 Sep 24;10:1103. doi: 10.3389/fphar.2019.01103.

*Bragança B contributed significantly to the experimental design, data acquisition, interpretation of the results obtained, as well as in drafting and revising the manuscript.

Other relevant publications within the scope of this thesis appeared as review papers in peer-reviewed JCR international journals:

Paper 3: **Bragança B[#],** and Correia-de-Sá P. (2020). Resolving the Ionotropic P2X4 Receptor Mystery Points Towards a New Therapeutic Target for Cardiovascular Diseases. Int J Mol Sci. 2020 Jul 15;21(14):5005. doi: 10.3390/ijms21145005.

Paper 4: Bessa-Gonçalves M[§], **Bragança B[#§],** Martins-Dias E, Correia-de-Sá P, Fontes-Sousa AP. (2018). Is the adenosine A_{2B} 'biased' receptor a valuable target for the treatment of pulmonary arterial hypertension? Drug Discov Today 2018 Jun;23(6):1285-1292. doi: 10.1016/j.drudis.2018.05.005. ([§]first co-authors)

[#]Bragança B contributed significantly to the conceptualization, writing, revising, and editing of the manuscript.

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PAPER 1

Front Pharmacol 2016 Mar 7;7:45.
doi: 10.3389/fphar.2016.00045.

Ion fluxes through $K_{Ca}2$ (SK) and Ca_v1 (L-type) channels contribute to chronoselectivity of adenosine A_1 receptor-mediated actions in spontaneously beating rat atria

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Abstract

Impulse generation in supraventricular tissue is inhibited by adenosine and acetylcholine via the activation of A_1 and M_2 receptors coupled to inwardly rectifying GIRK/K_{IR}3.1/3.4 channels, respectively. Unlike M_2 receptors, bradycardia produced by A_1 receptors activation predominates over negative inotropy. Such difference suggests that other ion currents may contribute to adenosine chronoselectivity. In isolated spontaneously beating rat atria, blockade of $K_{Ca}2$/SK channels with apamin and Ca_v1 (L-type) channels with nifedipine or verapamil, sensitized atria to the negative inotropic action of the A_1 agonist, R-PIA, without affecting the nucleoside negative chronotropy. Patch-clamp experiments in the whole-cell configuration mode demonstrate that adenosine, via A_1 receptors, activates the inwardly-rectifying GIRK/K_{IR}3.1/K_{IR}3.4 current resulting in hyperpolarization of atrial cardiomyocytes, which eventually reduces the repolarizing force and thereby prolong action potentials duration and Ca^{2+} influx into cardiomyocytes. Immunolocalization studies showed that differences in A_1 receptors distribution between the sinoatrial node and surrounding cardiomyocytes do not afford a rationale for adenosine chronoselectivity. Immunolabelling of K_{IR}3.1, K_{Ca}2.2, K_{Ca}2.3, and Ca_v1 was also observed throughout the right atrium. Functional data indicate that while both A_1 and M_2 receptors favor the opening of GIRK/K_{IR}3.1/3.4 channels modulating atrial chronotropy, A_1 receptors may additionally restrain K_{Ca}2/SK activation thereby compensating atrial inotropic depression by increasing the time available for Ca^{2+} influx through Ca_v1 (L-type) channels.

1. Introduction

Intravenous bolus of adenosine is clinically useful for prompt conversion of paroxysmal supraventricular tachycardia to sinus rhythm and to control ventricular contraction rate in atrial fibrillation (Savelieva and Camm, 2008; Lim et al., 2009). The use of the nucleoside is preferred mostly due to its rapid onset, short half-life (ranging from 1 to 10 s) and lesser hypotensive effect as compared to other previously recommended drugs, such as the Ca_v1 (L-type) channel blocker verapamil (Blomstrom-Lundqvist et al., 2003b). Adenosine affects many aspects of cardiac function, including heart rate, contractility and coronary flow through the activation of G protein-coupled A_1, A_{2A}, A_{2B}, and A_3 receptors (Mustafa et al., 2009). The A_1 receptor promoter is highly active in the atrium as compared to the ventricle, resulting in high A_1 receptor mRNA levels (Rivkees et al., 1999). This leads to a dominant localization of A_1 receptors in atrial cardiomyocytes where they exert direct inhibitory effects on chronotropy and dromotropy (Shryock and Belardinelli, 1997; Auchampach and Bolli, 1999), as well as indirect anti-β-adrenergic inotropic responses by opposing the responses of sympathetic nerves activation and β_1 receptors stimulation (Dobson, 1983; Romano et al., 1991).

Like muscarinic M_2 receptors which are responsible for the negative chronotropic and inotropic effects of acetylcholine, adenosine A_1 receptor effects are mediated by hyperpolarization of sinoatrial (SA) node cells as well as cells of the atrioventricular node primarily by inducing outward potassium currents through βγ subunits of G protein-coupled inwardly rectifying K^+ channels (GIRK or K_{IR} 3.1/3.4) (Belardinelli and Isenberg, 1983; Kurachi et al., 1986b; Yatani et al., 1987; Mubagwa and Flameng, 2001). Due to different expression levels of A_1 and M_2 receptors, the maximal GIRK/K_{IR} 3.1/3.4 current that can be activated by endogenous adenosine is smaller than the current triggered by cholinergic agonists (Kurachi et al., 1986b). Activation of GIRK/K_{IR}3.1/3.4 currents causes a reduction in the action potential duration, thereby decreasing the time available for Ca^{2+} influx through Ca_v1 (L-type) channels and, hence, heart rate and the force of muscle contraction in atrial myocardium (Urquhart et al., 1993; Belardinelli et al., 1995; Neumann et al., 1999). Negative inotropy may, however, be partially compensated by modulation of multiple downstream targets of G proteins, which may include Ca^{2+} influx through Cav1 (L-type) channels (Wang et al., 2013). The predominant and better-studied $Ca_v1.2$ channel isoform coexist with the $Ca_v1.3$ in the SA node and atrioventricular node where they participate in pacemaking by different mechanisms (Mangoni and Nargeot, 2008). Despite the extensive literature on this subject, several questions remain unanswered concerning the contribution of distinct K^+ and Ca^{2+} channels to differential pharmacological responses of atrial cardiomyocytes to both adenosine and acetylcholine.

Interestingly, it has been shown that the negative chronotropic and inotropic responses to adenosine may be differentiated at the postreceptor transduction level in the dog's heart (Oguchi et al., 1995). Supersensitive chronotropic and dromotropic effects have also been described for adenosine during isoproterenol-infusion in the human ventricle in vivo after heart transplantation, whereas the nucleoside did not reduce the isoproterenol-induced increase in contractility (Koglin et al., 1996). In this study, we show that the negative chronotropic effect caused by adenosine A_1 receptors activation is evidenced at much lower concentrations than the negative inotropic action of the nucleoside, which is in clear contrast to the M_2-receptormediated cardiodepression operated by acetylcholine. Given the clinical relevance of this finding and the lack of our knowledge regarding the contribution of K^+ and Ca^{2+} channel subtypes to adenosine chronoselectivity, we tested the effect of the nucleoside in the absence and in the presence of specific K^+ and Ca^{2+} channel blockers (see Table 3) in rat atria with intact SA rhythm and in voltage-clamp experiments using acutely dissociated atrial cadiomyocytes. For comparison purposes, we also evaluated whether these channel blockers modulate M_2 receptors activation, since this is the predominant cholinergic receptor subtype in atrial tissue of most mammalian species (Peralta et al., 1987; Hulme et al., 1990; Wang et al., 2001; Krejci and Tucek, 2002). Additionally, we investigated the regional distribution of the involved receptors (e.g., A_1 and M_2) and channels (e.g., Ca^{2+} and K^+) in the right atrium and SA node by immunofluorescence confocal microscopy.

2. Materials and Methods

2.1. Animals

Wistar rats (*Rattus norvegicus*; 250-300 g) of either sex (Charles River - CRIFFA, Barcelona, Spain; Vivarium ICBAS, Porto, Portugal) were housed in a temperature-controlled (21ºC) room with a regular 12:12-h light-dark cycle. The animals were provided free access to standard laboratory chow and water. Animal care and experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and followed the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health Guide for Care and Use of Laboratory animals (NIH Publications No. 80-23) revised 1996. All studies involving animals are reported in accordance with ARRIVE guidelines for reporting experiments involving animals (McGrath et al., 2010).

2.2. Isolated perfused spontaneously beating rat atria

Isolated perfused beating atria were prepared using a previously described method (Kitazawa et al., 2009), with some modifications. In brief, hearts were rapidly excised after

decapitation followed by exsanguination (Rodent guillotine, Stoelting 51330), and placed in a physiological solution (Tyrode's solution) composed of (mM): NaCl 137; KCl 2.7; $CaCl_2$ 1.8; $MgCl_2$ 1; NaH_2PO_4 0.4; $NaHCO_3$ 11.9; glucose 11.2 and gassed with 95% O_2 + 5% CO_2 (at pH 7.4). In some of the experiments the concentration of KCl was raised from 2.7 (see *e.g.* (De Biasi et al., 1989)) to 4.7 mM. Hearts were allowed to beat freely for a few seconds at room temperature, to empty its blood content. The paired rat atria with the SA node region were dissected out, cleaned of fatty tissues, and suspended in a 14-mL organ bath containing gassed Tyrode's solution at 37°C. Each auricular appendage was tied and connected with thread to the organ bath wall and to an isometric force transducer (MLT050/D; AD Instruments, Colorado Springs, CO, USA). Changes in isometric tension were recorded continuously using a PowerLab data acquisition system (Chart 5, version 4.2; AD Instruments, Colorado Springs, CO, USA). The preparations were allowed to equilibrate for 30–40 min. During this time, the preparations were superfused with Tyrode's solution and the tension was adjusted to 9.8 mN. This procedure allows atria (with intact SA node) to progressively recover rhythmic spontaneous beatings; preparations with spontaneous atrial rate below 200 beats min^{-1} or exhibiting rhythm variations above 10 beats min^{-1} during equilibrium were discarded to ensure measurements were made in atria with intact primary pacemaker SA node activity. None of the preparations exhibited noticeable signs of ectopic-activity caused by secondary pacemakers, usually related to asynchronous and abnormal contractions. Under these experimental conditions, spontaneously beating rat atria respond to muscarinic and β-adrenergic stimulation, but are unaffected by the application of atropine or propranolol alone used in concentrations high enough (10 μM) to prevent the effects of acetylcholine (100 μM) and isoproterenol (30 nM), respectively. Thus, myographic recordings reported in this study include rate (chronotropic effect) and contractile force (inotropic effect) of spontaneously beating atria measured in the absence of cholinergic and/or adrenergic tone.

In some of the experiments, isometric tension was tested at a fixed frequency of 240 beats min^{-1} commanded by electric field stimulation of the preparations as an index of inotropy measured without being affected by concurrent changes in chronotropy. Electric atrial pacing (4 Hz, 100 V, 0.5 ms) was performed using a Grass S48 Stimulator (Quincy, MA, USA) via two platinum electrodes positioned on each side of the preparations.

2.3. Experimental design

After reaching a steady-state (Control values shown in Table 4), the perfusion with Tyrode's solution was stopped and the preparations were incubated with increasing concentrations of R-(-)-N^6-(2-phenylisopropyl)adenosine (R-PIA, 0.001-1 μM), a stable adenosine A_1 receptor agonist, or oxotremorine (0.003-3 μM), a muscarinic M_2 receptor

agonist, either in the absence or in the presence of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 100 nM) or AF-DX 116 (10 μM), which respectively block A_1 and M_2 receptors. Adenosine (0.001-3 mM) and the adenosine A_{2A} receptor agonist, 2-p-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine (CGS 21680C, 0.003-1 μM), were also tested in some of the experiments. Agonists were added cumulatively into the bathing solution at 2 min intervals, as this time was considered sufficient for each concentration to equilibrate with the preparation and to cause a maximal response under the present experimental conditions. To examine the role of K^+ and Ca^{2+} channel currents in the effects of adenosine A_1 and muscarinic M_2 receptors activation, concentration-response curves to R-PIA (0.001-1 μM) and oxotremorine (0.003-3 μM) were established in the presence of the following inhibitors: 4-aminopyridine (4-AP, 10 μM), a nonspecific voltage-dependent K_v channel blocker, glibenclamide (10 μM), a selective blocker of ATP-sensitive $K_{ATP}/K_{IR}6$ channels, tertiapin Q (300 nM), a blocker of GIRK/K_{IR} channels with high affinity for K_{IR} 3.1/3.4 channels, apamin (30 nM), an inhibitor of small-conductance Ca^{2+}-activated K^+ ($K_{Ca}2$/SK) channels, nifedipine and verapamil (1 μM), selective blockers of high voltage-activated Ca_v1 (L-type) channels, and mibefradil (3 μM), a low voltage-activated Ca_v3 (T-type) channel blocker (see Table 4).

Table 3- List of used drugs and their pharmacological characteristics.

Drug	Target Selectivity	Concentration Range	Supplier
Adenosine	Endogenous adenosine receptor ligand	0.001-3 mM	Sigma-Aldrich
R-PIA	Selective A_1 receptor agonist	0.001-1 μM	Sigma-Aldrich
CGS21680C	Selective A_{2A} receptor agonist	0.003-1 μM	Sigma-Aldrich
DPCPX	Selective A_1 receptor antagonist	100 nM	Sigma Aldrich
Oxotremorine	Preferential M_2 receptor agonist (≥98%)[a]	0.003-3 μM	Tocris Cookson Inc.
AF-DX 116	Selective muscarinic M_2 receptor antagonist	10 μM	Tocris Cookson Inc.
4-AP	Nonspecific voltage-dependent K_v channel blocker[b]	10 μM	Sigma-Aldrich
Glibenclamide	Selective blocker of ATP-sensitive inward rectifier $K_{ATP}/K_{IR}6$ channels	10 μM	Ascent Scientific
Tertiapin Q	Potent blocker of inward rectifier GIRK/K_{IR} currents with high affinity for K_{IR} 3.1/3.4 channels[c]	300 nM	Ascent Scientific
Apamin	Selective blocker of small-conductance Ca^{2+}-activated $K_{Ca}2$/SK channels	30 nM or 0.003-1μM	Sigma-Aldrich
Nifedipine	Selective blocker of high-voltage Ca_v1 (L-type) channels	1 μM	Sigma-Aldrich
Verapamil	Selective blocker of high-voltage Ca_v1 (L-type) channels	1 μM or 0.03-10 μM	Tocris Cookson Inc.
Mibefradil	Moderately selective blocker of high-voltage Ca_v3 (T-type) channels. Displays IC_{50} values of 2.7 μM and 18.6 μM for T-type and L-type channels, respectively	3 μM	Tocris Cookson Inc.

[a]Used as a selective high-affinity antagonist for adenosine A_1 receptors (K_i~0.45 nM) with more than 700-fold selectivity over other adenosine receptors, namely the A_{2A} receptor (Lohse et al., *1987*).
[b]Cited as the predominant form of muscarinic receptors present in the heart of various mammalian species, including humans (Peralta et al., *1987*; Hulme et al., *1990*; Caulfield, *1993*).
[c]Used as a preferential M_2 (and M_4) receptor antagonist (pK_B 7.1–7.2), the most expressed receptor in the heart (Caulfield, *1993*).
[d]Used as voltage-dependent K_v channel blocker (e.g., Bardou et al., *2001*).
[e]Tertiapin Q is a high affinity and potent blocker of inward-rectifier K^+ currents that is widely used for inhibiting GIRK1/4 (K_{IR}3.1/3.4) channels in the nanomolar concentration range (see e.g., Jin and Lu, *1998*; Whorton and MacKinnon, *2011*).
[f]Prototypical potent and highly selective inhibitor of the small-conductance Ca^{2+}-activated K^+-channel ($K_{Ca}2$, SK); complete blockade of $K_{Ca}2$ (SK) currents were observed at 100 nM apamin (Hugues et al., *1982*).

For the sake of data normalization, all the inhibitors were allowed to equilibrate with the preparations for 15 min before application of R-PIA or oxotremorine; values for atrial rate (chronotropic effect) and contractile force (inotropic effect) after reaching the

equilibrium with the receptor antagonists or ion channel inhibitors are shown in Table 4. The concentrations of the inhibitors in this study were within the range of channel selectivity described in the literature. Since, blockade of K^+ channels can stimulate Ca^{2+} influx through voltage-sensitive Ca_v1 (L-type) channels, we examined the responses of the rat spontaneously beating atria to cumulative application of verapamil (0.03-10 µM) in the absence and presence of apamin (30 nM) or tertiapin Q (300 nM). In another group of experiments, the myographic effects of increasing concentrations of apamin (0.003-1 µM) in the absence and presence of verapamil (1 µM) were also recorded. The protocol for drug application was identical to that described for the studies using R-PIA and oxotremorine (see Figure 5A).

Table 4. Influence of receptor antagonists and ion channel inhibitors on chronotropism and inotropism of spontaneously beating rat atria.

Protocols	Chronotropism (*beats.min*^{1})	Inotropism (*mN*)	*n*
R-PIA (0.001-1 µM)			
+ Control	**200 ± 5**	**3.9 ± 0.7**	**40**
+ DPCPX (100 nM)	220 ± 4	3.8 ± 0.2	4
+ 4-AP (10 µM)	210 ± 15	5.1 ± 0.4*	6
+ Glibenclamide (10 µM)	214 ± 26	4.8 ± 0.6*	5
+ Tertiapin Q (300 nM)	206 ± 14	3.7 ± 0.3	5
+ Apamin (30 nM)	221 ± 11	4.5 ± 0.5	8
+ Nifedipine (1 µM)	148 ± 26*	3.7 ± 1.1	5
+ Verapamil (1 µM)	160 ± 17*	3.8 ± 0.6	5
+ Mibefradil (3 µM)	169 ± 16*	3.5 ± 0.2	7
Oxotremorine (0.003-3 µM)			
+ Control	**213 ± 5**	**3.5 ± 0.3**	**17**
+ AF-DX 116 (10 µM)	225 ± 13	3.2 ± 0.1	6
+ 4-AP (10 µM)	209 ± 21	4.6 ± 0.4*	5
+ Glibenclamide (10 µM)	220 ± 26	4.1 ± 0.4*	6
+ Tertiapin Q (300 nM)	228 ± 15	3.6 ± 0.5	5
+ Apamin (30 nM)	220 ± 13	4.0 ± 0.5	7
+ Nifedipine (1 µM)	142 ± 26*	3.6 ± 0.5	5
+ Verapamil (1 µM)	165 ± 17*	3.4 ± 0.4	6
+ Mibefradil (3 µM)	164 ± 15*	3.3 ± 0.2	6
Verapamil (0.03-10 µM)			
+ Control	**205 ± 11**	**3.3 ± 0.3**	**14**
+ Apamin (30 nM)	210 ± 19	3.9 ± 0.4	7
+ Tertiapin Q (300 nM)	202 ± 11	3.4 ± 0.2	5
Apamin (0.003-1 µM)			
+ Control	**218 ± 6**	**3.4 ± 0.3**	**9**
+ Verapamil (1 µM)	157 ± 20*	3.5 ± 0.8	7

Receptor antagonists and ion channel inhibitors were allowed to equilibrate with the preparations during 15 min before R-PIA (0.001–1 µM), oxotremorine (0.003–3 µM), verapamil (0.03–10 µM), or apamin (0.003–1 µM) application. Shown are values for atrial rate (chronotropic effect) and contractile force (inotropic effect) in the absence (Control or vehicle) and in presence of receptor antagonists and ion channel inhibitors after reaching the equilibrium, i.e., measured immediately before incubation with R-PIA, oxotremorine, verapamil, and apamin. The data are expressed as mean ± SEM from an n number of individual experiments (animals).
*P < 0.05 compared with the control situation before incubation with receptor antagonists or ion channel inhibitors.

2.4. Isolation of atrial cardiomyocytes

Atrial cardiomyocytes were obtained by enzymatic digestion using a Langendorff perfusion apparatus. Rat hearts were quickly removed and washed in HEPES buffer solution (composition in mM: NaCl 135, KCl 5, $MgSO_4$ 1.5, NaH_2PO_4 0.33, HEPES 10, $CaCl_2$ 0.5, D-glucose 15, adjusted to pH 7.35 with NaOH) containing heparin (50 UI/mL) at room temperature (~20 °C). The excised hearts were then catheterized through the aorta and superfused retrogradely at a flow rate of 7 $ml.min^{-1}$ with a nominally calcium-free HEPES buffer solution gassed with 100% O_2 at 37ºC. Five minutes after initiating heart perfusion, the superfusion fluid was supplemented with collagenase II (Worthington Biochemical Corp., 148 U/ml) and protease XIV (Sigma-Aldrich, 10 U/mL) and the calcium

concentration was raised to 0.2 mM. As soon as the heart became soft to the touch, the superfusion was stopped (for about 15 min) and atria were dissected free from the ventricles. Isolated atria were then gently minced into small pieces with microdissecting scissors and further digested using a plastic transfer pipette to release single myocytes. To separate single myocytes from non-digested tissue, the cellular suspension was filtered through a 500 µm mesh. The cellular suspension was centrifuged 3 times at 18 g for 3 min. The resulting pellet of each centrifugation was then re-suspended in fresh physiological solution (described above) containing 10 mM 2,3-butanedione monoxime and increasing concentrations of $CaCl_2$ to steeply raise the extracellular calcium to a final concentration of 1 mM. This isolation procedure yields ~60-70% of Ca^{2+} tolerant atrial cadiomyocytes with clear 63yofibrillar striations. Acutely dissociated atrial cardiomyocytes were kept at room temperature and used up to 6 hours after their isolation.

2.5. Patch-clamp experiments in isolated atrial cardiomyocytes

Acutely dissociated atrial cardiomyocytes were placed onto 35 mm plastic petri dishes (Nunclon™ Δ surface; Nunc, Roskilde, Denmark), which were used as recording chambers mounted on the stage of an inverted microscope. Myocytes were allowed to adhere to the bottom of chamber for 10 min. A gravity-fed system was used for the exchange of extracellular solution (2–3 $ml.min^{-1}$), which had the following composition (in mM): NaCl 135, KCl 5, $MgSO_4$ 1.5, NaH_2PO_4 0.33, HEPES 10, $CaCl_2$ 1, D-glucose 15, adjusted to pH 7.35 with 1 mM NaOH. The bathing solution was bubbled with 100% O_2 at room temperature. Atrial cardiomyocytes were voltage-clamped using the whole cell patch-clamp configuration, as described previously (Vicente et al., 2010). Briefly, the patch pipettes (2.80 ± 0.09 MΩ, n = 19) were pulled from borosilicate glass capillaries (Science Products GmbH, GB150T-8P) and filled with an internal solution containing (in mM): potassium gluconate 135, KCl 5, NaCl 5, $Na_{1/2}$HEPES 10, $MgCl_2$ 1, EGTA 0.1, Na_2ATP 2, NaGTP 0.4 (pH 7.3 adjusted with 1 mM KOH; 305 ± 5 mOsm). Only rod-shaped myocytes with no spontaneous contractions at rest were used for experiments. The estimated junction potential for the filling and bathing solution combinations mentioned above is -8.9 mV (calculated with JPCalc 2.00, School of Physiology and Pharmacology University of New South Wales). Data were not corrected for the junction potential. Currents were recorded with an Axopatch 200B electrometer (Axon Instruments Inc., USA) and stored on a PC using the pClamp 6.0.3 software (Axon Instruments Inc., USA) and an analogue digital interface (Digidata 1200; Axon Instruments, USA). Signals were acquired at a sampling rate of 5 kHz and filtered at 2 kHz (-3 dB, four pole Bessel). Quantification of currents were made by measuring the peak current 30 ms after the initial voltage step of the command pulse, which accounts for an approximate measure of the peak current but, away enough from the

occurrence of the fast I_{Na}, avoiding the contamination of the sodium currents. Current amplitudes were measured in respect to “zero current,” which was given by the current value at −40 mV, a potential where the “expected” physiological current should be zero. Whole-cell capacitance (132 ± 6 pF, n = 9) was calculated from the area under the curve fitted to the transient capacitive current produced by 5 mV test depolarizing step from a holding potential of −70 mV. Cells with significant leak currents were rejected. Also, series resistance (5.5 ± 0.3 MΩ, n = 14) were monitored throughout the experiments and only recordings with variation<10% were considered valid. The holding potential (V_H) was kept at −70 mV, unless otherwise specified. The Ca^{2+} dependence of outward K+ currents were assessed using a two-pulse protocol. A prepulse lasting 50 ms to −10 mV was delivered to trigger Ca^{2+} influx through voltage-activated channels. The prepulse was immediately followed by a second depolarizing pulse to +40 mV for 750 ms to elicit the outward K+ current. Before evaluating the voltage-dependence of activation, the current stability was monitored with a set of two-pulse protocols applied every 2 min. Drugs were diluted previously and then included in the superfusion fluid.

2.6. Immunofluorescence staining and confocal microscopy studies

Rat hearts were excised (see above) and placed in oxygenated Tyrode’s solution at room temperature. Following heart excision, the right atrium containing the SA node region and surrounding atrial muscle was accurately isolated. Tissue fragments were stretched to all directions, pinned flat onto cork slices and embedded in Shandon cryomatrix (Thermo Scientific) before frozen in a liquid nitrogen-isopentane bath. Frozen sections with 8 µm thickness were cut perpendicular to the *crista terminalis* of the right atrium (see Figure 4A). Once defrosted, tissue sections were fixed in phosphate buffered saline (PBS) containing 50% acetone and 2% paraformaldehyde. Following fixation, the preparations were washed three times for 10 min each using 0.1 M PBS and incubated with a blocking buffer, consisting in fetal bovine serum 10%, bovine serum albumin 1%, Triton X-100 0.3% in PBS, for 2 h. After blocking and permeabilization, samples were incubated with selected primary antibodies (Table 5) diluted in incubation buffer (fetal bovine serum 5%, serum albumin 1%, Triton X-100 0.3% in PBS), overnight at 4ºC. For double immunostaining, antibodies were combined before application to tissue samples. Following the washout of primary antibodies with PBS (3 cycles of 10 min) tissue samples were incubated with species-specific secondary antibodies (Table 5) in the dark for 2 hours, at room temperature. Negative controls were carried out by replacing the primary antibodies with non-immune serum; cross-reactivity of the secondary antibodies was tested in control experiments in which primary antibodies were omitted. Finally, tissue samples were mounted on optical-quality glass slides using VectaShield as antifade mounting media (H-

1200; Vector Labs) and stored in the dark at 4 ºC. Observations were performed and analyzed with a laser-scanning confocal microscope (Olympus Fluo View, FV1000, Tokyo, Japan).

Table 5- Primary and secondary antibodies used in immunohistochemistry experiments.

Antigen	Code	Host	Dilution	Supplier
Primary antibodies				
Connexin 43 (Cx43)	ab11370	Rabbit (rb)	1:700	Abcam
Neurofilament 160 (NF-160)	ab7794	Mouse (ms)	1:1000	Abcam
Vimentin	M0725	Mouse (ms)	1:150	Dako
Adenosine receptor A_1	AB 1587P	Rabbit (rb)	1:100	Chemicon
Muscarinic receptor M_2	AMR-002	Rabbit (rb)	1:200	Alomone
Small-conductance Ca^{2+}-activated K^+ channel ($K_{Ca}2.2$)	AP10032PU-N	Goat (gt)	1:400	Abcam
Small conductance Ca^{2+}-activated K^+ channels ($K_{Ca}2.3$)	ab83737	Rabbit (rb)	1:300	Abcam
High voltage-activated Ca^{2+} (L-type) channels ($Ca_v\alpha_2\delta$)	ab2864	Mouse (ms)	1:50	Abcam
G protein-coupled inwardly rectifying K^+ channels ($K_{ir}3.1$)	ab61191	Rabbit (rb)	1:500	Abcam
Secondary antibodies				
Alexa Fluor 488 anti-rb	A-21206	Donkey	1:1500	Molecular Probes
Alexa Fluor 568 anti-ms	A-10037	Donkey	1:1500	Molecular Probes
Alexa Fluor 633 anti-gt	A-21082	Donkey	1:1500	Molecular Probes

The SA node region is found in close proximity to subepicardial sinus node artery (arrow in Figure 1B). SA node is characterized by a large number of neurofilament 160 (NF-160) positive neuronal fires (Dobrzynski et al., 2005) and small-size cardiomyocytes that are negative against connexin-43 (Cx43) staining, a gap junction protein ubiquitously expressed in the heart apart from in nodal tissue (van Kempen et al., 1991) (Figure 4C). Cells within the SA node are surrounded by dense connective tissue; collagen fibers (light blue staining) can be differentiated from cardiomyocytes (red staining) with the Masson's trichrome staining (Figure 4B). Vimentin was used as a fibroblast-cell marker in immunofluorescence confocal microscopy studies (see inserts in Figure 4E). Semi-quantification of immunofluorescence signals was performed using the FluoView software; at least three images per section of the right atrium and the SA node obtained with a 600X magnification (640 × 640 pixels resolution) were processed. The images were stored in TIFF format with the same resolution and, subsequently, analyzed in 3D Objects Counter plugin for ImageJR software version 1.50d (National Institutes of Health). This plugin allowed us to measure the integrated pixel density of automatically generated regions of interest (ROI). The average integrated pixel density of all generated ROIs of each image was used to estimate brightness intensity of the specific immunolabelling. Fluorescence intensity ratio for each pair of sections was used as a semi-quantitative approach to evaluate the relative expression of receptors and ionic channels in the right atrium and the SA node. Of note, the acquisition settings on the confocal microscope were kept constant in all optical sections of the right atrium and the SA node from the same animal. Representative images of immunofluorescence staining were used to create tridimensional models representing the immunoreactivity intensity by means of the Interactive 3D surface plot v2.33 plugin for ImageJ. Plugin settings were kept constant in all tridimensional

assemblies. The peak height and color represent the magnitude of immunoreactivity intensity. One researcher conducted all semi-quantitative image analysis blindly.

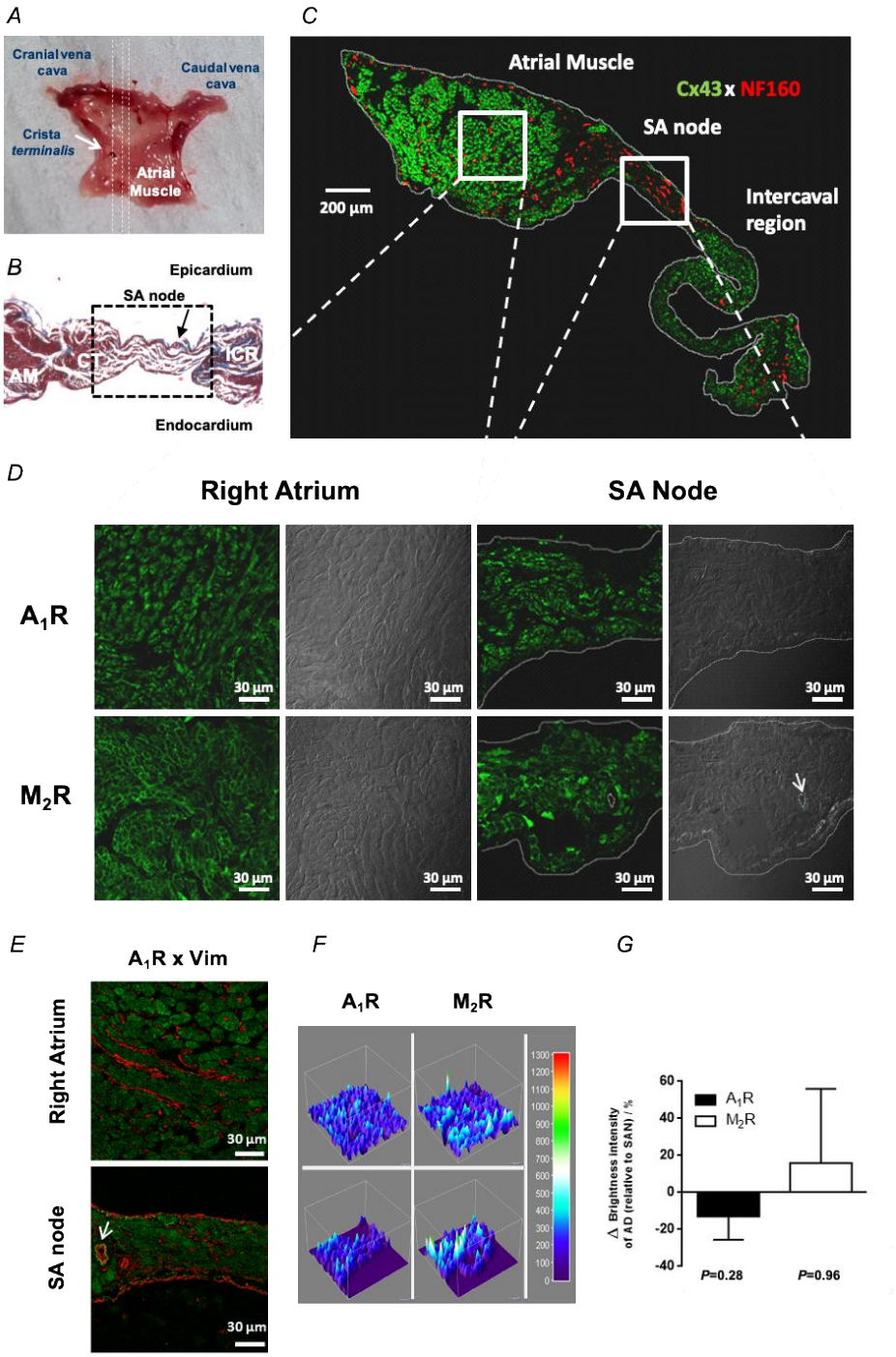

Figure 4. Anatomical and molecular identification of the rat SA node (A–C). (A) Endocardial view of a typical rat atrial muscle-SA node preparation. Dashed lines indicate the orientation of cuts performed on the right atrial appendage for Masson's trichrome staining and immunofluorescence analysis. (B) High-magnification (400x) images of Masson's trichrome staining of SA node (dashed box) and surrounding regions; AM, atrial muscle; CT, crista terminalis; ICR, intercaval region. Red staining, myocytes; blue staining, connective tissue. Black arrow indicates the SA node artery. (C) Montage of confocal optical sections showing immunolabelling for connexin 43 protein (Cx43, green signal) and neurofilament 160 (NF160, red signal) throughout the right atrial appendage from the rat. Boxes depict representative areas of the right atrium muscle and SA node used for

immunolocalization of receptors and ion channels. (D) Representative confocal micrographs of the rat SA node and neighboring atrial muscle showing immunofluorescence labeling of A_1 (upper panels) and M_2 (lower panels) receptors; the corresponding differential interference contrast (DIC) images are also shown for comparison. (E) Please note that immunolabelling of A_1 receptors (green) did not co-localize with vimentin (Vim) staining (red), which was used as a fibroblast-cell marker. Similar results were obtained in six additional experiments. The white arrow indicates the SA node artery. (F) Tridimensional surface modeling representing immunoreactivity of images depicted in panel (D). Color bar represents relative fluorescence intensity map. (G) Graph depicting semi-quantitative analysis of A_1R and M_2R expression in right atria and SA node; the ordinates are immunofluorescence intensity ratio between A_1R and M_2R staining in paired samples from the right atrium and SA node keeping the image acquisition settings constant. Positive and negative values indicate staining predominance in contractile myocardium and SA node of the right atrium, respectively. Values are mean ± SEM; at least 3 microscopic fields were analyzed per section of the right atrium and SA node obtained from three to seven rats. $P > 0.05$ indicates that no significant differences were found in the expression of A_1R and M_2R between the two atrial regions.

2.7. Solutions and chemicals

Adenosine, 4-aminopyridine (4-AP), apamin, 2,3-butanedione monoxime, 2-p-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine (CGS21680C), 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX), nifedipine, propranolol, protease XIV, **R**-(-)-N^6-(2-phenylisopropyl)adenosine (R-PIA) were from Sigma-Aldrich (St. Louis, MO, USA); AF-DX 116, oxotremorine sesquifumarate, mibefradil and verapamil were from Tocris Cookson Inc. (Bristol, UK); glibenclamide and tertiapin Q were from Ascent Scientific (Bristol, UK); Dimethylsulfoxide (DMSO), serum albumin and Triton X-100 were from Merck (Darmstadt, Germany); collagenase II was from Worthington Biochemical Corp (Lakewood, NJ, USA). AF-DX 116 and glibenclamide were made up in DMSO stock solution. DPCPX was made up in 99% DMSO/1% NaOH 1 mmol L^{-1} (v/v). R-PIA, verapamil and nifedipine were made up in ethanol; these solutions were kept protected from the light to prevent photodecomposition. Other drugs were prepared in distilled water. All stock solutions were stored as frozen aliquots at -20ºC. Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used (0.5% v/v), were observed. The pH of the superfusion solution did not change by the addition of the drugs in the maximum concentrations applied to the preparations.

2.8. Presentation of data and statistical analysis

The isometric contractions were recorded and analyzed before and after the addition of each drug at the desired concentration. Results were presented as percentages of variation compared to baseline, obtained before the administration of the evaluated drug. Concentration-response curves were fitted by non-linear regression using GraphPad Prism 5.04 software (La Jolla, CA, USA) function: log(inhibitor) vs. response. We assumed that both data share best-fit values for top and bottom and a Hill slope equal to 1; in the case of

drugs (e.g., R-PIA) showing an intermediate increase in inotropy, we considered that data share best-fit values for bottom (constrained to −100%) in order to estimate pIC50 values. Fitting used the least squares method. The data are expressed as mean ± SEM, with n indicating the number of animals used for a particular group of experiments. With the exception of patch-clamp experiments using acutely dissociated atrial cardiomyocytes, each rat was used to test only one pair of drug and antagonist/inhibitor. Comparison between concentration-response curves obtained in the absence and in the presence of a receptor antagonist or ion channel inhibitor was performed using two-way ANOVA followed by the Sidak's multiple comparison test. Individual pairs of data were compared using paired Student's t-test when parametric data was considered. The means of unmatched groups were compared using unpaired Student's t-test with Welch's correction when parametric data was considered. For multiple comparisons, one-way ANOVA followed by Dunnett's modified t-test was used. $P < 0.05$ (two-tailed) values were considered to show significant differences between means.

3. Results

3.1. Adenosine acting via A_1 receptors is a chronoselective atrial depressant as compared to the muscarinic M_2 receptor agonist, oxotremorine

Figure 5 shows that activation of adenosine A_1 and muscarinic M_2 receptors with R-PIA (0.001–1 μM) and oxotremorine (0.003–3 μM), respectively, decreased in a concentration-dependent manner the rate (negative chronotropic effect) and the force (negative inotropic effect) of spontaneous contractions of rat atria. The negative chronotropic effect of R-PIA (pIC_{50} 7.26 ± 0.04) was evidenced at much lower ($P < 0.01$) concentrations than its negative inotropic action (pIC_{50} 6.14 ± 0.07) (Figure 5Di), whereas oxotremorine-induced depression of both rate and tension of spontaneously beating atria was observed in the same concentration range (pIC50 6.97 ± 0.03 and 6.81 ± 0.10, respectively; $P > 0.05$) (Figure 5Ei).

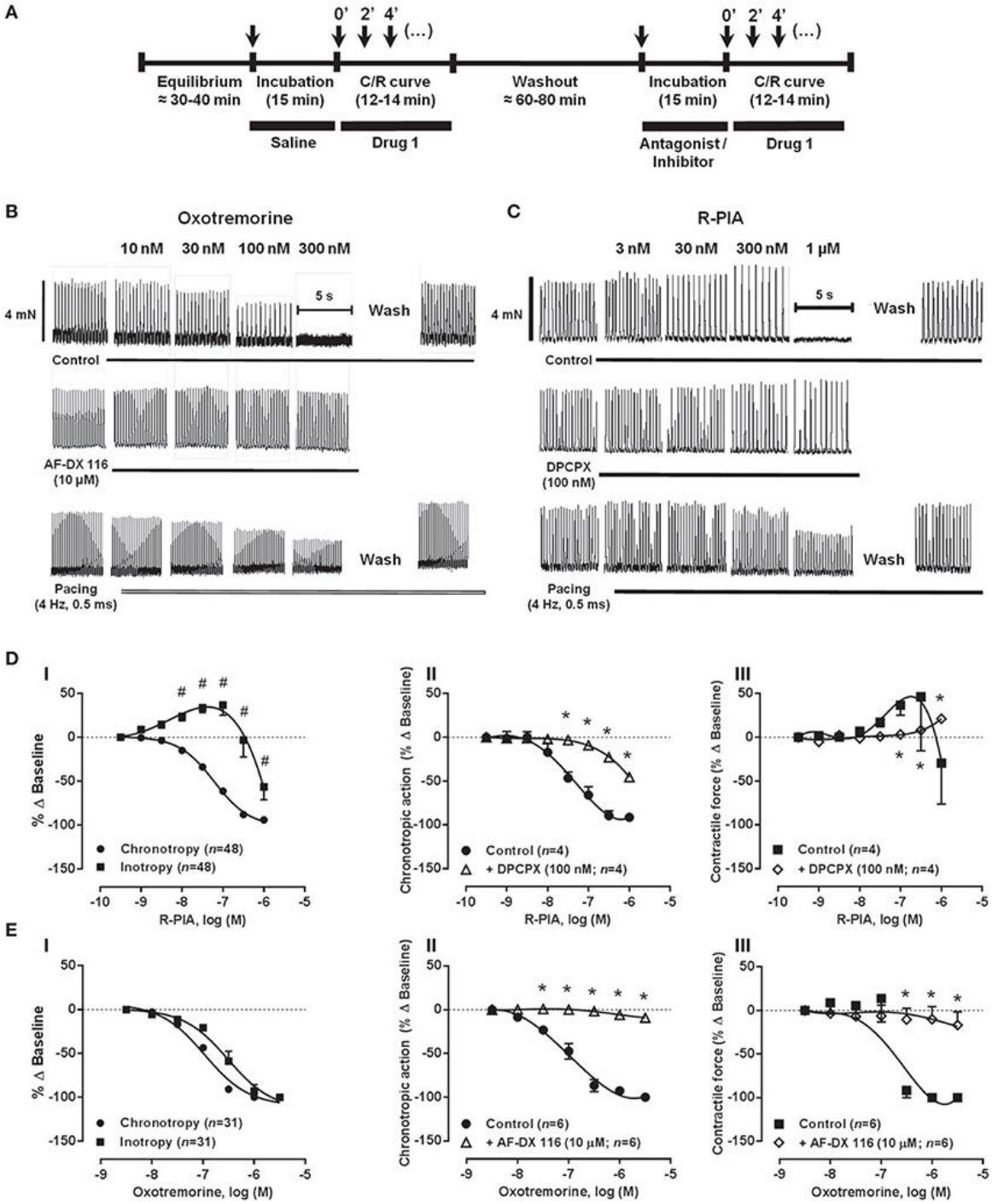

Figure 5. Schematic representation of the protocol used for drug applications **(A).** Concentration-response C/R curves of oxotremorine (**B,E**) and R-PIA (**C,D**) on the spontaneously beating rat atria in the absence (Control) and in the presence of selective M2 (AF-DX 116) and A1 (DPCPX) receptor antagonists. The effects of oxotremorine and R-PIA in rat atria electrically-paced at a constant frequency of 240 beats per min (4 Hz) are also shown for comparison (spontaneous atrial rate in control conditions was 218 ± 4 beats.min−1, n = 10). Oxotremorine (0.003–3 µM) and R-PIA (0.001–1 µM) were applied once every 2 min at increasing concentrations; AF-DX 116 (10 µM) and DPCPX (100 nM) were added to the incubation fluid 15 min before application of oxotremorine or R-PIA. The ordinates are percentage of variation of spontaneous contraction rate (chronotropic effect, ii) and mechanical tension (inotropic effect, iii) compared to baseline values obtained before application of the corresponding agonist. The data are expressed as mean ± SEM from an n number of individual experiments. $^{\#}P < 0.05$ compared with agonist-induced percentage of baseline variation on chronotropy; *P < 0.05 compared with the effect of oxotremorine or R-PIA in the absence of receptor antagonists AF-DX 116 and DPCPX, respectively.

Adenosine (0.001–3 mM) mimicked the negative chronotropic and inotropic effects of the full A_1 agonist, R-PIA (0.001–1 µM), but the effect of the natural nucleoside was 4 log units less potent than R-PIA (Figure 6).

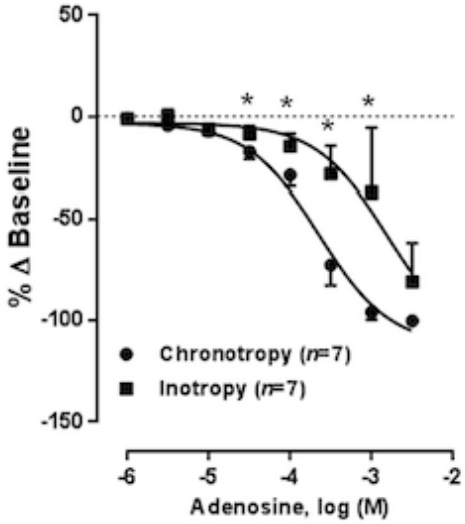

Figure 6. Concentration-response curves of adenosine on rate (chronotropy) and mechanical tension (inotropy) of spontaneously beating atria. Adenosine (0.001–3 mM) was applied once every 2 min at increasing concentrations. The ordinates are percentage of variation from baseline spontaneous contractions. The data are expressed as mean ± SEM from an *n* number of individual experiments. $^{*}P < 0.05$ compared with adenosine-induced percentage of baseline variation on chronotropy.

Reduction of atrial rate produced by adenosine (pIC_{50} 3.68 ± 0.09) was preferential compared to its ability to decrease tension of spontaneously beating atria (pIC_{50} 2.60 ± 0.16, n = 9, P < 0.02 vs. negative chronotropy). Unlike adenosine, R-PIA transiently increased (P < 0.05) atrial contractile force when applied in 0.03 and 0.1 µM concentrations (Figure 2Di). Likewise, the selective adenosine A_{2A} receptor agonist, CGS21680C, produced a mild positive inotropic effect (maximal increase, ~18% at 300 nM) on spontaneously beating rat atria, when this compound was applied in a similar concentration range (0.003–1 µM) as R-PIA (n = 5, data not shown) (see also (Dobson and Fenton, 1997)). These findings suggest that the A_{2A} receptor has limited importance in the response to adenosine in the isolated spontaneously beating rat atria, yet it may contribute to increase atrial contractile force when significant negative chronotropism concurrently occurs as observed with R-PIA (see Figure 2Di).

Figures 5Dii,iii show that selective blockade of adenosine A_1 receptors with DPCPX (100 nM), significantly shifted to the right the concentration-response curves for R-PIA (0.001–1 µM) in the spontaneously beating rat atria. The blocking effect of DPCPX was dependent on the concentration (2.5, 10, and 100 nM, n = 4−6), thus indicating that the A_1 receptor must be the dominant receptor involved in the negative chronotropic and inotropic actions of R-PIA. Likewise, data from Figures 5Eii,iii show that depression of rate and

tension of spontaneous atrial contractions caused by oxotremorine (0.003–3 μM) were both completely prevented by pre-incubation with the muscarinic M_2 receptor antagonist, AF-DX 116 (10 μM). Immunolocalization studies showed that A_1 and M_2 receptors are evenly expressed in SA node and atrial cardiomyocytes (Figures 4D,F,G), thus indicating that differences in the receptors regional distribution do not account for adenosine chronoselectivity in spontaneously beating rat atria. A yet unknown postreceptor transduction mechanism to explain the negative chronotropic supersensitivity to adenosine in the dog heart has been hypothesized (Oguchi et al., 1995). Figure 7 shows that adenosine negative chronoselectivity might depend on potassium ionic currents since it was significantly ($P < 0.05$) reduced by raising the extracellular concentration of KCl from 2.7 to 4.7 mM (see e.g., (De Biasi et al., 1989)). Under these conditions, the negative inotropic effect of R-PIA (Figure 7D) became evident at the same concentration range (0.003–1 μM) as that needed to slow down atrial rate (Figure 7C), yet no changes were detected in the atrial effects caused by the muscarinic receptor agonist, oxotremorine (0.003–3 μM) (Figures 7A,B).

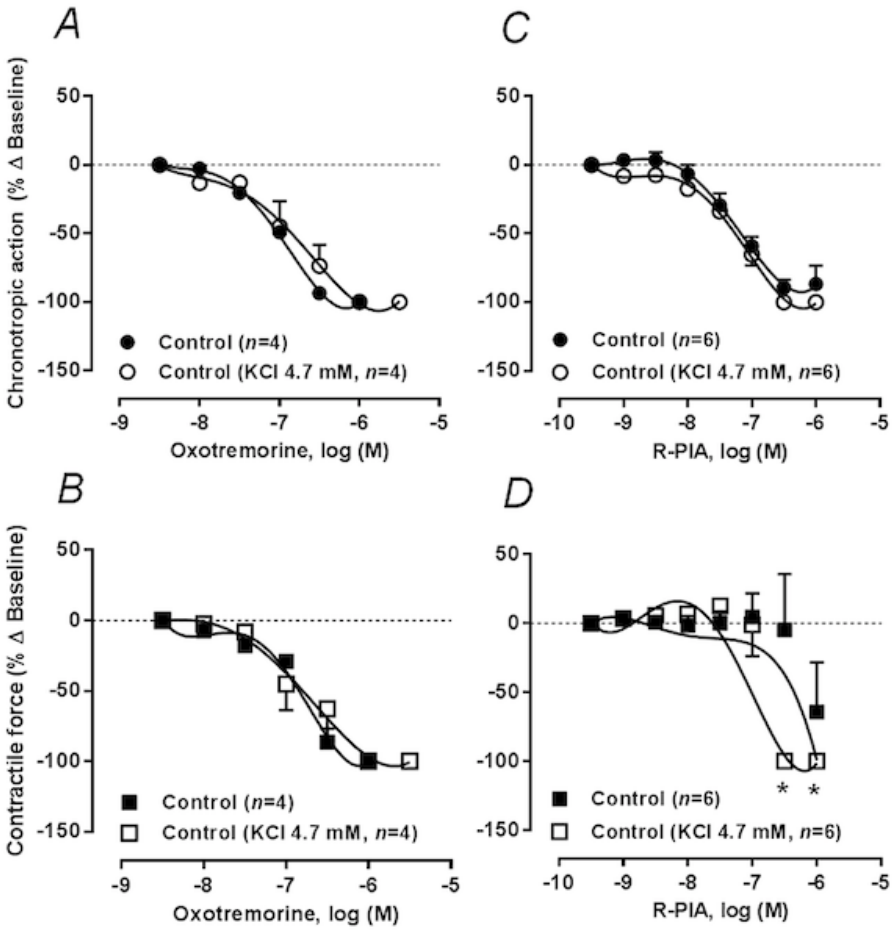

Figure 7. Effect of increasing the extracellular concentration of potassium from 2.7 (Control) to 4.7 mM on the negative chronotropic and inotropic effects of oxotremorine (A,B) and R-PIA (C,D) on spontaneously beating rat atria. The ordinates are percentage of variation of spontaneous

contraction rate (chronotropic effect, A,C) and mechanical tension (inotropic effect, B,D) compared to baseline values obtained before increasing the KCl concentration to 4.7 mM. Data are expressed as mean ± SEM from an *n* number of individual experiments. $^{*}P < 0.05$ compared with the effect of oxotremorine or R-PIA in control conditions.

3.2. Blockage of G protein-coupled inwardly rectifying K^+ (GIRK/K_{IR}) channels, but not of K_v and K_{ATP}/K_{IR}6 channels, counteracts atrial depression caused by A_1 and M_2 receptor agonists

Figures 8 and 9 show the concentration-response curves of R-PIA (0.001-1 μM) and oxotremorine (0.003-3 μM), respectively, in the spontaneously beating rat atria obtained in the absence (control) and in the presence of subtype-specific K^+ channel blockers.

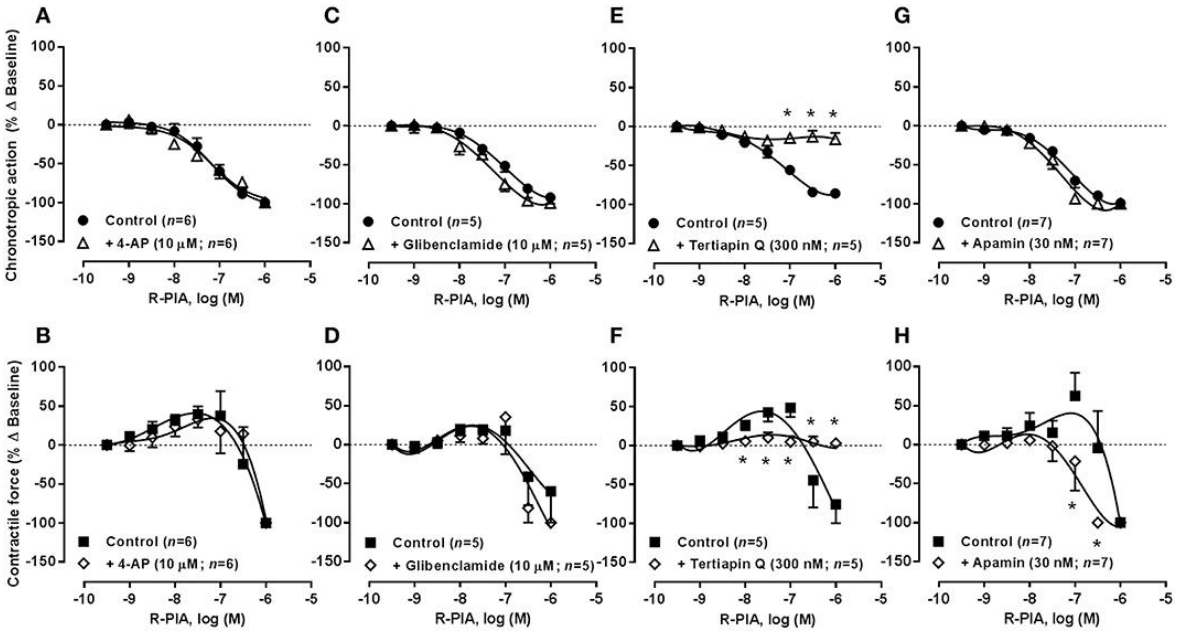

Figure 8. Concentration-response curves of R-PIA (0.001–1 μM) on the spontaneously beating rat atria in the absence (Control) and in the presence of potassium channels blockers: 4-AP (10 μM, A,B), glibenclamide (10 μM, C,D), tertiapin Q (300 nM, E,F) and apamin (30 nM, G,H). Drug applications followed the protocol depicted in Figure 5A. The ordinates are percentage of variation of spontaneous contraction rate (chronotropic effect, A,C,E,G) and mechanical tension (inotropic effect, B,D,F,H) as compared to baseline values obtained before application of R-PIA. The data are expressed as mean ± SEM from an n number of individual experiments. *P < 0.05 compared with the effect of R-PIA in the absence of potassium channel blockers.

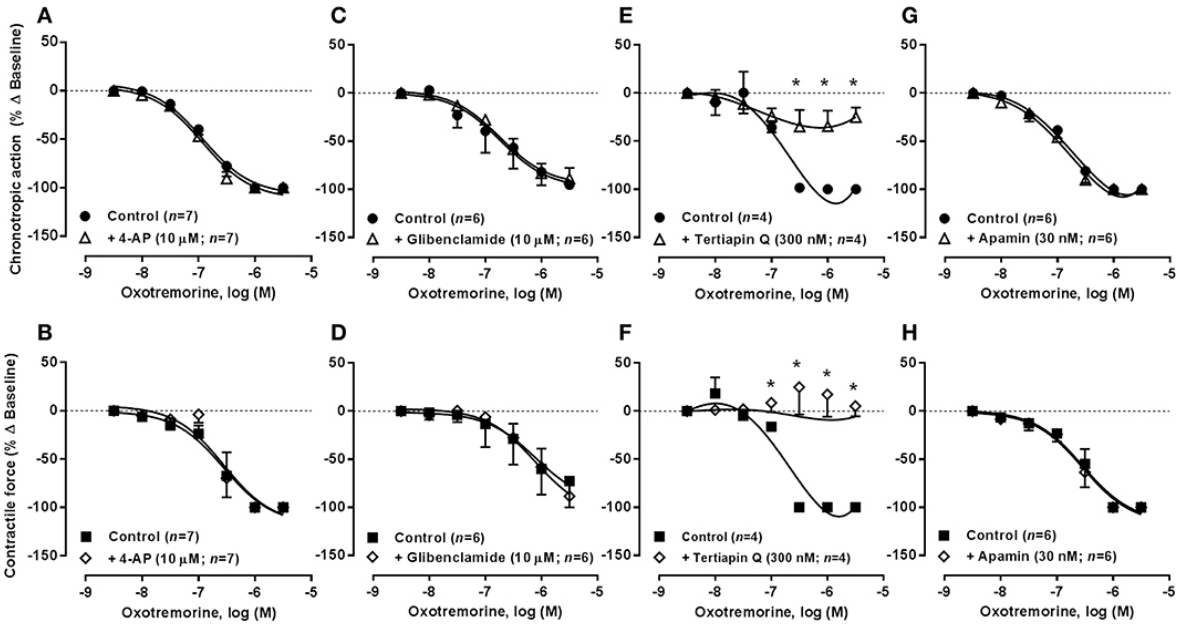

Figure 9. Concentration-response curves of oxotremorine (0.003–3 μM) on the spontaneously beating rat atria in the absence (Control) and in the presence of potassium channels blockers: 4-AP (10 μM, A,B), glibenclamide (10 μM, C,D), tertiapin Q (300 nM, E,F), and apamin (30 nM, G,H). Drug applications followed the protocol depicted in Figure 5A. The ordinates are percentage of variation of spontaneous contraction rate (chronotropic effect, A,C,E,G) and mechanical tension (inotropic effect, B,D,F,H) as compared to baseline values obtained before application of oxotremorine. The data are expressed as mean ± SEM from an n number of individual experiments. *P < 0.05 compared with the effect of oxotremorine in the absence of potassium channel blockers.

As observed in the guinea-pig atria (De Biasi et al., 1989), blockade of voltage-gated K_v and ATP-sensitive K_{ATP}/K_{IR}6 channels respectively by 4-aminopyridine (4-AP, 10 μM) and glibenclamide (10 μM), respectively, had no significantly (*P*>0.05) effects on the negative chronotropic and inotropic actions of R-PIA and oxotremorine. Similar results were obtained when the concentration of 4-AP was increased from 10 to 100 μM (*n*=6). On their own, 4-AP (0.01-3 mM, *n*=5) and glibenclamide (1-100 μM, *n*=4) increased atrial inotropism (to a maximum of 40% above control) in a concentration-dependent manner, without affecting the rate of spontaneous atrial contractions; when used at a 10 μM concentration, these inhibitors raised the force of atrial contractions by no more than 25% (see Table 4). The positive inotropic effects of 4-AP and glibenclamide were prevented by blocking β-adrenoceptors with propranolol (10 μM), agreeing with the hypothesis that these drugs may depolarize cardiac sympathetic nerve terminals favoring endogenous noradrenaline release, which may be responsible for the increase in the force of atrial contractions (data not shown).

In contrast to the voltage-gated K^+ channels, inwardly rectifier potassium channels (K_{ir}) are more permeable to K^+ during hyperpolarization than during depolarization. Activation of G protein-coupled inwardly rectifying K^+ channels (GIRK or K_{IR} 3.1/3.4) by acetylcholine hyperpolarizes the resting membrane potential, thereby reducing the

probability of action potential generation, while contributing to shorten atrial action potentials and the effective refractory period (ERP). Tertiapin Q (300 nM), a blocker of GIRK/K_{IR} channels with high affinity for K_{IR} 3.1/3.4 channels (Kodama et al., 1996; Yamada, 2002), prevented the inhibitory effects of R-PIA and oxotremorine on spontaneous atrial contractions (Figures 8D and 9D, respectively). Application of Tertiapin Q did not affect spontaneous atrial contractions when this drug was applied alone in the 0.03 to 1 μM concentration range (*n*=4, see Table 4), thus confirming that atrial rate (chronotropic effect) and contractile force (inotropic effect) is not under the control of adenosine and acetylcholine endogenous tonus in the present experimental conditions ((Han et al., 2010); but see e.g., (Wang et al., 2013)).

3.3. Blockage of $K_{Ca}2$/SK channels with apamin sensitized atria to the negative inotropic action of R-PIA, but failed to affect oxotremorine-induced atrial depression

SK channels (small conductance calcium-activated potassium channels) are a subfamily of Ca^{2+}-activated K^+ channels. Their activation limits the firing frequency of action potentials and is important for regulating after hyperpolarization in many types of electrically excitable cells. This is accomplished through the hyperpolarizing leak of positively charged potassium ions along their concentration gradient into the extracellular space. Blockade of Ca^{2+}-activated $K_{Ca}2$/SK channels with apamin (30 nM) shifted to the left (P<0.05) the concentration-response of R-PIA (0.001-1 μM) regarding the negative inotropic component of the A_1 receptor action (pIC_{50} 7.05 ± 0.23, n = 7, P < 0.05 vs. control) (Figure 8H), without much affecting the action of the nucleoside on the rate of atrial rate (pIC_{50} 7.48 ± 0.09, n = 7, P > 0.05 vs. control) (Figure 8G). That is, pre-treatment with apamin (30 nM) sensitized atria to the negative inotropic effect of R-PIA in a way that the negative chronotropic and inotropic actions of the A_1 receptor agonist became evident in the same range of concentrations (pIC_{50} 7.48 ± 0.09 and 7.05 ± 0.23 for chronotropism and inotropism, respectively, n = 7, P > 0.05) (see above). Coincidently, a similar result was obtained upon reducing the K^+ gradient across the plasma membrane by raising the extracellular concentration of KCl from 2.7 to 4.7 mM (see Figure 7C,D). These findings suggest that the bradycardic and the negative inotropic actions of adenosine may be dissociated in terms of the intracellular mechanisms being involved. Contrariwise, apamin (30 nM), as well as changes in the extracellular concentration of KCl, failed to affect the depressant effects of oxotremorine (0.003-3 μM) on spontaneously beating rat atria under the same experimental conditions (Figure 9G,H; see also Figure 7A,B).

Figure 10 confirms that both SA node and atrial cardiomyocytes exhibit immunoreactivity against K_{IR}3.1, K_{Ca}2.2, K_{Ca}2.3, and Ca_v1 channels. Differences are evident

in the distribution of apamin-sensitive $K_{Ca}2.2$ and $K_{Ca}2.3$ channels, being the latter more abundant in the SA node compared to atrial cardiomyocytes, while the opposite was observed regarding the $K_{Ca}2.2$ channel.

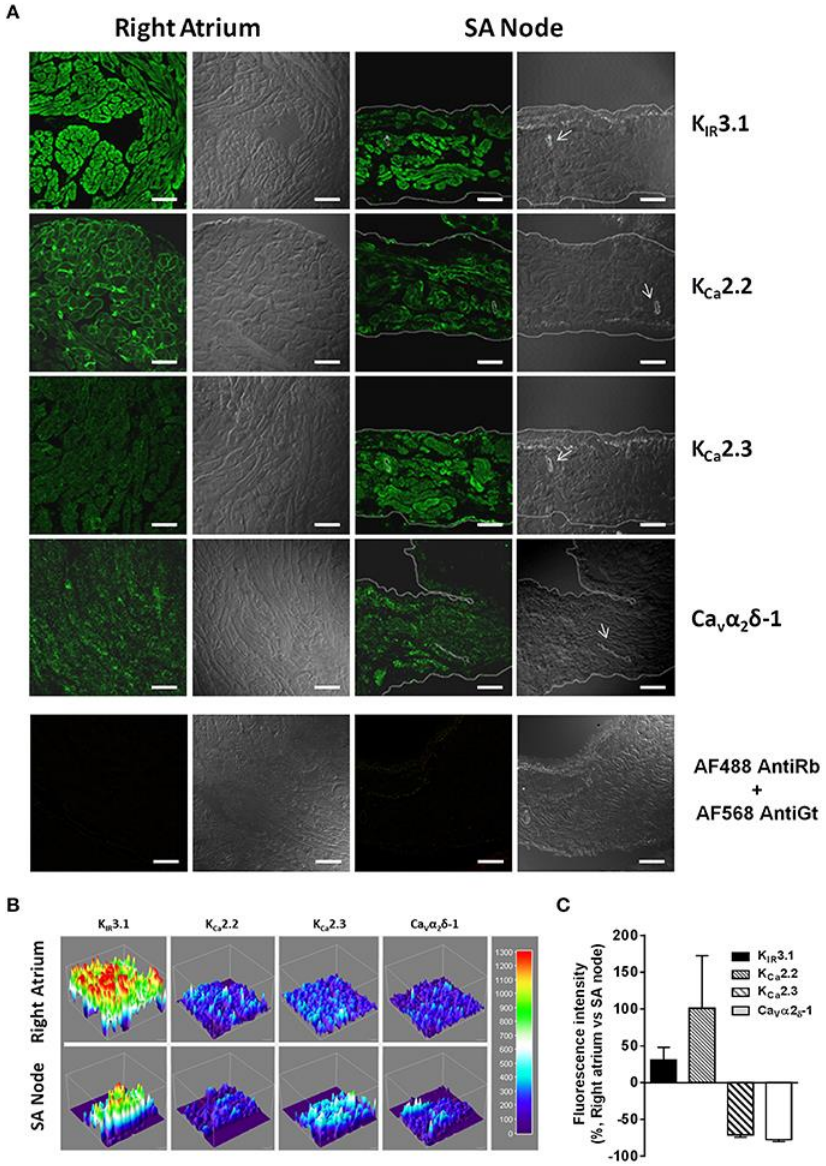

Figure 10. Representative confocal micrographs of rat right atrium and SA node sections showing immunofluorescence labeling for $K_{IR}3.1$ (GIRK1), $K_{Ca}2.2$ (SK2), $K_{Ca}2.3$ (SK3), and $Ca_V\alpha_{2\delta-1}$ channel subunits (first and third columns); the corresponding differential interference contrast (DIC) images are also shown for comparison (second and fourth columns) (A). Last row shows cross-reactivity of secondary antibodies, Alexa Fluor 488 anti-rabbit (AF488 anti-Rb), and Alexa Fluor 568 anti-goat (AF568 anti-Gt), in which primary antibodies were omitted (see Table 5). During documentation the settings on the confocal microscope were adjusted appropriately to show immunoreactivity for sections containing both primary and secondary antibodies and these settings were maintained when documenting cross-reactivity of secondary antibodies ran in parallel to minimize biases during capture and printing of digital images. White arrows indicate SA node arteries. Similar results were obtained in five additional experiments. Horizontal bar = 30 μm. (B) Tridimensional surface modeling representing immunoreactivity of images depicted in panel (A). Color bar represents relative fluorescence intensity map. (C) Graph depicting semi-quantitative analysis of $K_{IR}3.1$, $K_{Ca}2.2$, $K_{Ca}2.3$, and $Ca_V\alpha_{2\delta-1}$ expression in right atria and SA node; the ordinates are immunofluorescence intensity ratio between $K_{IR}3.1$, $K_{Ca}2.2$, $K_{Ca}2.3$, and $Ca_V\alpha_{2\delta-1}$ staining in paired samples from the right atrium

and SA node keeping the image acquisition settings constant. Positive and negative values indicate staining predominance in contractile myocardium and SA node of the right atrium, respectively. Values are mean ± SEM; at least 3 microscopic fields were analyzed per section of the right atrium and SA node obtained from three to five rats.

High magnifications of these confocal micrographs counterstained with DAPI (nuclear dye) are shown in Figures 11 and 12. Results show that although these ion channels exhibit a mixed membranar and cytosolic pattern, ion channels specific immunostaining was absent from the nucleus. The lack of more convincing sarcolemmal staining, except for the $K_{Ca}2.2$ channel, is a limitation of the present study. Intracellular immunostaining pattern of highly expressed plasma membrane proteins is often seen in fixed cells, as primary antibodies can enter permeabilized cells and bind to target proteins localized in the Golgi during their trafficking to the plasma membrane or in caveolae and/or endosomes when subject to recycling from the plasma membrane, as part of membrane plasticity phenomena. Fluorescent immunostaining of receptors and ion channels may also be differently distributed at the periphery of myocytes and in transverse tubule (T-tubule) invaginations of the sarcolemmal membrane, both in living and permeabilized cardiomyocytes (Balijepalli et al., 2003). Prominent T-tubule-staining pattern can be recognized as fine fluorescent spots inside cardiac myocytes. The functional significance of intracellular localization of plasma membrane receptors and ion channels certainly deserves future investigations, which are beyond the scope of this study.

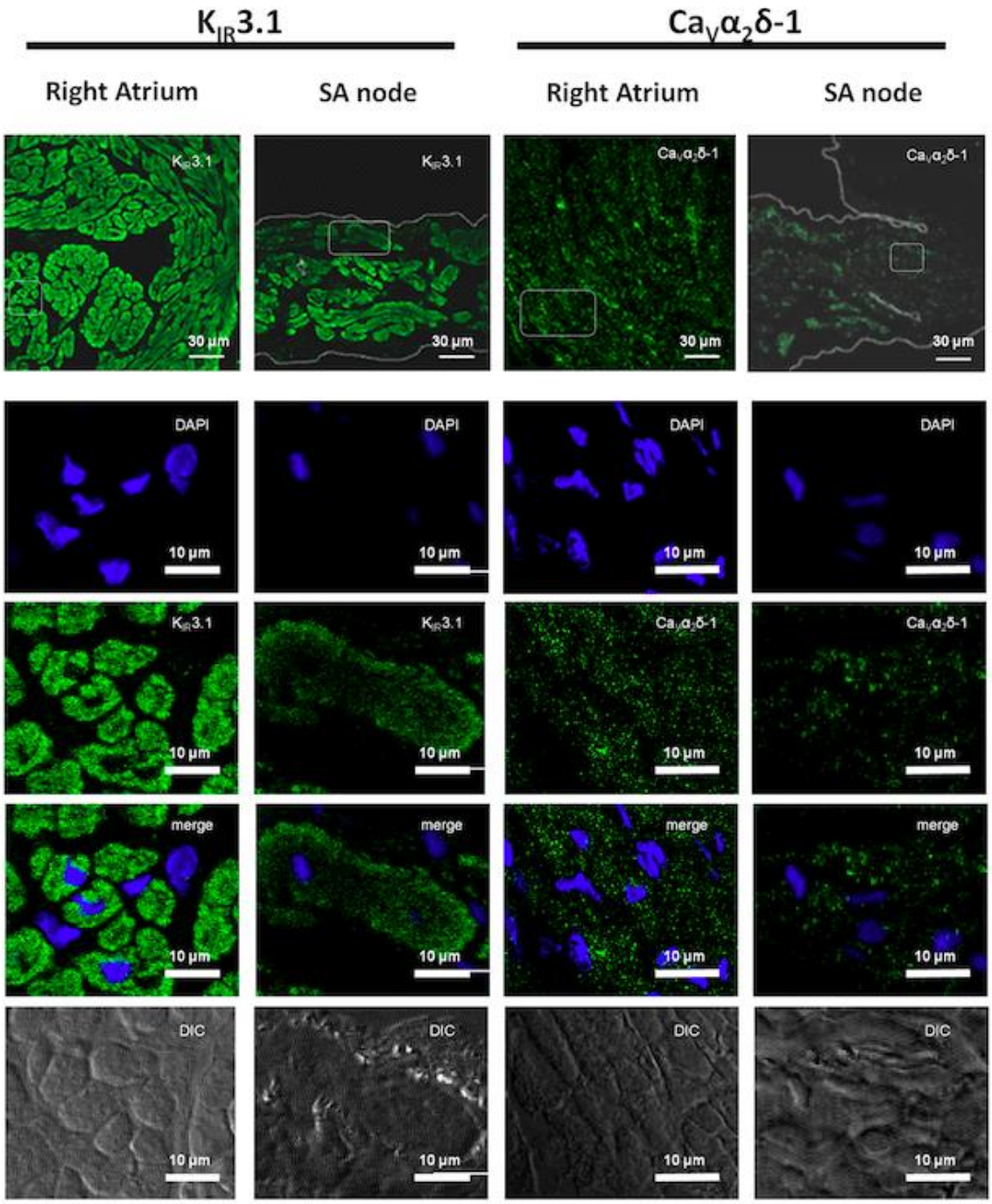

Figure 11. Higher-magnification representative confocal micrographs of rat right atrium and SA node sections shown in Figure 10. Immunofluorescence stainings for $K_{IR}3.1$ (GIRK1) and $Ca_V\alpha_{2\delta-1}$ channel subunits appear in green. Cell nuclei are stained in blue with DAPI. The corresponding differential interference contrast (DIC) images are also shown for comparison (last row). Similar results were obtained in five additional experiments.

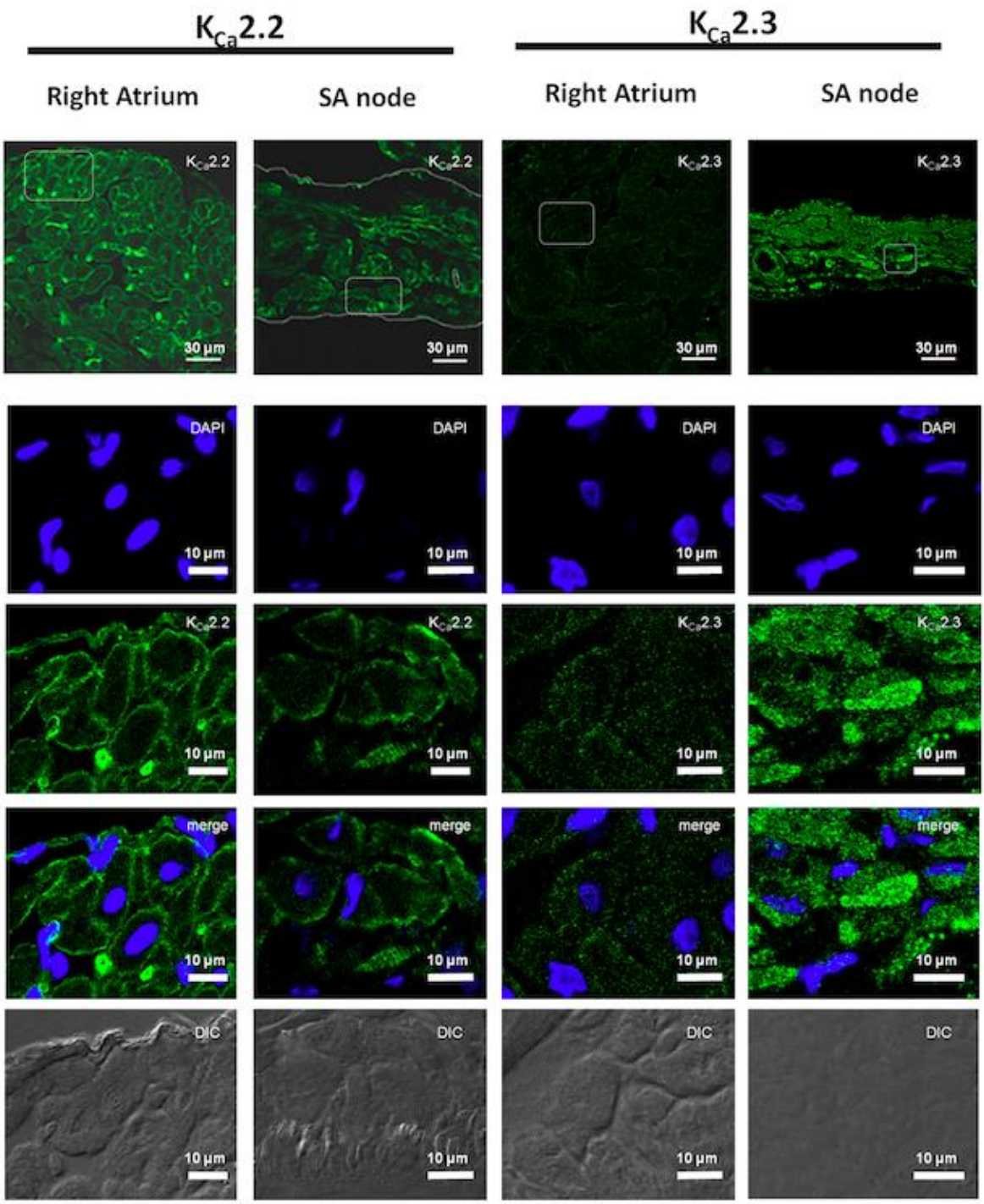

Figure 12. Higher-magnification representative confocal micrographs of rat right atrium and SA node sections shown in Figure 10. Immunofluorescence stainings for KCa2.2 (SK2) and KCa2.3 (SK3) channel subunits appear in green. Cell nuclei are stained in blue with DAPI. The corresponding differential interference contrast (DIC) images are also shown for comparison (last row). Similar results were obtained in five additional experiments.

3.4 Isolated atrial cardiomyocytes exhibit a delayed outward K^+ current that is dependent on Ca^{2+} influx through Ca_V1 (L-type) channels

The functionality of small conductance Ca^{2+}-activated $K_{Ca}2$/SK outward channels were tested by performing voltage-clamp experiments in acutely dissociated rat atrial cardiomyocytes using the whole-cell patch-clamp configuration. To evaluate the current-voltage dependence, we covered a wide range of potentials by setting 10 mV steps (of 260 ms) from −130 to +60 mV, while keeping the holding potential at −70 mV (Figure 13A). The corresponding voltage-intensity curves revealed three main components (Figures 13Ai,ii). An obvious inward rectifying component was resolved in the range of −130 to −70 mV. From −50 to 0 mV, we detected an inward "hump," whereas a typical delayed outward K^+ current was observed above +10 mV. Unfortunately, this protocol could only be applied a limited number of times, as patch seals tend to break down due to contraction of cardiomyocytes particularly at the most depolarized steps, thus precluding further pharmacological manipulations. The use of cardioplegic drugs, such as 2,3- butanedione monoxime, after the isolation procedure was out of the question as it affects several ion channels. Lowering Ca^{2+} beyond a certain threshold and/or increasing the cell Ca^{2+} buffering capacity was also disadvantageous because it could preclude investigation of Ca^{2+}-activated K^+ currents. Hence, to investigate outward K^+ currents over long periods of time as required in this study (see below), a less abrasive single voltage step (to +40 mV) was performed (Figure 13B). This protocol consisted in a brief depolarization (50 ms) to −10 mV aiming at Ca^{2+} influx through voltage-activated channels, which was immediately followed by a longer depolarizing step (750 ms) to +40 mV. This was used because the resolution of the outward current was greater when preceded by a brief depolarizing step to −10 mV as compared to the situation where the voltage of preceding pulses was raised to −40 mV or to +20 mV; the same trend was observed in cells from three different animals. Moreover, the outward K+ current recorded at +40 mV was greater if the duration of the prepulse (to −10 mV) was increased (data not shown; see e.g., (Marrion and Tavalin, 1998)).

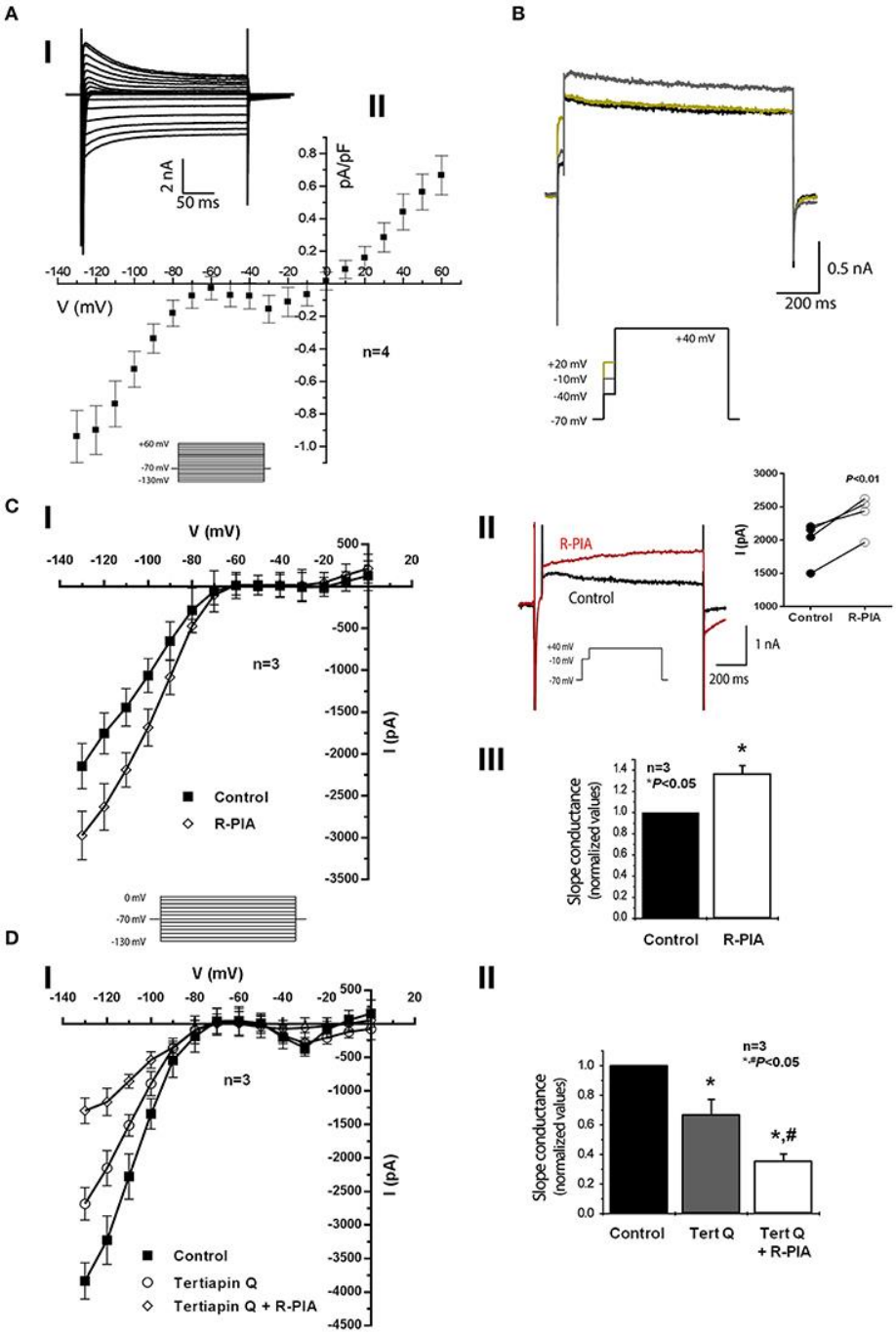

Figure 13. Effects of adenosine A_1R activation on whole-cell voltage-clamp recordings in rat atrial myocytes. (Ai) Representative currents following a set of voltage pulses (260 ms), covering a wide range of potentials, with incremental depolarization steps (10 mV) from −130 to +60 mV (holding voltage −70 mV) (see inset). (Aii) Corresponding current density-voltage relationship showing three different components in terms of voltage dependence: a strong inward rectifier, an inward "hump," and a delayed outward current. Data are expressed as mean ± SEM of four animals; recordings from three to five isolated atrial cardiomyocytes were averaged per experimental animal. (B) Representative current traces from a triple set of double depolarizing pulses: one first step to −40, −10, and +20 mV, lasting 50 ms, followed by a second pulse to +40 mV, lasting 750-ms (see inset). One can notice a larger outward current at +40 mV when preceded by a prepulse to −10 mV, which suggests a Ca2+-dependent current component. Panels (C,D) show current-voltage relationships obtained from currents recorded following a set of voltage steps (−130 to 0 mV, 10 mV steps, holding voltage −70 mV, 260 ms duration each) in the absence (Control) and in the presence of R-PIA (300 nM, Ci) and tertiapin Q (300 nM, Di) with or without R-PIA (300 nM). Data are expressed as mean ± SEM of three animals; recordings from four to five isolated atrial cardiomyocytes were averaged per

experimental animal. Panel (Cii) shown are typical recording traces showing that activation of the adenosine A1 receptor with R-PIA (300 nM) increases the Ca2+-dependent outward current obtained following application of the double-pulse protocol consisting of one pre-pulse of 50 ms duration to −10 mV immediately followed by a second pulse to +40 mV lasting 750-ms (see inset). This experiment was repeated using four cardiomyocytes isolated from three different animals (right-hand side panel); *P < 0.01 (paired Student's t-test) represent significant differences from control. (Ciii) Refers to normalized values of slope conductance (calculated with measurements of −120 to −80 mV) in cells obtained from three different animals in the absence (Control) and in the presence of R-PIA (300 nM). Panel (Dii) shows similar experiments as (Ciii), but in this case tertiapin Q (300 nM) with or without R-PIA (300 nM) was used instead of R-PIA alone. Error bars represent SEM of three animals. *,#P < 0.05 (unpaired Student's t-test with Welch's correction) represent significant differences from control or from tertiapin Q alone, respectively.

The inward current “hump” detected in the current-voltage relationship peaking at −30 and −10 mV (Figure 13A) suggests the activation of voltage-gated calcium channels allowing Ca^{2+} influx mainly through high-voltage Cav1 (L-type) channels (Grant, 2009). Even though we did not perform experiments to directly evaluate calcium dynamics, it is likely that activation of voltage-activated calcium channels may account for the inward current “hump” observed here. Thus, Ca^{2+} recruitment through voltage-gated channels, most probably via high-voltage Cav1 (L-type) channels, ensures coupling to small-conductance Ca^{2+}-activated K^+ outward currents to occur as described in central neurons (Marrion and Tavalin, 1998) and, most importantly, in cardiac myocytes (Lu et al., 2007). In an attempt to elucidate the differential shape of action potentials between atrial and ventricular myocytes, Baro and Escande found long lasting Ca^{2+}-activated K^+ outward currents in atrial myocytes, which were sensitive to apamin (Baro and Escande, 1989). Consistent with this report, we show here that the outward K^+ current was clearly voltage-dependent with the maximal amplitude obtained when atrial cardiomyocytes were depolarized with a prepulse reaching −10 mV, i.e., close to the maximum activation of high-voltage Cav1 (L-type) currents.

3.5. The adenosine A_1 receptor plays a dual role in atrial cardiomyocytes by activating an inward-rectifying GIRK/K_{IR}3.1/K_{IR}3.4 current and by inhibiting a Ca^{2+}-activated K_{Ca}2/SK outward current depending on cell depolarization

Incubation of isolated atrial cardiomyocytes with the adenosine A_1 receptor agonist, R-PIA (300 nM), augmented the inward current (Figure 13Ci), by increasing the slope conductance 1.36 fold above the control value (Figure 13Ciii). Figure 13Cii shows that R-PIA (300 nM) further increased the magnitude of the outward current when this was preceded by a brief (50 ms) depolarizing step to −10 mV allowing Ca^{2+} influx through high-voltage Cav1 (L-type) channels (Grant, 2009); this trend was observed in four out of seven cells from three different animals (right-hand side panel), but the magnitude of the outward current in the presence of R-PIA varied considerably reflecting heterogeneity of the cells

that compose atrial muscle. Interestingly, R-PIA (300 nM) changed significantly the kinetics of the outward current recorded at +40 mV (Figure 13Cii). Interestingly, R-PIA (300 nM) changed significantly the kinetics of the outward current recorded at +40 mV (Figure 13Cii). Stimulation of A_1 receptors in atrial myocytes is known to activate inwardly-rectifying GIRK/K_{IR}3.1/K_{IR}3.4 channels (Belardinelli and Isenberg, 1983; Kurachi et al., 1986b; Yatani et al., 1987; Mubagwa and Flameng, 2001), which may lead to the appearance of a slowly activating component in the outward current (Belardinelli and Isenberg, 1983; Kurachi et al., 1986b; Banach et al., 1993; Wellner-Kienitz et al., 2000; Lomax et al., 2003). To test this hypothesis, we investigated the effect of the adenosine A_1 receptor agonist, R-PIA (300 nM), under blockage of GIRK/K_{IR}3.1/K_{IR}3.4 channels. Pretreatment with tertiapin Q (300 nM) decreased the whole cell conductance (Figures 13D, 14A). More relevant to the present context, subsequent activation of A_1 receptors with R-PIA (300 nM), still in the presence of tertiapin Q (300 nM), caused a further decrease, instead of an increase, of the whole-cell outward current (Figures 13Di,ii). This suggests that upon activation of the adenosine A_1 receptor, two potassium currents are altered. While the inwardly-rectifying GIRK/K_{IR}3.1/K_{IR}3.4 current is enhanced, a second current subsisting after blockage of GIRK/K_{IR}3.1/K_{IR}3.4 channels with tertiapin Q is inhibited by the A_1 receptor agonist.

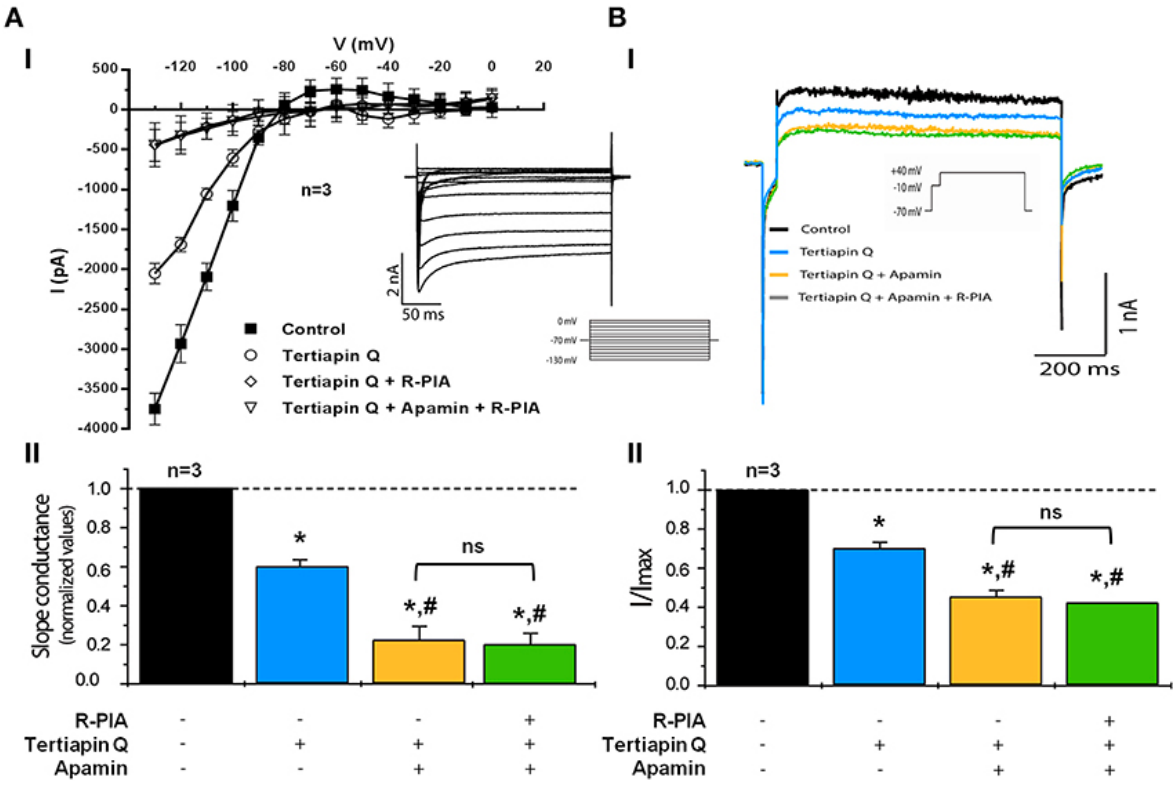

Figure 14. Activation of adenosine A_1R inhibits Ca^{2+}-activated outward K_{Ca}2/SK current in isolated rat atrial myocytes when inwardly rectifying GIRK/K_{IR}3 channels are blocked with tertiapin Q. (Ai) Current-voltage relationship showing strong inward rectification obtained from currents recorded following a set of voltage steps (−130 to 0 mV, 10 mV steps, holding voltage −70 mV, 260 ms duration each) in the absence (Control) and in the presence of tertiapin Q (300 nM), apamin (30 nM) and R-

PIA (300 nM), applied cumulatively (see the inset for representative currents). Data are expressed as mean ± SEM of three different animals; recordings from three to five isolated atrial cardiomyocytes were averaged per experimental animal. (Aii) Bar-graphs representing pooled data from three different animals in which the average slope conductance of the inward current was normalized to the maximum obtained in the control situation, without test drugs. In panel (B) shown are representative whole-cell voltage-clamp recording traces from double depolarization protocol held at holding potential of −70 mV using the same cell as in (Ai). Currents were elicited by a single 10 ms depolarizing pulse from −70 to −10 mV followed by a 750-ms pulse to +40 mV (see inset in panel Bi). Panel (Bi) shows the effects on Ca^{2+}-activated outward $K_{Ca}2$/SK currents of cumulative applications of tertiapin Q (300 nM), apamin (30 nM), and R-PIA (300 nM). Sodium currents were truncated to facilitate visualization of effects. (Bii) Bar-graphs representing pooled data from three similar experiments in which peak current was normalized to the maximum current obtained in the absence of added drugs (Control). All experiments were performed in the presence of E4031 (10 μM) to prevent rapid delayed rectifier potassium currents operated by hERG channels from being activated. Error bars represent SEM of three animals. *, #P < 0.05 (unpaired Student's t-test with Welch's correction) represent significant differences from the control or from tertiapin Q alone, respectively; ns, not significant.

We, then, set up to investigate the nature of the R-PIA sensitive outward current observed under conditions of GIRK/K_{IR}3.1/K_{IR}3.4 channel blockade (Figure 14). In the presence of tertiapin Q (300 nM), application of apamin (30 nM) to the superfusion fluid decreased further the Ca^{2+}-dependent delayed outward K+ current (Figure 14B). At 30 nM concentration the current mediated by small conductance Ca^{2+}-activated $K_{Ca}2$/SK channels are supposed to be substantially blocked by apamin (Xu et al., 2003; Tuteja et al., 2005). Under these pharmacological restraining conditions, subsequent application of R-PIA (300 nM) did not cause further modifications (P > 0.10) in the outward current (Figure 14B). Overall, these results show that (1) there is a component of the outward current that is mediated by small conductance Ca^{2+}-activated $K_{Ca}2$/SK channels, and (2) such $K_{Ca}2$/SK component is not modified further by A_1 receptors activation, since R-PIA failed to alter the whole-cell current in the presence of apamin (30 nM). Figure 14A shows data obtained when evaluating whole-cell conductance using a similar pharmacological approach. The reduction in the magnitude of the inwardly-rectifying current caused by tertiapin Q (300 nM) was amplified by blocking $K_{Ca}2$/SK channels with apamin (30 nM). Notably, blockade of GIRK/K_{IR}3.1/K_{IR}3.4 and $K_{Ca}2$/SK channels with tertiapin Q (300 nM) and apamin (30 nM), respectively, prevented further changes in whole-cells conductance by application of the A1 receptor agonist, R-PIA (300 nM) (Figures 14A,B). Thus, electrophysiological data strongly indicate that besides activation of inwardly rectifying GIRK/K_{IR}3.1/K_{IR}3.4 currents (Belardinelli and Isenberg, 1983; Kurachi et al., 1986b; Yatani et al., 1987; Mubagwa and Flameng, 2001), stimulation of adenosine A_1 receptors leads to inhibition of apamin-sensitive Ca^{2+}-activated $K_{Ca}2$/SK channels in atrial cardiomyocytes.

3.6. Loss of atrial chronoselectivity of adenosine A_1 receptors by blocking high voltage-activated Ca_v1 (L-type) currents with nifedipine or verapamil

The $K_{Ca}2$/SK channel gating mechanism is controlled by intracellular Ca^{2+} levels entering via voltage-activated calcium channels (Ca_v) (Marrion and Tavalin, 1998). Figure 15 shows that blockade of Ca_v1 (L-type) channels with nifedipine or verapamil, applied in a concentration (1 μM) that reduced atrial chronotropy roughly by 25% (see Table 4), sensitized atria to the negative inotropic action of R-PIA (0.001–1 μM) without much affecting the action of the A_1 receptor agonist on atrial rate. The negative chronotropic and inotropic actions of R-PIA were both evident at the same concentration range in the presence of Ca_v1 (L-type) channel blockers, nifedipine (pIC50 7.51 ± 0.10 and 7.14 ± 0.14 for chronotropism and inotropism, respectively, n = 5, P > 0.05) or verapamil (pIC50 7.80 ± 0.20 and 7.34 ± 0.24 for chronotropism and inotropism, respectively, n = 5, P > 0.05), thus resulting in a loss of the atrial chronoselectivity of adenosine A1 receptors (see above).

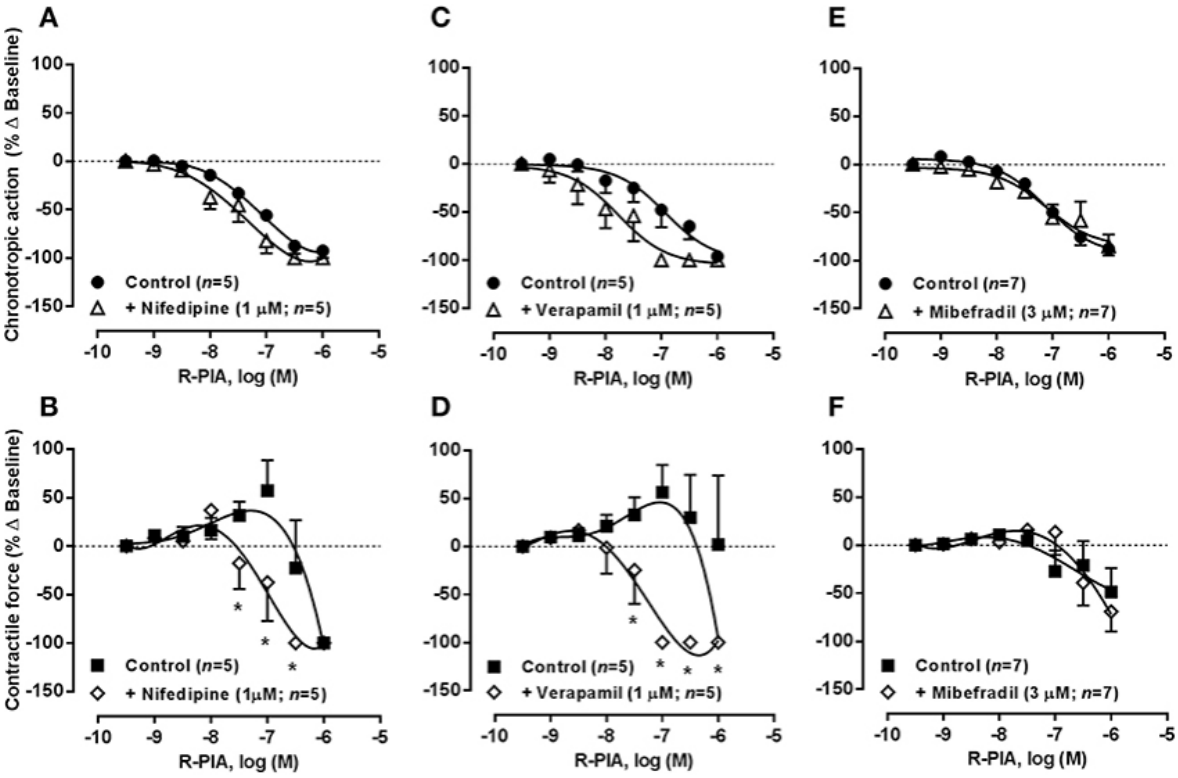

Figure 15. Concentration-response curves of R-PIA (0.001–1 μM) on the spontaneously beating rat atria in the absence (Control) and in the presence of voltage-sensitive calcium channels inhibitors: nifedipine (1 μM, A,B), verapamil (1 μM, C,D), and mibefradil (3 μM, E,F). Drug applications followed the protocol depicted in Figure 5A. The ordinates are percentage of variation of spontaneous contraction rate (chronotropic effect, A,C,E) and mechanical tension (inotropic effect, B,D,F) as compared to baseline values obtained before application of R-PIA. The data are expressed as mean ± SEM from an n number of individual experiments. *P < 0.05 compared with the effect of R-PIA in the absence of voltage-sensitive calcium channels inhibitors.

Under similar experimental conditions, pre-treatment with acetylcholine did not significantly ($P > 0.05$) modify the depressing effects of R-PIA (0.001–1 μM) on spontaneously beating rat atria, when the cholinergic agonist was applied in a concentration (30 μM) that mimicked the negative chronotropic effect (25% reduction from control, n = 3–4) of nifedipine or verapamil. Conversely, blockade Cav1 (L-type) channels with 1 μM nifedipine or verapamil failed to modify oxotremorine (0.003–3 μM)-induced depression in the rat spontaneously beating atria (Figure 16). These findings contrast with those obtained with the adenosine A_1 receptor agonist, suggesting that the negative chronotropic and inotropic actions of oxotremorine are independent on Ca^{2+} influx through Ca_v1 (L-type) channels and on apamin-sensitive $K_{Ca}2$/SK channels, relying most probably on the control of K^+ currents via G protein-coupled inwardly rectifying K^+ channels (GIRK or $K_{IR}3.1/3.4$) sensitive to tertiapin Q. Blockade of low voltage-activated Ca_v3 (T-type) channels with mibefradil (0.1–10 μM) concentration-dependently reduced atrial chronotropy to a maximum of 30% (n = 7), without affecting the force of spontaneous atrial contractions (see Table 4). Mibefradil (3 μM) failed to modify the effects of both R-PIA (0.001–1 μM, Figures 15E,F) and oxotremorine (0.003–3 μM, Figures 16E,F) in the spontaneously beating rat atria (Belardinelli et al., 1995).

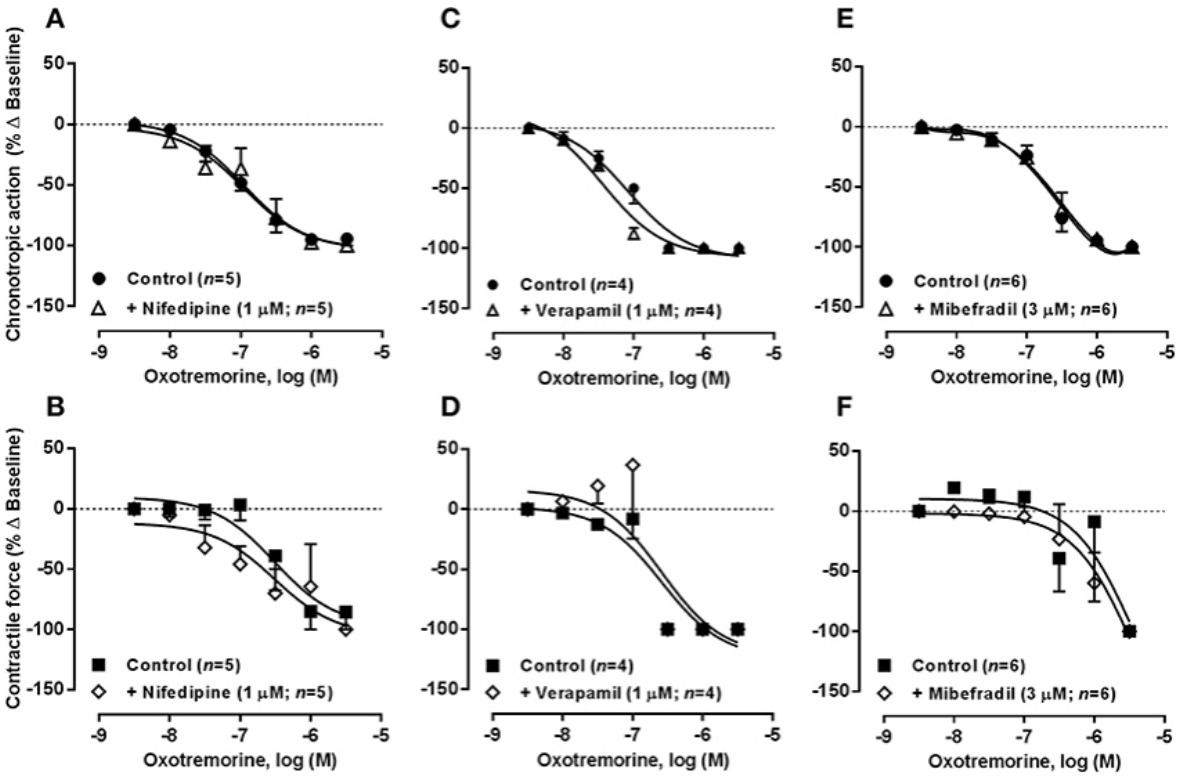

Figure 16. Concentration-response curves of oxotremorine (0.003–3 μM) on the spontaneously beating rat atria in the absence (Control) and in the presence of voltage-sensitive calcium channels inhibitors: nifedipine (1 μM, A,B), verapamil (1 μM, C,D), and mibefradil (3 μM, E,F). Drug applications followed the protocol depicted in Figure 5A. The ordinates are percentage of variation of spontaneous contraction rate (chronotropic effect, A,C,E) and mechanical tension (inotropic effect,

B,D,F) as compared to baseline values obtained before application of oxotremorine. The data are expressed as mean ± SEM from an n number of individual experiments.

3.7. Interplay between $K_{Ca}2$/SK, GIRK/K_{IR} and Ca_v1 (L type) channels on spontaneously beating rat atria

Figure 17 shows the responses of spontaneously beating rat atria to the cumulative application of verapamil (0.03–10 μM) in the absence and presence of apamin (30 nM) or tertiapin Q (300 nM). Verapamil (0.03–10 μM), as well as nifedipine (0.03– 30 μM, data not shown), decreased the rate of contractions of the rat spontaneously beating atria in a concentration-dependent manner. Similarly to adenosine A_1 receptor agonist, the negative chronotropic effect of verapamil (0.03–10 μM, Figures 17A,C) was evidenced in concentrations unable to decrease the magnitude of atrial tension (Figures 17B,D). It is worth noting that the negative inotropic potency of R-PIA (Figure 5C) and verapamil (data not shown) was not significantly ($P > 0.05$) modified in atria paced electrically at a constant frequency of 240 beats.min^{-1} ; for instance, the pIC_{50} values obtained for the negative inotropic action of R-PIA were not different ($P > 0.05$) in spontaneously beating (6.14 ± 0.07, n = 38) and electrically-paced (6.20 ± 0.05, n = 7) rat atria. A similar situation was detected regarding the negative inotropic effect of oxotremorine in spontaneously beating (pIC_{50} value of 6.81 ± 0.10, n = 31) and electrically-paced (pIC50 value of 7.22 ± 0.15, n = 7) atria (Figure 5B). These findings were obtained notwithstanding the maximal reduction of myographic recordings amplitude induced by pacing in the presence of R-PIA and oxotremorine did not go beyond 30% of that observed in spontaneously beating atria (see Figure 5B). Thus, data indicate that sustained inotropy is not a direct consequence of the intracellular residual Ca^{2+} accumulation due to slowing down the rhythm of atrial contractions (negative dromotropic/chronotropic effects) in response to drug applications. However, in contrast to the adenosine A_1 receptor agonist, blockade of $K_{Ca}2$/SK and GIRK/K_{IR} channels respectively with apamin (30 nM) and tertiapin Q (300 nM) failed to modify atrial effects produced by verapamil (0.03–10 μM). In another set of experiments, we tested the effects of increasing concentrations of apamin (0.003–1 μM) in the absence and in the presence of verapamil (1 μM) (Figure 18).

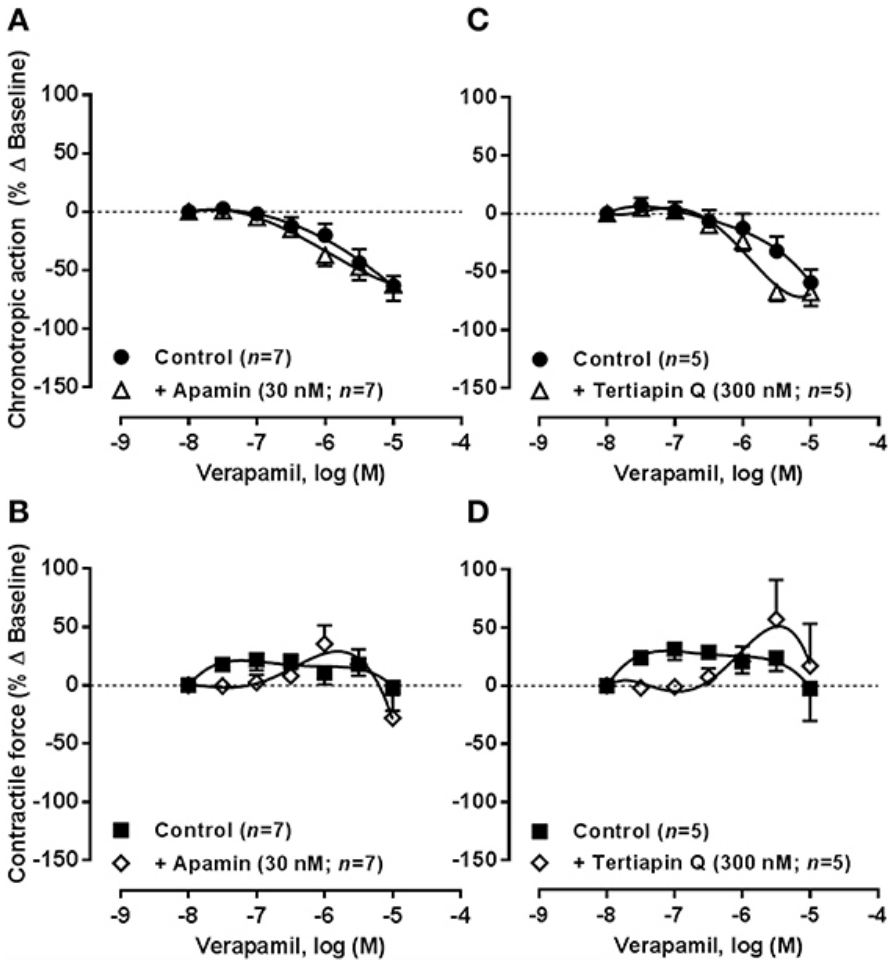

Figure 17. Concentration-response curves of the Ca_V1 (L-type) channel inhibitor, verapamil (0.03–10 μM), on the spontaneously beating rat atria in the absence (Control) and in the presence of apamin (30 nM, A,B) and tertiapin Q (300 nM, C,D), which selectively block $K_{Ca}2$/SK and GIRK/K_{IR} channels, respectively. Drug applications followed the protocol depicted in Figure 2A. The ordinates are percentage of variation of spontaneous contraction rate (chronotropic effect, A,C) and mechanical tension (inotropic effect, B,D) as compared to baseline values obtained before application of verapamil. The data are expressed as mean ± SEM from an n number of individual experiments.

Blockade of Ca^{2+}-activated $K_{Ca}2$/SK channels with apamin (0.003–1 μM) did not significantly ($P > 0.05$) change the spontaneous atrial rate (Figure 18A), but we observed a moderate positive inotropic effect (maximal increase ~20% at 0.1 μM, Figure 18B) in line with that occurring with other K^+ channel blockers, 4-AP and glibenclamide (see above). Interestingly, the mild positive inotropic effect of apamin (0.003–1 μM) reproduced the transient increase in atrial contractile force caused by the adenosine A_1 receptor agonist, R-PIA, in a wide range of concentrations (1–100 nM, see Figure 5), but the $K_{Ca}2$/SK channel inhibitor had no comparable effect on chronotropy. Verapamil (1 μM) reversed the positive inotropic effect of apamin leading to a mild negative inotropic effect (Figure 18B). Data suggest that strengthening spontaneous atrial contractions by blocking $K_{Ca}2$/SK channels requires Ca^{2+} influx through verapamil-sensitive Ca_V1 (L type) channels.

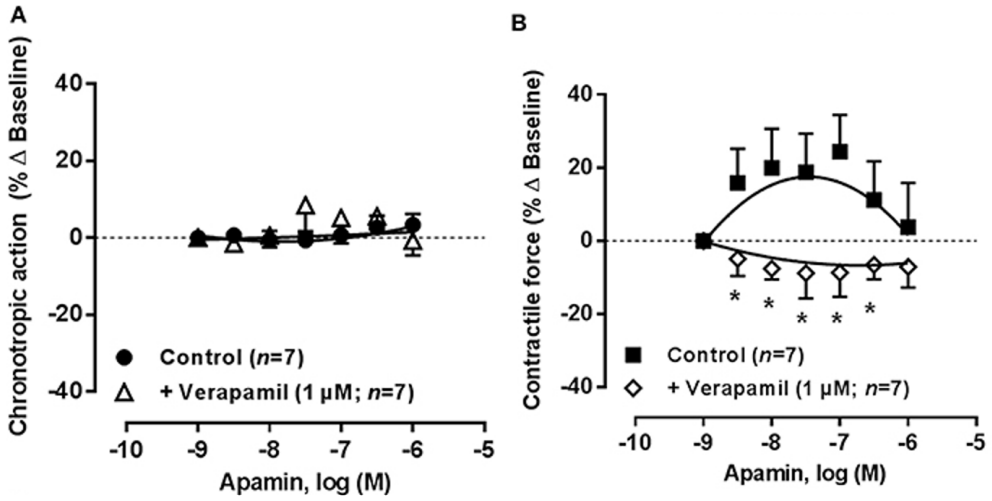

Figure 18. Concentration-response curves of the KCa2/SK channel blocker, apamin (0.003–1 µM), on the spontaneously beating rat atria in the absence (Control), and in the presence of verapamil (1 µM). Drug applications followed the protocol depicted in Figure 5A. The ordinates are percentage of variation of spontaneous contraction rate (chronotropic effect, A) and mechanical tension (inotropic effect, B) as compared to baseline values obtained before application of apamin. The data are expressed as mean ± SEM from an n number of individual experiments. *P < 0.05 compared with the effect of apamin in the absence of verapamil.

4. Discussion

The present study demonstrates that adenosine and its stable analog, R-PIA, acting via inhibitory A_1 receptors are chronoselective atrial depressants affecting inotropy in a much lesser degree compared to the muscarinic M_2 receptor agonist, oxotremorine, in spontaneously beating rat atria. Likewise, intravenous R-PIA caused a marked and sustained sinus bradycardia without affecting myocardial contractility leading to a reduction in the mean arterial blood pressure in anesthetized pigs (Wainwright and Parratt, 1993). A different result was obtained by Lee et al., since the A_1 receptor agonist, N6 - cyclopentyladenosine (CPA), was about equipotent on electrically-driven left atria (negative inotropic action) and on spontaneously beating right atria (negative chronotropic action) (Lee et al., 1993). However, these authors used a pacing frequency of 1 Hz (60 beats.min^{-1}) that is far too low compared to the spontaneous atrial rate (200–220 beats.min^{-1}) as verified in our study using both right and left atria with the SA node intact (see Table 4). We show here that activation of the A_1 receptor with R-PIA decreases the contractile force of atria paced at 4 Hz frequency (just slightly above the spontaneous atrial rate) by a similar extent to that observed in spontaneously beating atria. In anesthetized pigs, right atrial pacing with a frequency just above the spontaneous sinus rate in the pig (110 beats.min^{-1}) abrogated R-PIA-induced decrease in blood pressure, showing that the fall in blood pressure seen in unpaced animals was a result of decreased cardiac rate and was not due to a reduction in myocardial contractility and/or to peripheral vasodilatation. These findings

indicate that negative chronotropic-dependent inotropy, which has been pointed out as a major drawback of experiments performed in spontaneously beating isolated atria (Stemmer and Akera, 1986), does not account significantly to adenosine chronoselectivity. Supersensitive negative chronotropic and dromotropic effects of adenosine have been described in the presence of isoproterenol in the transplanted human heart in which adenosine failed to attenuate the isoproterenol-induced increase in contractility whereas it produced an exaggerated negative chronotropic and dromotropic effect (Koglin et al., 1996). Isolated spontaneously beating atria as used in the present study are devoid of counterregulatory inotropic actions of the sympathetic nervous system. A remaining stimulation of β_1-adrenoceptors can also not afford a valid explanation for adenosine chronoselectivity in our study since atria were unaffected by the application of propranolol (Dobson, 1983; Romano et al., 1991). Immunofluorescence confocal microscopy showed no significant differences in the distribution of the A_1 receptor protein between SA node and atrial cardiomyocytes suggesting that it can also not afford a rationale for adenosine chronoselectivity (see Figure 4D). This was observed despite the demonstration that the A_1 receptor mRNA expression was higher in the right atrium than in the SA node, with no differences found on M_2 receptor mRNA levels between distinct atrial regions (Chandler et al., 2009). These findings suggest that the negative chronotropic and inotropic atrial responses to adenosine, via A_1 receptors, are differentiated at the post-receptor transduction level (see e.g., (Oguchi et al., 1995)). While both A_1 and M_2 receptors promote K^+ efflux through βγ subunits of G protein-coupled inwardly rectifying (tertiapin Q-sensitive) GIRK/K_{IR}3 channels modulating SA node automatism (Belardinelli and Isenberg, 1983; Kurachi et al., 1986b; Yatani et al., 1987; Mubagwa and Flameng, 2001), we demonstrated here for the first time that activation of adenosine A_1 receptors concurrently inhibit small conductance outward K_{Ca}2/SK currents probably via G protein α subunit. Cardiac K^+ channels have been recognized as potential targets for the actions of neurotransmitters, hormones and class III antiarrhythmic drugs that prolong action potential duration and refractoriness; potassium channel inhibitors can effectively prevent/inhibit cardiac arrhythmias (Li and Dong, 2010). Among all K^+ channel inhibitors used in this study, namely 4-aminopyridine, glibenclamide, and apamin, only the selective GIRK/K_{IR}3.1/3.4 channel blocker, tertiapin Q, produced no significant effects on spontaneously beating atria when used alone. It is, therefore, reasonable to conclude that the primary effect of tertiapin Q on spontaneously beating atria is limited to blockade of muscarinic- and adenosine-activated GIRK/K_{IR}3.1/3.4 currents and that, in the absence of endogenous ligands, both atrial rate and contractile force are not controlled by constitutive A_1 and M_2 receptors activity. Scarcity of intrinsic A_1 receptor tone in the absence of endogenous adenosine was also inferred from the lack of effect of DPCPX (2.5–100 nM) alone on spontaneous atrial performance, as this

compound has inverse agonist properties in systems with constitutive A_1 receptor activity (Searl and Silinsky, 2012; He et al., 2013). Besides tertiapin Q, the repercussion of K^+ channels blockade on atrial activity may reflect a positive inotropic effect operated either directly due to cardiomyocyte depolarization, or indirectly by promoting noradrenaline release from depolarized sympathetic nerves associated to blockage of outward K^+ currents at resting membrane potential (Lawson, 1996; Xu et al., 2003; Tuteja et al., 2005; Grant, 2009). The participation of noradrenaline release from sympathetic nerves was assumed because the inotropic effect of K^+ channel inhibitors was prevented by the β-receptor blocker, propranolol. Moreover, the resting heart rate in unrestrained conscious wild-type and GIRK4 knockout mice was virtually identical, indicating that other signaling pathways involved in heart rate regulation might balance the missing IK_{ACh} current (Wickman et al., 1998). These authors showed that the diminished bradycardic response of the GIRK4 knockout mice to A_1 receptor activation was only fifty percent of the heart rate decrease in response to adenosine and acetylcholine in vivo. Therefore, they hypothesized that the residual bradycardic effect of adenosine and acetylcholine in the knockout mice may be due to decreases in slow depolarizing currents, namely cationic (I_f), and sustained inward (IST) currents, which are directly or indirectly modulated by the cyclic AMP pathway. The opposite was however detected in our hands, where blockade of GIRK/K_{IR}3.1/3.4 currents with tertiapin Q reduced, instead of increasing, the whole-cell inward current caused by the A1 receptor agonist, R-PIA, in rat atrial cardiomyocytes (Figure 13). Besides ivabradine analogs (e.g., zatebradine), which produce use-dependent inhibition of hyperpolarization-activated mixed Na^+-K^+ inward current If (cyclic nucleotide-gated HCN channel) in sinoatrial node cells but also significantly reduce voltage-gated outward K^+ currents (IK) at the same concentrations, there are no other specific pharmacological manipulators to dissect the influence of these currents in the spontaneously beating rat atria. Yet, we found no differences in spontaneous atrial rate and inotropy upon applying the cyclic AMP-specific phosphodiesterase type 4 inhibitor rolipram (1–100 μM) (unpublished observations), showing that under the present experimental conditions cyclic nucleotide-gated currents play a minor role. Moreover, in vivo data supports a greater role for If currents in His-Purkinje fibers vs. SAN tissue. In support of our hypothesis, immunolocalization studies with an antibody specific for GIRK1/K_{IR}3.1 demonstrates that the distribution of this channel subtype follows a similar pattern of both A1 and M2 receptors in SA node and atrial cardiomyocytes (Figure 10).

Blockade of voltage-dependent Kv with 4-AP and ATP-sensitive K_{ATP}/K_{IR}6 channels with glibenclamide failed to modify atrial depression caused by adenosine A_1 and muscarinic M_2 receptor agonists in spontaneously beating rat atria, when the K^+ channel inhibitors were applied in concentrations as high as 10 μM. Likewise, 4-AP and blockade of

the rapid delayed rectifier potassium current (I_{Kr}) operated by hERG channels with E4031 did not modify cardiac responses to adenosine in isolated blood perfused atria of the dog (Oguchi et al., 1995). These findings rule out the involvement of voltage-dependent K_v cannels on the inhibitory effects of A_1 and M_2 agonists and confirm data from interaction studies between 4-AP (0.3–3 mM) and R-PIA in the guinea-pig using spontaneously beating and electrically-driven atria (De Biasi et al., 1989). Concerning the lack of effect of glibenclamide in antagonizing the responses to A_1 and M_2 receptor agonists in rat atria, our results agree with previous reports in the guinea-pig indicating that cardiodepression by these agents are not operated by ATP-sensitive K_{ATP}/K_{IR}6 channels (Urquhart et al., 1993; Ford and Broadley, 1999).

Due to high sensitivity of K_{Ca}2/SK channels for Ca^{2+} activation yielding half maximal activation at ~300 nM [Ca^{2+}]i with a Hill coefficient between 4 and 5 (Xia et al., 1998), these channels aid in integrating changes in intracellular free Ca^{2+} concentration with membrane potential in the late phase of cardiac repolarization. The small conductance Ca^{2+}-activated SK potassium channel subfamily differs in apamin sensitivity, with the SK2 (K_{Ca}2.2) being more sensitive (IC50 ~ 70 pM) than the SK3 (K_{Ca}2.3) (IC50 ~ 0.63–6 nM), while a more pronounced expression of these channels exist in atria compared with ventricle myocytes of different species (Xu et al., 2003; Jager and Grissmer, 2004; Tuteja et al., 2005). Data from immunolocalization studies indicate that the apamin-sensitive SK2 (K_{Ca}2.2) channel seems to be more abundant in atrial cardiomyocytes than in the SA node (Figure 10), while the opposite is observed with the SK3 (K_{Ca}2.3) channel (cf. (Tuteja et al., 2005)). Preferential co-localization of SK2 (K_{Ca}2.2) channels and A_1 receptors in atrial cardiomyocytes, rather than in SA node, might explain why apamin sensitized (by more than 10-fold) atria to the negative inotropic action of the A_1 receptor agonist, R-PIA, without significantly affecting the chronotropic effect of the nucleoside. In theory, the leftward shift caused by apamin of the concentration-response curve of R-PIA regarding its negative inotropic effect was unexpected, considering that inhibition of repolarizing K_{Ca}2/SK currents should prolong the action potential duration and, thereby, increase the time available for Ca^{2+} influx through Ca_v1 (L-type) channels leading to a positive inotropic response. In fact, apamin alone caused a mild (<20%) positive inotropic response (with no effect on chronotropy) which was counteracted by the Cav1 (Ltype) channel inhibitor, verapamil (Figure 18). Controversy still exists in the literature regarding the effect of K_{Ca}2/SK channel blockers on action potential duration that derive from interspecies differences, experimental idiosyncrasies (e.g., paced vs. unpaced, high vs. low pacing rates), uneven expression of channels throughout atria and unusual pharmacological profiles due to SK2-SK3 channels heteromerization (see e.g., (Xu et al., 2003; Nagy et al., 2009; Hancock et al., 2015)).

Apart from this dispute, we agree with other authors that action potential repolarization is controlled by a, not yet fully characterized, fine balance between various transmembrane ionic currents that are essential to determine the duration of the cardiac action potential. One possible explanation for the above disparity could be that blockade of $K_{Ca}2$/SK channels stabilizes the K^+ gradient across the cell membrane at a higher level (Xu et al., 2003), leading to increased GIRK/KIR3.1/3.4-mediated K^+ efflux and cell hyperpolarization per G protein-coupled receptor activated, thus affecting the relative potency of the A_1 receptor agonist. If this were the case, apamin should have also potentiated the negative inotropic effect of the M_2 receptor agonist in a similar manner to that observed with the A_1 agonist, taking into consideration that both receptors couple to GIRK/K_{IR}3.1/3.4 channels (Kurachi et al., 1986b). However, atrial depression caused by oxotremorine was not affected by apamin, indicating that Ca^{2+}-activated $K_{Ca}2$/SK channels are not involved in muscarinic M_2 receptors activity. Activation of $K_{Ca}2$/SK currents causes a hyperpolarizing leak of potassium ions from the cell in favor of its concentration gradient. Therefore, increases in the extracellular potassium concentration should mimic blockage of these currents by apamin. Coincidently, both apamin and augmentation of the extracellular concentration of potassium (from 2.7 to 4.7 mM) sensitized atria to the negative inotropic effect of the A_1 receptor agonist, without affecting adenosine negative chronotropy. Neither apamin nor increases in the extracellular K^+ concentration affected significantly oxotremorine-induced cardiodepression in spontaneously beating rat atria.

We are aware that increases in extracellular K^+ has many effects on excitable cells, yet under our experimental conditions this approach positively discriminated the A_1 receptor-mediated negative inotropic effect by shifting to the left the concentration-response curve of the adenosine analog, R-PIA, without affecting the muscarinic atrial depression. The most plausible explanation might be that inactivation of $K_{Ca}2$/SK outward currents caused by adenosine A_1 receptors (via G protein α subunit) in the late phase of atrial repolarization may prolong the action potential duration and shift the resting membrane potential of cardiomyocytes toward more depolarized potentials resulting in an increase in the net Ca^{2+} influx through Ca_v1 (L-type) channels (cf. (Lu et al., 2007; Skibsbye et al., 2015)). This hypothesis may be challenged by a recent report showing that in right atria paced at 5 Hz frequency both acetylcholine and the A_1 receptor agonist, CPA, shortened action potential duration at 90% repolarization (APD90) and the ERP in a tertiapin Q-sensitive manner (Wang et al., 2013). However, in contrast to our work using spontaneously beating atria, these authors found a basal GIRK/K_{IR}3 current that was active without exogenous receptor activation, which may be a confounder of results interpretation in the two studies. Moreover, application of tertiapin Q after receptor activation caused APD90 and ERP return close to baseline values but it did not gave an overshoot that would be

expected if the activation by adenosine and acetylcholine only affected GIRK/K_{IR}3-mediated currents. This phenomenon was interpreted as a reflection of multiple downstream targets of Gi receptors, which may include activation of Ca_v1.3 (L-type) channels that would contribute to sustain inotropy (Wang et al., 2013).

The molecular mechanisms underlying the coupling of Cav1 and KCa2/SK channels are unknown. Functional coupling of Ca_v1.3 (L-type) and K_{Ca}2/SK channels has been described in atrial myocytes via the cytoskeletal protein α-actinin2, an F-actin cross-linking protein directly bridging C-terminal regions of both channels (Lu et al., 2007). An indirect pathway requiring binding of Ca^{2+} to calmodulin (CaM), which then bounds to a CaM-binding domain on the intracellular subunit of the SK channel, has also been proposed. Here, we showed that adenosine A_1 receptors exerts a dual role by activating GIRK/K_{IR}3 and inactivating K_{Ca}2/SK mediated outward currents, with the latter being revealed at a test potential of +40 mV after transient activation of high-voltage Ca_v1 (L-type) channels by a brief (50 ms) depolarizing pulse to −10 mV (Grant, 2009). Upon blocking GIRK/K_{IR}3.1/3.4 channels with tertiapin Q, the A_1 receptor agonist decreased, rather than increased, the outward component and this effect was fully prevented by co-application of the K_{Ca}2/SK channel blocker apamin. These findings clearly indicate that the net GIRK/K_{IR}3.1/3.4 outward current triggered by adenosine that is responsible for reducing the SA node automatism may be partially counteracted by the inactivation of an apamin-sensitive Ca^{2+}-activated K_{Ca}2/SK repolarizing current leading to prolongation of action potential duration and to Ca^{2+} influx into atrial cardiomyocytes via voltage-gated Cav1 (L-type) channels. On its own, Ca^{2+} influx into cardiomyocytes may also influence action potential duration through Ca^{2+}-sensitive ionic currents, such as the sodium-calcium exchanger and the calcium-sensitive chloride current. Although there are several studies addressing the effects of adenosine on potassium currents in atrial myocytes (Kurachi et al., 1986b; Banach et al., 1993; Wellner-Kienitz et al., 2000; Lomax et al., 2003), to the best of our knowledge there is no report showing that activation of the adenosine A_1 receptor leads to inactivation of an outward K^+ current carried out by K_{Ca}2/SK channels in these cells. The (patho)physiological role of apparent opposite effects on potassium currents caused by adenosine A_1 receptors activation is under debate. Our study suggests that inhibition of K_{Ca}2/SK channels by adenosine plays a remarkable role on atrial inotropy. These findings seem to be reliable because blockade of K_{Ca}2/SK channels cause a delay in the late phase of the cardiac repolarization (Xu et al., 2003; Tuteja et al., 2005; Nagy et al., 2009; Hancock et al., 2015). According to the most accepted hypothesis to explain negative inotropic effects upon GIRK/K_{IR}3.1/3.4 channels (Wang and Belardinelli, 1994; Ford and Broadley, 1999), inhibition of cardiac repolarizing K^+ currents, such as the apamin-sensitive small conductance Ca^{2+}-activated K_{Ca}2/SK current, may increase the time available for Ca^{2+} influx

via Ca_v1 (L-type) channels due to action potential prolongation and this might counteract the negative inotropic effects promoted by K^+ efflux through GIRK/K_{IR}3.1/3.4 channels. In addition to the enrolment of $K_{Ca}2$/SK channels on atrial inotropic mechanisms, these channels have been identified as key players in the course of supraventricular arrhythmias (Li et al., 2009; Yu et al., 2012), which is particularly interesting in a context of anti- and proarrhythmic properties of adenosine and its derivatives (Kabell et al., 1994; Bertolet et al., 1997; Lim et al., 2009). Further studies remain to be performed to elucidate if $K_{Ca}2$/SK channels are exclusively modulated by adenosine A_1 receptors, but at this moment our data suggests that cardiac muscarinic M_2 receptors do not play a role in this mechanism.

Besides the involvement of various K^+ channel subtypes, the electrophysiological activity of atrial myocytes involves Ca^{2+} influx by Ca_v1 (L-type) voltage-sensitive channels (Amin et al., 2010). Novel studies describe the $Ca_v1.3$ channel as a determinant of human heart rate but not of ventricular excitation-contraction coupling, which depends mainly on Ca^{2+} influx through the $Ca_v1.2$ channel isoform (Baig et al., 2011). Confocal microscopy data showed that immunoreactivity against Ca_v1 (L-type), using an antibody specific for the regulatory $\alpha_{2\delta}$ subunit of the channel that does not target subtype-specific α_1 subunits, was consistently distributed in both SA node and right atrium. Voltage-sensitive Ca_v1 (L-type) channels are blocked by organic antagonists. These include dihydropyridines (nifedipine) and phenylalkylamines (verapamil), but only the second has cardio-selective activity with useful clinical indications to normalize tachycardia rhythms (Grant, 2009). However, little is known about the functional interactions between adenosine and Ca_v1 (L-type) channel blockers on atrial activity despite the two compounds are often used in line to revert supraventricular dysrhythmias.

Our findings clearly demonstrate for the first time that blockade of Ca_v1 (L-type) channels by nifedipine and verapamil predisposes atria to the negative inotropic action of adenosine probably by uncoupling A_1-receptor-mediated $K_{Ca}2$/SK channel inhibition from its effector system, the Ca_v1 (L-type) subtype channel. Further studies are required to investigate the interplay of the two channels vis a vis A_1 receptors activation. Contrariwise, nifedipine and verapamil were devoid of effect on muscarinic M_2-receptor-mediated actions on spontaneously beating rat atria. This disparity is in agreement with literature. Unlike the A_1 receptor, activation of the muscarinic M_2 receptor in the heart might not affect Ca_v1 (L-type) currents (De Biasi et al., 1989; Song and Belardinelli, 1994; Belardinelli et al., 1995), although both receptors may co-localize and share a common pathway leading to hyperpolarization of atrial myocytes via K^+ efflux through GIRK/K_{IR}3.1/3.4 channels (Belardinelli and Isenberg, 1983; Kurachi et al., 1986b).

Receptor reserve refers to a phenomenon whereby stimulation of only a fraction of the whole receptor population apparently elicits the maximal effect achievable in a particular

tissue depending on agonists efficacy and on the pathways activated to cause signal amplification (reviewed in (Dhalla et al., 2003)). If the receptor reserve is small for a given agonist, the agonist will only elicit the effect in a significant extent when used at high concentrations, while the same agonist can produce the effect even in low concentrations if the receptor reserve is high. Previous studies indicate that atrial cardiomyocytes possess a substantial A_1 receptor reserve for the direct negative inotropy, which is greater than the A_1 receptor reserve for any other effects in the guinea-pig atrium paced electrically at 3 Hz-frequency (Gesztelyi et al., 2013; Kiss et al., 2013). Taking this into account, one would predict that enzymatically stable full A_1 receptor agonists, such as R-PIA, should be more potent in decreasing the contractile force than the atrial rate. Yet, we show here that bradycardia produced by R-PIA predominates over the negative inotropic effect of the A_1 receptor agonist in spontaneously beating isolated rat atria. Whether increases in atrial A_1 receptor reserve specifically for the direct negative inotropy occur in the presence of the $K_{Ca}2$/SK blocker, apamin, or of Ca_v1 (L-type) channel inhibitors, nifedipine or verapamil, deserve future investigations as they may sensitize atria to mechanical depression in clinical conditions requiring administration of adenosine followed by verapamil for conversion of paroxysmal supraventricular tachycardia.

5. Conclusion

In conclusion, this study contributes to elucidate the pharmacology of the ionic mechanisms responsible for adenosine chronoselectivity via adenosine A_1 receptors activation in spontaneously beating rat atria as compared with other cardiodepressant agents, like those activating muscarinic M_2 receptors and inhibiting voltage-sensitive Ca_v1 (L-type) channels. Activation of the adenosine A_1 receptor decreases SA node automatism mainly by promoting K^+ efflux through βγ subunits of G protein-coupled inwardly rectifying GIRK/$K_{IR}3$ channels. This effect may be counteracted by inhibition of $K_{Ca}2$/SK currents (probably via G protein α subunit) leading to a subsequent prolongation of atrial repolarization. The increase in the time available for Ca^{2+} influx through voltage-sensitive Ca_v1 (L-type) channels may be essential to sustain inotropy in the presence of the adenosine A_1 receptor agonist in concentrations causing significant negative chronotropic actions. We are aware that this hypothesis is not consensual therefore detailed electrophysiological studies are warranted to determine precisely whether or not activation of the adenosine A_1 receptor contributes to prolongation of action potentials in atrial cardiomyocytes. Although Ca^{2+} transients are certainly important determinants of atrial contractility, further studies are required to investigate how they integrate with mechanisms regulating the ultimate step of cardiac contractility, the myofilament Ca^{2+} sensitivity and cross-bridging, which may also conceivably be altered by A_1 receptors stimulation (see e.g.,

(Strang et al., 1995)). Even so, our findings might be of clinical relevance, as conversion of paroxysmal supraventricular tachycardia to sinus rhythm may involve different sequences of intravenous drugs administrations including adenosine (or its stable derivative, tecadenoson) and/or Ca_v1 (L-type) channel inhibitors (e.g., verapamil) (Lim et al., 2009). Thus, loss of adenosine chronoselectivity and atrial sensitization to the negative inotropic action of A_1 receptors by Ca_v1 (L-type) channel blockers may have deleterious effects in critical patients whenever adenosine is used concomitantly with verapamil.

PAPER 2

Front Pharmacol 2019 Sep 24;10:1103.
doi: 10.3389/fphar.2019.01103.

The ionotropic P2X4 receptor has unique properties in the heart by mediating the negative chronotropic effect of ATP while increasing the ventricular inotropy

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Abstract

Background: Mounting evidence indicates that reducing the sinoatrial node (SAN) activity may be a useful therapeutic strategy to control of heart failure. Purines, like ATP and its metabolite adenosine, consistently reduce the SAN spontaneous activity leading to negative cardiac chronotropy, with variable effects on the force of myocardial contraction (inotropy). Apart from adenosine A_1 receptors, the human SAN expresses high levels of ATP-sensitive ionotropic P2X4 receptors (P2X4R), yet their cardiac role is unexplored.

Methods: Here, we investigated the activity of P2 purinoceptors on isolated spontaneously beating atria (chronotropy) and on 2 Hz-paced right ventricular (RV, inotropy) strips from Wistar rats.

Results: ATP (pEC_{50}=4.05) and its stable analogue ATPγS (pEC_{50}=4.69) concentration-dependently reduced atrial chronotropy. Inhibition of ATP breakdown into adenosine by NTPDases with POM-1 failed to modify ATP-induced negative chronotropy. The effect of ATP on atrial rate was attenuated by a broad-spectrum P2 antagonist, PPADS, as well as by 5-BDBD, which selectively blocks the P2X4R subtype; however, no effect was observed upon blocking the A_1 receptor with DPCPX. The P2X4R positive allosteric modulator, ivermectin, increased the negative chronotropic response of ATP. Likewise, CTP, a P2X agonist that does not generate adenosine, replicated the P2X4R-mediated negative chronotropism of ATP. Inhibition of the Na^+/Ca^{2+} exchanger (NCX) with KB-R7943 and ORM-10103, but not blockage of the HCN channel with ZD7288, mimicked the effect of the P2X4R blocker, 5-BDBD. In paced RV strips, ATP caused a mild negative inotropic effect, which magnitude was 2 to 3-fold increased by 5-BDBD and KB-R7943. Immunofluorescence confocal microscopy studies confirm that cardiomyocytes of the rat SAN and RV co-express P2X4R and NCX1 proteins.

Conclusions: Data suggest that activation of ATP-sensitive P2X4R slows down heart rate by reducing the SAN activity while increasing the magnitude of ventricular contractions. The mechanism underlying the dual effect of ATP in the heart may involve inhibition of

intracellular Ca^{2+}-extrusion by bolstering NCX function in the reverse mode. Thus, targeting the P2X4R activation may create novel well-tolerated heart-rate lowering drugs with potential benefits in patients with deteriorated ventricular function.

1. Introduction

Heart rate is primarily set in the right atria by spontaneous generation of rhythmic actions potentials in the sinoatrial node (SAN) (Boyett et al., 2000). The autonomous activity of SAN cardiomyocytes is orchestrated by activation of several ion channels and regulating proteins, which interplay to generate effective action potentials in a regular time basis (Yaniv et al., 2015; Fabbri et al., 2017). The unstable resting membrane potential characteristic of SAN cardiomyocytes is mainly due to mutual influence of intracellular Ca^{2+} "clocks" and membrane potential oscillations. The unstable resting membrane potential and the spontaneous firing of SAN cardiomyocytes are mainly attributed to "funny" currents carried by hyperpolarization-activated cyclic nucleotide-gated channels (HCN) and by the electrogenic Na^{+}/Ca^{2+} exchanger (NCX) functioning in the forward Ca^{2+}-extrusion mode (Tsutsui et al., 2018; Vinogradova et al., 2018b). NCX operates as an integrator of intracellular Ca^{2+} with the membrane potential, in a way that it is responsible by a slow depolarizing current that drives the diastolic depolarization phase in response to calcium leakage from sarcoplasmatic reticulum and other subsidiary Ca^{2+} stores (Bogdanov et al., 2001; Sanders et al., 2006; Groenke et al., 2013; Herrmann et al., 2013). The NCX dynamically fluctuates between forward and reverse modes, thereby extruding or importing Ca^{2+} to subcellular regions, respectively (Samanta et al., 2018). Impairment of the NCX forward-mode by genetic ablation, pharmacological inhibition or even by simply changing its electrochemical gradient consistently induces bradycardia, supporting NCX as a key element in SAN pacemaker activity (Kurogouchi et al., 2000; Bogdanov et al., 2001; Sanders et al., 2006; Groenke et al., 2013; Herrmann et al., 2013).

The SAN, like other regions of the heart, is under control of countless number of signaling molecules, including adenosine triphosphate (ATP) and its derivatives, namely adenosine (Erlinge and Burnstock, 2008; Mangoni and Nargeot, 2008; Burnstock and Pelleg, 2015). Since the pioneering work of Drury and Szent-Gyorgyi almost a century ago (1929), the protective role of purine nucleotides and nucleosides as retaliatory mediators released in response to hypoxia and stressful stimuli in the vascular system has been expanded to the myocardium (Forrester and Williams, 1977), where purines engage energy-saving negative chronotropic, dromotropic and inotropic actions (Versprille and van Duyn, 1966; Lundberg et al., 1984; Pelleg et al., 1987; Belardinelli et al., 1995). Apart from cellular damage, ATP release is not stochastic but rather a fine regulated process (Lazarowski, 2012), involving (1) electrodiffusional movement through membrane ion

channels, including pannexin- and connexin-containing hemichannels; (2) facilitated diffusion by nucleotide-specific ATP-binding cassette (ABC) transporters; and (3) cargo-vesicle trafficking and exocytotic granule secretion (Clarke et al., 2009; Pinheiro et al., 2013a; Pinheiro et al., 2013b; Timoteo et al., 2014).

Once in the extracellular milieu, ATP builds up its effects through activation of P2 purinoceptors, which comprise seven ionotropic (P2X1-7) and eight metabotropic ($P2Y_{1,2,4,6,11,12,13,14}$) receptors. Extracellular ATP can also indirectly activate purinoceptors of the P1 family (A_1, A_{2A}, A_{2B} and A_3) after its conversion into adenosine by cascade of ectonucleoside triphosphate diphosphohydrolases (NTPDases) (Yegutkin, 2008). Intravenous ATP produces negative chronotropy, as well as several other acute cardiovascular effects that resembles adenosine application, almost neglecting ATP-sensitive receptors as active and functional regulators of the cardiac function (Pelleg and Belhassen, 2010). Apart from a massive presence of adenosine A_1 receptors (Braganca et al., 2016), the SAN from both rodents and humans expresses several P2 purinoceptor subtypes, including ionotropic P2X4, P2X7, and metabotropic $P2Y_1$, $P2Y_2$ and $P2Y_{14}$ (Musa et al., 2009), yet their role is still largely unknown. Besides species differences, the type and relative abundance of P2 purinoceptors in the heart varies with location and disease conditions (Musa et al., 2009).

Relatively recent evidence called our attention showing that the highly expressed ionotropic P2X4 in the human SAN may be an important modulator of NCX function (Shen et al., 2014). Activation of ionotropic P2X receptors, including the P2X4, triggers the influx of Na^+ that is subsequently exchanged by Ca^{2+} via the NCX (Jarvis and Khakh, 2009). Using various models of heart failure in rodents, it has been demonstrated that reversion of the NCX function by P2X4 activation may lead to improvements of ventricular performance and clinical outcome (Hu et al., 2001; Mei and Liang, 2001; Yang et al., 2004; Yang et al., 2014; Yang et al., 2015). Indeed, pharmacological inhibition of Na^+/K^+-ATPase with digitalis interferes with cardiac NCX function, resulting in both negative chronotropy and positive inotropy, thus explaining the rationale for usage of digitalis-like drugs in the management of patients with tachyarrhythmias and ventricular contractile incompetence (Blomstrom-Lundqvist et al., 2003a; Ponikowski et al., 2016).

In this study, we aimed at characterizing the effect of the P2X4 on heart rate using isolated spontaneously beating rat atria strips. Taking into consideration that most currently available negative chronotropic drugs are associated with a negative inotropic impact as a major drawback (Ponikowski et al., 2016), experiments were designed to assess the effect of the P2X4 on the magnitude of paced ventricular contractions.

2. Material and Methods

2.1. Animals

Animal care and experimental procedures were conducted in strict accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123), Directive 2010/63/EU and Portuguese rules (DL 113/2013). All experimental protocols involving animals were approved by the competent national authority Direção Geral de Alimentação e Veterinária, and by the ICBAS Animal Ethical Committee (No. 224/2017). All efforts were made to minimize animal suffering and to reduce the number of animals used according to the ARRIVE guidelines. Wistar rats (*Rattus norvegicus*; 250-300 g; Charles River, Barcelona, Spain) of either sex were kept at a constant temperature (21ºC) and a regular light (06:30-19:30 h) – dark (19:30-06:30 h) cycle, with food and water provided *ad libitium*.

2.2. Isolated spontaneously beating atria

Isolated spontaneously beating atria were prepared using a previously described method (Braganca et al., 2016), with some modifications. In brief, hearts were rapidly excised after decapitation followed by exsanguination (Rodent guillotine, Stoelting 51330), and placed in a physiological solution (Tyrode's solution) composed of (mM): NaCl 137; KCl 4.7; $CaCl_2$ 1.8; $MgCl_2$ 1; NaH_2PO_4 0.4; $NaHCO_3$ 11.9; glucose 11.2 and gassed with 95% O_2 + 5% CO_2 (at pH 7.4). Hearts were allowed to beat freely for a few seconds at room temperature, to empty its blood content. The paired rat atria with the SAN region were dissected out, cleaned of fatty tissues, and suspended in a 14-mL organ bath containing gassed Tyrode's solution at 37ºC. Each auricular appendage was tied and connected with thread to the organ bath wall and to an isometric force transducer (MLT050/D; AD Instruments, Colorado Springs, CO, USA). Changes in isometric tension were recorded continuously using a *PowerLab* data acquisition system (Chart 5, version 4.2; AD Instruments, Colorado Springs, CO, USA). The preparations were allowed to equilibrate for 30–40 min. During this time, the preparations were continuously superfused with Tyrode's solution (1 mL/min) and the tension was adjusted to 9.8 mN. This procedure allows atria (with intact SA node) to progressively recover rhythmic spontaneous beatings (average of 247±5 beats min^{-1} at the beginning of the experimental protocol, n=79); preparations with spontaneous atrial rate below 200 beats min^{-1} or exhibiting rhythm variations above 10 beats min^{-1} during equilibrium were discarded to ensure measurements were made in atria with intact primary pacemaker SAN activity. None of the preparations exhibited noticeable signs of ectopic-activity caused by secondary pacemakers, usually related to asynchronous and abnormal contractions.

Under these experimental conditions, spontaneously beating rat atria respond to muscarinic and β-adrenergic stimulation, but are unaffected by the application of atropine or atenolol alone used in concentrations high enough (1 μM and 3 μM, respectively) to prevent the effects of acetylcholine (100 μM) and isoproterenol (30 nM), respectively (data not shown). Thus, myographic recordings reported in this study include rate (chronotropic effect) and contractile force (inotropic effect) of spontaneously beating atria measured in the absence of cholinergic and/or adrenergic tone.

2.3. Isolated paced right ventricle strips

Following the isolation procedures described above for spontaneously beating atria, we also obtained right ventricular (RV) strips (2 mm wide, 8-10 mm long and 1.5 mm thick) by cutting RV-free wall longitudinally to its surface. A pair of ventricular strips was used from each right ventricle. RV strip ends were tied and connected with thread to the 14-mL organ bath hook and to an isometric force transducer (MLT050/D; AD Instruments, Colorado Springs, CO, USA). Changes in the isometric tension of RV strips, measured both by the active tension (mN/mg of wet tissue weight) and by the derivative of developed force over time (+dF/dt, mN/s), were tested at a fixed frequency of 120 beats per min commanded by electric field stimulation of the preparations, so that inotropy was measured without being affected by concurrent changes in chronotropy. Electric pacing (2 Hz, +50% voltage above threshold, 2 ms) was generated by independent Grass S48 stimulators (Quincy, MA, USA) and delivered via two platinum electrodes positioned on each side of the preparations. Equilibrium of the preparations was performed as described above for spontaneously beating atria. Only ventricular preparations exhibiting rhythmic contractions with similar amplitude were used.

2.4. Experimental design

After reaching a steady-state, the Tyrode's solution flow through the organ bath was stopped and the preparations were incubated for an additional period of 15 min before drug applications. The concentration-response curves for ATP and related nucleotides were performed by non-cumulative application of increasing concentrations of the nucleotides during 5 min followed by a washout period with Tyrode's solution (15 mL/min) to avoid biases resulting from accumulation of their metabolites and to prevent receptors desensitization. To shorten the experimental duration and, thus, to increase results reproducibility, the majority of the preparations were incubated for 5 min with a fixed concentration (near the EC_{50} value) of the nucleotide either in the absence and in the presence of drug modulator (e.g. receptor antagonist, channel inhibitor); the latter contacted with the preparations at least for 15 min before application of the nucleotide and we kept 2-

hour washout intervals between testing again the same nucleotide to exclude biases related to P2 purinoceptors desensitization (controls not shown). To avoid damage of RV strips performance by prolonged pacing at 2 Hz, parallel experiments were performed using the two strips coming from the same animal to test the effect of ATP in the absence and in the presence of any modulator.

2.5. Immunofluorescence staining and confocal microscopy studies

Rat hearts were excised (see above) and placed in oxygenated Tyrode's solution at 33-34ºC. Following heart excision, the right atrium (RA) containing the SAN region and RV were accurately isolated through the interauricular and interventricular septa and clean from tissue debris. Tissue fragments were placed over a small lung lobule fragment with the endocardial layer facing down, stretched to all directions, pinned flat onto cork slices and embedded in Shandon cryomatrix (Thermo Scientific) before frozen in a liquid nitrogen-isopentane; frozen samples were stored at -80ºC until use. Frozen sections with 8 µm thickness were cut perpendicular to the crista terminalis of the RA and parallel to the long axis in the case of RV (see (Braganca et al., 2016)). Following fixation, the preparations were washed three times for 10 min each using 0.1 M PBS and incubated with a blocking buffer, consisting in fetal bovine serum 10%, bovine serum albumin 1%, Triton X-100 0.3% in PBS, for 2 h. After blocking and permeabilization, samples were incubated with selected primary antibodies (Table 6) diluted in incubation buffer (fetal bovine serum 5%, serum albumin 1%, Triton X-100 0.3% in PBS), overnight at 4ºC. For double immunostaining, antibodies were combined before application to tissue samples. Following the washout of primary antibodies with PBS (3 cycles of 10 min) tissue samples were incubated with species-specific secondary antibodies (Table 6) in the dark for two hours, at room temperature. Finally, VectaShield mounting medium with 4´-6-diamidino-2-phenylindole (DAPI) to stain the nuclei (H-1200; Vector Labs) was used, before cover-slipping the glass slides. Observations were performed and analyzed with a laser-scanning confocal microscope (Olympus FluoView, FV1000, Tokyo, Japan).

Table 6. List of primary and secondary antibodies used in immunohistochemistry experiments.

Antigen	Code	Host	Dilution	Supplier
Primary antibodies				
Cx43	Ab11370	Rabbit (rb)	1:700	Abcam
NF-160	Ab7794	Mouse (ms)	1:1000	Abcam
HCN4	Agp-004	Guinea-pig (gp)	1:150	Alomone
P2X4 (C-terminus)	Apr-002	Rabbit (rb)	1:200	Alomone
P2X4 (extracell. loop)	Apr-024	Rabbit (rb)	1:200	Alomone
NCX1	Anx-011	Rabbit (rb)	1:50	Alomone
Secondary antibodies				
Alexa Fluor 488 anti-rb	A-21206	Donkey	1:1500	Molecular probes
Alexa Fluor 568 anti-ms	A-10037	Donkey	1:1500	Molecular probes
TRITC 568 anti-gp	706-025-148	Donkey	1:150	Jackson Immuno Res.

The SAN was characterized as described in a previous study from our group (Braganca et al., 2016). SAN identification was also facilitated by observation of the sinus node artery surrounded by small-size cardiomyocytes positive for the hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) and negative against connexin-43 (Cx43), a gap junction protein ubiquitously expressed in the heart apart from in nodal tissue. In some of the experiments, the existence of a large number of neurofilament 160 (NF-160) positive neuronal fibers was also used to identify the SAN region (Tellez et al., 2006; Braganca et al., 2016).

2.6. Solutions and Chemicals

Adenosine-5′-triphosphate (ATP); adenosine 5′-[γ-thio]-triphosphate (ATPγS); cytidine-5'-triphosphate (CTP); 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX); 22,23-dihydroavermectin B1 (ivermectin); and 2-[(3,4-dihydro-2-phenyl-2H-1-benzopyran-6-yl)oxy]-5-nitro-pyridine (ORM-10103) were obtained from Sigma (Poole, U.K.). 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one (5-BDBD); 3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine hydrochloride (A438079); 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate (KR-R7943) and sodium metatungstate (POM-1) were from Tocris Cookson Inc. (Bristol, UK). Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and ZD7288 were from Ascent Scientific (Bristol, UK). Dimethylsulfoxide (DMSO), serum albumin and Triton X-100 were from Merck (Darmstadt, Germany). DPCPX was made up in 99% DMSO/1% NaOH 1 mM (v/v); 5-BDBD, A438079, Ivermectin, ORM-10103, KB-R7943 were made up in DMSO. Other drugs were prepared in distilled water. All stock solutions were stored as frozen aliquots at −20°C. Dilutions of these stock solutions were made daily and kept protected from the light to prevent photodecomposition. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used (0.5% v/v), were observed. The pH of the Tyrode's solution did not change by the addition of the drugs in the maximum concentrations applied to the preparations.

2.7. Presentation of data and statistical analysis

The isometric contractions were recorded and analyzed before and after the addition of each drug at the desired concentration. Results were presented as percentages of variation compared to baseline (% Δ baseline), obtained before application of the test drug. Data are expressed as mean ± SEM, with *n* indicating the number of animals used for a particular group of experiments. Graphical data are expressed as box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage of variation from baseline. Since modulation of ATP-mediated responses were unpredictable,

we did not apply any power calculations to pre-determine sample size, thus we pre-specified a number of 4-7 experiments for each condition. Concentration-response curves were analyzed by fitting four-parameter logistic sigmoidal functions to the experimental data to estimate pEC_{50} for negative chronotropy and inotropy of the nucleotides. All curve fitting procedures, graphical and statistical analyses were carried out using GraphPad Prism 7.04 for Windows software (La Jolla, USA). Spontaneous or electrically-evoked mechanical tension (inotropic effect) and contraction rate (chronotropic effect) were evaluated using the Student's *t*-test for paired samples assuming a Gaussian distribution of data. Given that significant variability (ranging from 15 to 35%) was observed amongst animals for the negative chronotropic action of ATP (100 µM), changes on the nucleotide effect in the presence of a modulator was always compared to its absence in the same animal or preparation to make differences between paired values consistent. A value of $p<0.05$ was considered to represent a significant difference.

3. Results

3.1. Effects of ATP on sinoatrial chronotropy and right ventricular inotropy

Non-cumulative application of ATP (0.001-1 mM) concentration-dependently decreased atrial chronotropy and right ventricular inotropy (Figure 19). The onset of ATP response was readily visible in about 30 s for both myocardial preparations; it reached a sustained maximal effect roughly 1 min after application and lasted while the nucleotide was kept in the incubation fluid, *i.e.* at least for 5 min. Spontaneously beating atria were slightly more sensitive ($p<0.05$) to the inhibitory effect of ATP compared to paced RV strips (Figure 19C). The estimated pEC_{50} for the negative chronotropic and inotropic ATP responses were 4.05 and 3.45, respectively. Of note, ATP exhibited a biphasic effect on atrial inotropy, which was characterized by an initial decrease in the magnitude of atrial contractions followed by a gradual recovery to levels above the baseline (Figure 19A), as reported by other authors (Froldi et al., 1994; Gergs et al., 2008b).

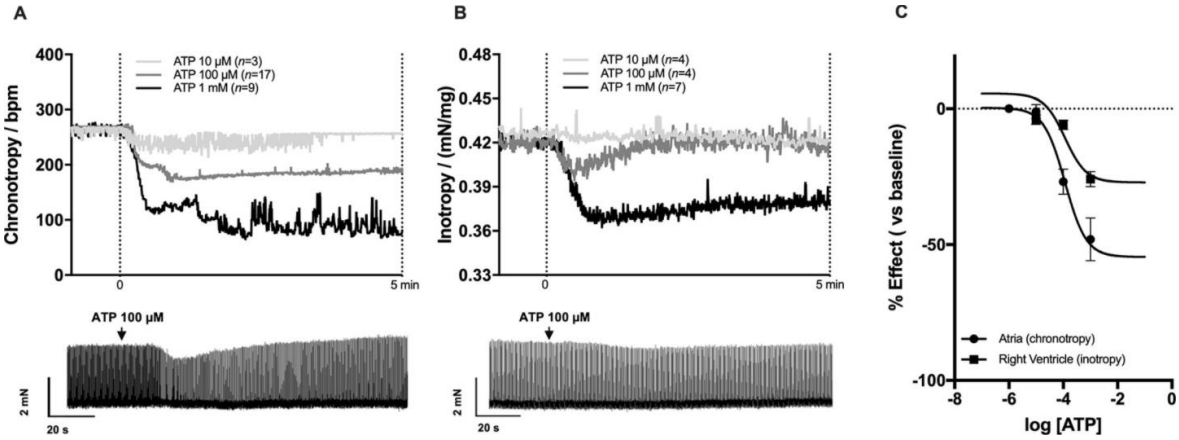

Figure 19. Effects of ATP on spontaneously beating atria (A, chronotropy, beats min−1) and 2 Hz-paced right ventricular (B, inotropy, mN/mg of tissue) strips from Wistar rats. ATP (0.01–1 mM) was applied non-cumulatively for 5 min followed by a washout period to avoid biases resulting from bath accumulation of metabolites and from receptors desensitization. Upper panels (A) and (B) show average values; bottom panels show typical myographical recordings of atrial and ventricular preparations challenged with ATP (100 µM). Panel (C) shows the concentration-response curves for ATP (0.001–1 mM) fitted by four-parameter logistic sigmoidal functions used to estimate pEC50 values for the negative chronotropic and inotropic effects of the nucleotide. Data are expressed as mean ± SEM from an n number of animals indicated in upper panels (A) and (B), respectively.

3.2. The negative chronotropic effect of ATP depends on P2 purinoceptors activation

The negative chronotropic effect of ATP following intravenous application of the nucleotide resembles that obtained after administration of adenosine (Pelleg and Belhassen, 2010), the end product of ATP hydrolysis by the ectonucleotidase cascade (Cardoso et al., 2015). To know whether ATP is acting directly on P2 purinoceptors or indirectly via P1 receptors after its extracellular conversion into adenosine, we tested the effect of ATPγS, an enzymatically-stable ATP analogue. ATPγS (100 µM) decreased sinoatrial chronotropy (-19±5%, n=6) by a similar extent to that observed for ATP (100 µM, -18±5%, n=6) (Figure 20A); the estimated pEC_{50} for the negative chronotropic effect of ATPγS was 4.69 (Figure 21A). Moreover, the broad-spectrum P2 receptor antagonist PPADS (10 µM) significantly attenuated the negative chronotropic response of ATP (-20±2% vs -11±4%, n=7, p<0.001) (Figure 20B); the blocking effect of PPADS was more evident upon increasing the concentration of the P2 receptor antagonist to 100 µM (-17±4% vs -2±2%, n=4) (see Figure 21D).

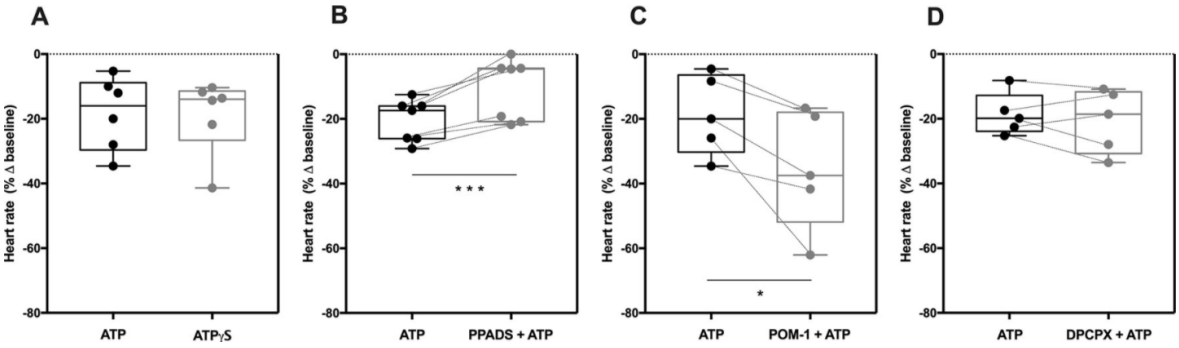

Figure 20. The negative chronotropic effect of ATP depends on P2 purinoceptors activation. The negative chronotropic effect of ATP (100 µM) was tested either in the absence or in the presence of the non-selective P2 receptor antagonist, PPADS (10 µM, B), the NTPDase inhibitor, POM-1 (100 µM, C), and the selective adenosine A1 receptor antagonist, DPCPX (3 nM, D). The negative chronotropic effect of the enzymatically stable ATP analogue, ATPγS (100 µM, A), is also shown for comparison. Represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage (%) of variation from baseline; horizontal lines inside boxes indicate the corresponding medians. Each data point represents the result of a single experiment; data from the same experiment are connected by lines. *p < 0.05, ***p < 0.001 (Student's t-test for paired samples) represent significant differences when compared to the effect of ATP alone.

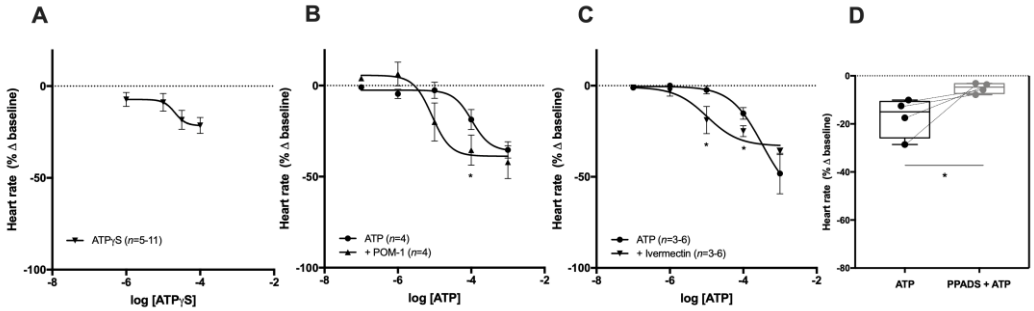

Figure 21- ATP (0.1 µM–1 mM) concentration-dependently decrease the spontaneous atrial rate. The negative chronotropic effect of ATP was mimicked by its enzymatically stable analogue, ATPγS (0.001–0.1 mM, A), and its potency was increased after pretreatment of the preparations with POM-1 (100 µM, a non-selective NTPDase inhibitor, B) and with ivermectin (30 µM, a positive allosteric modulator of the P2X4 receptor, C). For comparison purposes, we show in panel D that ATP (100 µM)-induced negative chronotropy was fully blocked by PPADS (100 µM). In panels A–C, data are expressed as mean ± SEM from an n number of animals. In panel D, represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage (%) of variation from baseline; horizontal lines inside boxes indicate the corresponding medians; each data point represents the result of a single experiment; data from the same experiment are connected by lines. *p < 0.05 (Student's t-test for paired samples) represent significant differences when compared to the effect of ATP alone.

Blockage of ATP breakdown by NTPDases with POM-1 (100 µM) significantly (p<0.05) potentiated the negative chronotropic response of ATP (100 µM) (-19±6% vs -35±8%, n=5, p<0.05) (Figure 20C), whereas the selective adenosine A_1 receptor antagonist, DPCPX (3 nM) (Lohse et al., 1987), was ineffective (-19±3% vs -21±4%, n=5; p>0.05) (Figure 20D). Indeed, the NTPDase inhibitor, POM-1 (100 µM), shifted to the left (pEC_{50}=5.10; p<0.05) the concentration-response curve of ATP (0.001-1 mM) without significantly modifying the Hill slope (see Figure 21B).

On their own, PPADS (10 μM and 100 μM), POM-1 (100 μM) and DPCPX (3 nM) were virtually devoid of effect on spontaneous atrial beating rate (see Figure 22). It is also worth noting that blockage of muscarinic ACh receptors with atropine (1 μM) did not modify the negative chronotropic effect of ATP (100 μM) (data not shown), ruling out putative changes in the cholinergic tone operated by ATP.

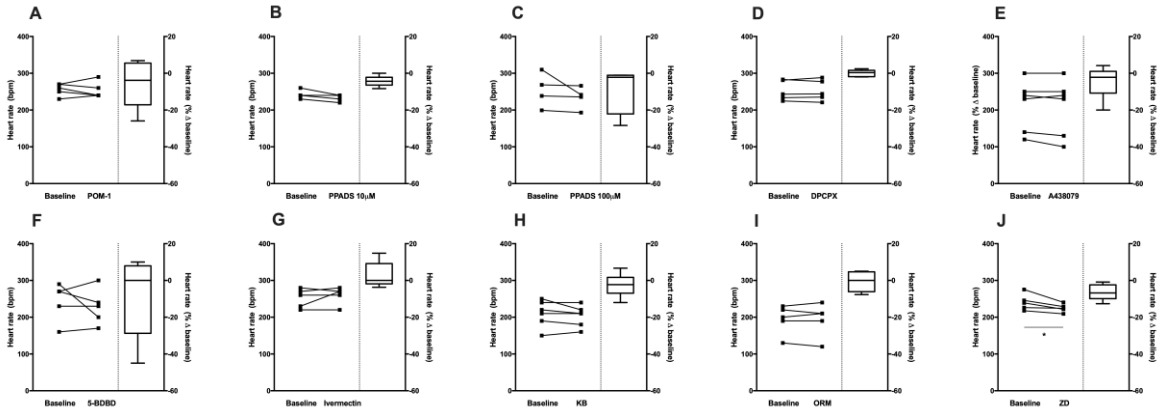

Figure 22. Effects of receptor antagonists/allosteric modulators, NTPDase inhibitor and ion transport blockers on spontaneously beating rat atria (A–J). POM-1 (100 μM, A), PPADS (10 μM, B; 100 μM, C), DPCPX (3 nM, D), A438079 (3 μM, E), 5-BDBD (10 μM, F), ivermectin (30 μM, G), KB-R7943 (3 μM, H), ORM-10103 (3 μM, I), and ZD7288 (300nM, J) contacted with the preparations at least for 15 min; the rate of spontaneous atrial contractions (beats min−1) was measured immediately before ATP applications (see Figures 20, 23 and 24) and compared to baseline conditions in the absence of any drug. Each data point represents the result of a single experiment; points from the same experiment are connected by lines. On the right hand-side of each panel, represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage (%) of variation from baseline; horizontal lines inside boxes indicate the corresponding medians. *p < 0.05 (Student's t-test for paired samples) represent significant differences when compared to baseline.

3.3. The negative chronotropic effect of ATP is mediated by P2X4 receptors activation

The P2 purinoceptors expression in the SAN is species specific. For instance, in humans the rank order of expression of ionotropic P2X receptors is the following: P2X4>P2X7>>P2X1>P2X5 (P2X2 and P2X3 are absent), while in rats it is P2X5>>P2X7>>P2X4~P2X1~P2X2>P2X3 (Musa et al., 2009). Regrettably, there are no specific pharmacologic agonists or antagonists to the P2X5 receptor. It is worth noting that the P2X7 receptor is negatively modulated by extracellular Ca^{2+} and shows low affinity (0.1–1mM) for ATP. This contrasts with the estimated EC_{50} values in the low micromolar range for ATP and ATPγS that is characteristic of the most abundant P2X4 receptor in the human SAN (Soto et al., 1996) (Michel et al., 1997). Interestingly, both P2X4 and P2X7 receptor pores are able to translocate extensive amounts of Na^+ into the cells. These premises

prompted us to test whether these receptors could be involved in the negative chronotropic effect of ATP.

Selective blockage of the P2X7 receptor with A438079 (3 µM, Figure 23A) failed to modify the negative chronotropy effect of ATP (100 µM) (-31±5% vs -26±9%, n=6, p>0.05), whereas the potent and selective P2X4 receptor antagonist, 5-BDBD (10 µM) (Coddou et al., 2019), significantly attenuated ATP-induced negative chronotropism (-31±7% vs -17±5%, n=6, p<0.05) (Figure 23B). The negative chronotropic action of ATP (100 µM) was potentiated by ivermectin (30 µM) (-16±3% vs -25±3%, n=5, p<0.05) (Figure 23C), a drug that acts as positive allosteric modulator of the P2X4 receptor via a dual mechanism that involves potentiation and delayed inactivation of its currents, exhibiting selectivity over other P2X receptors (Khakh et al., 1999b). The concentration-response curve of ATP (0.001-1 mM) was shifted to the left (pEC_{50}=4.99; p<0.05) by ivermectin (30 µM) compared to the effect of ATP alone (see Figure 21C). Please note that, on their own, A438079 (3 µM, -5±3%, n=6), 5-BDBD (10 µM, -8±9%, n=5) and ivermectin (30 µM, 3±3%, n=5) were virtually devoid of effect on the spontaneous atrial frequency (see Figure 22).

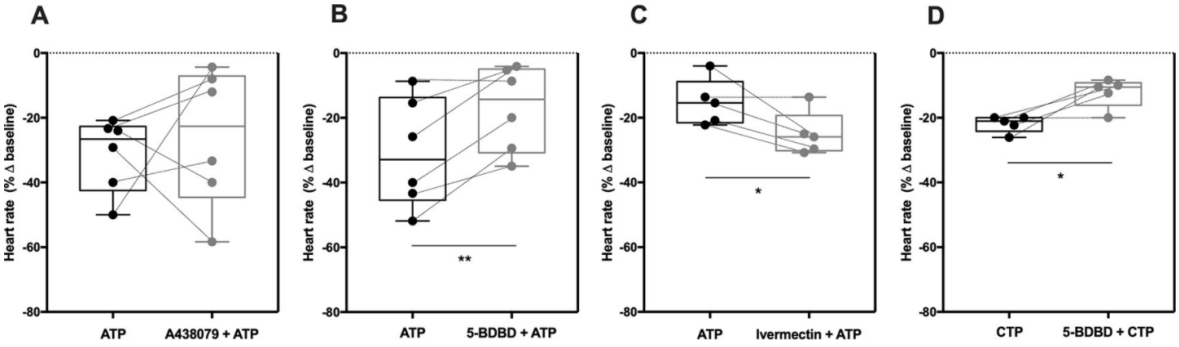

Figure 23. The negative chronotropic effect of ATP is mediated by activation of P2X4. The negative chronotropic effect of ATP (100 µM) was tested either in the absence or in the presence of the P2X7 receptor antagonist, A438079 (3 µM, A), the P2X4 receptor antagonist, 5-BDBD (10 µM, B) and the positive allosteric modulator of the P2X4 receptor, ivermectin (30 µM, C). The negative chronotropic effect of CTP (1 mM, D) either in the absence or in the presence of 5-BDBD (10 µM), is also shown for comparison. Represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage (%) of variation from baseline; horizontal lines inside boxes indicate the corresponding medians. Each data point represents the result of a single experiment; data from the same experiment are connected by lines. *p < 0.05, **p < 0.01 (Student's t-test for paired samples) represent significant differences when compared to the effects of ATP or CTP alone, respectively.

In order to further explore the potential involvement of the P2X4 receptor, we used CTP as a preferential P2X receptor agonist whose hydrolysis does not directly generate adenosine or other adenine nucleotides. Despite the fact that the P2X4 receptor exhibits low affinity for CTP compared to ATP (Soto et al., 1996; Kasuya et al., 2017), CTP (1 mM) decreased the spontaneous atrial rate by 22±1% (n=5) and this effect was also antagonized by 5-BDBD (10 µM, -12±2%, n=5, p<0.05) (Figure 23D).

3.4. The P2X4-mediated negative chronotropic effect of ATP involves NCX, but not HCN

ATP binding to the P2X4 receptor dramatically increases Na^+ and Ca^{2+} influx through the receptor pore, which may interfere with NCX function as one of the sarcolemma controllers of the SAN pacemaker activity (Shen et al., 2014). Besides NCX, the normal sinus rhythm also depends on HCN channels mediating I_f currents (Bogdanov et al., 2001; Sanders et al., 2006; Groenke et al., 2013; Herrmann et al., 2013). Therefore, we thought it was relevant to evaluate the P2X4 receptor influence on downstream activation of NCX and/or HCN membrane transporters, which are essential to control heart rate. To this end, we used two different compounds known to inhibit NCX activity, namely KB-R7943 and the recently developed ORM-10103 (Jost et al., 2013). Since both inhibitors have putative negative chronotropic actions, we performed concentration-response curves to determine the minimal concentration beyond that reduction of chronotropy would be a problem in interaction experiments (data not shown). Incubations of KB-R7943 and ORM-10103 for 15 min at a final concentration of 3 µM had no effect on spontaneously beating atria strips (-3±3%, n=6, p>0.05 vs baseline; -1±2%, n=5, p>0.05 vs baseline; respectively) (Figure 22).

The negative chronotropic effect of ATP (100 µM) was attenuated by KB-R7943 (3 µM; -19±4% vs -8±3%, n=6, p<0.05) and by ORM-10103 (3 µM; -29±4% vs -17±5%, n=5, p<0.05) (Figure 24A, B); the inhibitory effect of these compounds had a similar magnitude to that observed with the P2X4 antagonist, 5-BDBD (10 µM) (see Figure 23B). Pre-incubation with the ivabradine-like HCN channel inhibitor ZD7288 (300 nM) did not significantly modify the chronotropic effect of ATP (100 µM) (-12±2% vs -11±3%, n=5, p>0.05) (Figure 24C). On its own, ZD7288 (300 nM) decreased the spontaneous atrial rate only by 6±2% below the control (n=5, p<0.05) (Figure 22).

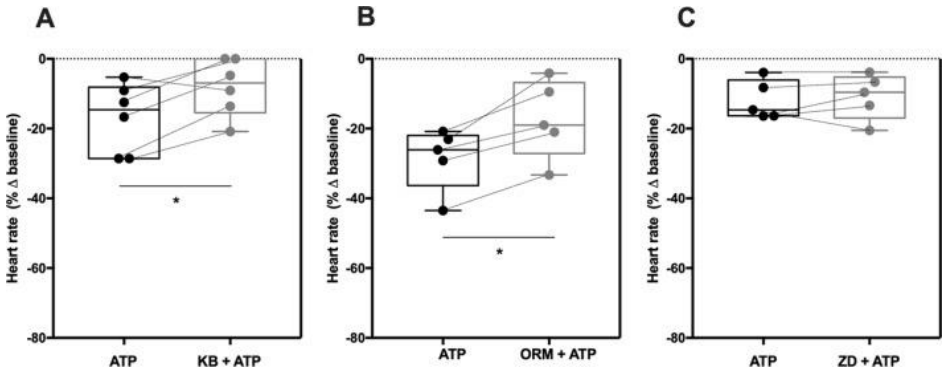

Figure 24. The negative chronotropic effect of ATP involves ion exchange via pacemaker NCX, but not HCN. ATP (100 µM)-induced negative chronotropism was tested either in the absence or in the presence of two NCX inhibitors, KB-R7943 (3 µM, A) and ORM-10103 (3 µM, B), and of a HCN channel inhibitor, ZD7288 (300 nM, C). Represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage (%) of variation from baseline; horizontal lines inside boxes indicate the corresponding medians. Each data point represents the result of a single experiment; data from the same experiment are connected by lines. *p < 0.05

(Student's t-test for paired samples) represent significant differences when compared to the effect of ATP alone.

3.5. P2X4-induced NCX transport reversal counteracts the negative inotropic effect of ATP in paced ventricular strips

Apart from digitalis, heart rate slowing drugs used in clinical practice decrease cardiac inotropism as a major drawback (Ponikowski et al., 2016). As shown in Figure 19B, ATP (0.001-1 mM) concentration-dependently decreased the amplitude of paced RV contractions. Figure 25 shows that blockage of P2X4 receptors with 5-BDBD (10 µM) augmented the negative inotropic effect of ATP (100 µM) measuring the percent variation of the active tension (-6±2% vs -19±5%, n=5, p<0.05; Figure 25A) or of the derivative of developed force over time (+dF/dt) (-4±2% vs -18±6%, n=5, p<0.05; Figure 25B) in paced RV strips. The effect of 5-BDBD (10 µM) was mimicked by KB-R7943 (3 µM), *i.e.* inhibition of NCX sensitized RV strips to the negative inotropic effect of ATP (100 µM) calculated also measuring the percent variation of the active tension (-5±2% vs -19±5%, n=5, p<0.05; Figure 25C) or of the derivative of developed force over time (+dF/dt) (-2±2% vs -12±4%, n=5, p<0.05; Figure 25D). On their own, 5-BDBD (10 µM) and KB-R7943 (3 µM) marginally reduced ventricular inotropy by 14±4% (n=5, p<0.05) and by 11±19% (n=5, p>0.05), respectively (Figure 26). These findings suggest that activation of the P2X4 partially counteracts the negative inotropic effect of ATP probably by reversing the NCX electrogenic current to pump Na^+ out and Ca^{2+} into ventricular cardiomyocytes.

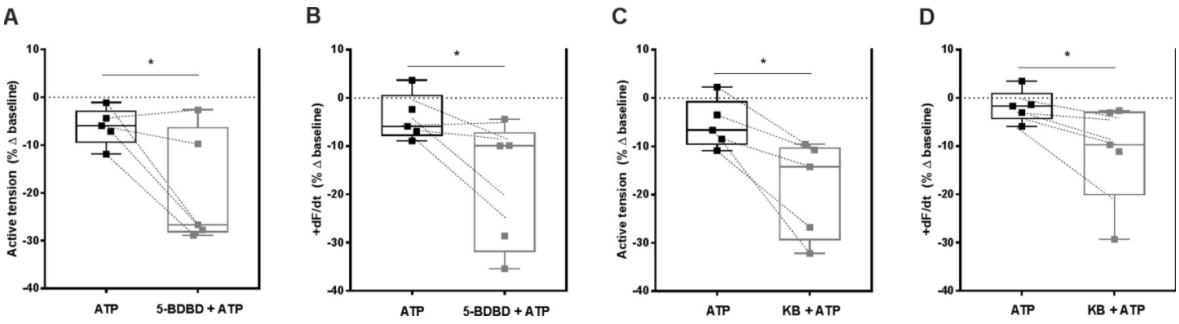

Figure 25. Selective blockage of P2X4 and of NCX transporter partially offsets the negative inotropic effect of ATP in paced rat ventricular strips. The negative inotropic effect of ATP (100 µM) was tested either in the absence or in the presence of the P2X4 receptor antagonist, 5-BDBD (10 µM, A and B) and of the NCX inhibitor, KB-R7943 (3 µM, C and D). Represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage (%) of variation from the baseline isometric tension of RV strips, measured as the active tension (mN/mg of wet tissue weight, panels A and C) and the derivative of developed force over time (+dF/dt, mN/s, panels B and D); horizontal lines inside boxes indicate the corresponding medians. Each data point represents the result of a single experiment; data from the same experiment are connected by lines. *p < 0.05 (Student's t-test for paired samples) represent significant differences when compared to the effect of ATP alone.

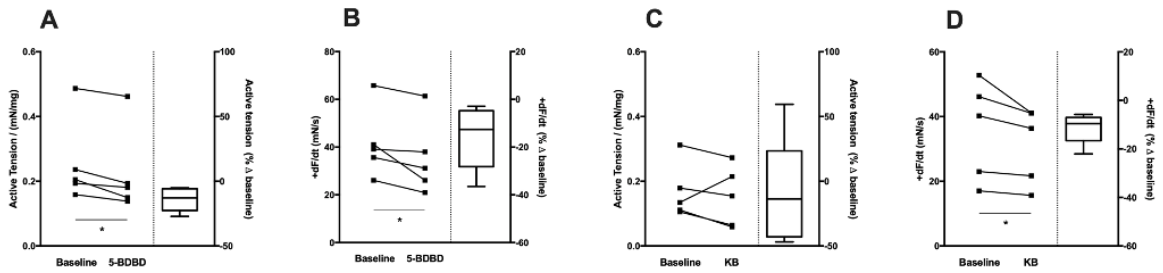

Figure 26. Effects of the P2X4 receptor antagonist, 5-BDBD (10 µM, A and B) and of the NCX inhibitor, KB-R7943 (3 µM, C and D) on paced right ventricular contractions. Drugs contacted with the preparations at least for 15 min; the amplitude of 2 Hz-paced ventricular contractions measured as active tension (mN/mg of wet tissue weight, panels A and C) and as the derivative of developed force over time (+dF/dt, mN/s, panels B and D) was measured immediately before ATP applications (see Figure 25) and compared to baseline conditions in the absence of any drug. Each data point represents the result of a single experiment; points from the same experiment are connected by lines. On the right hand-side of each panel, represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values calculated as a percentage (%) of variation from baseline; horizontal lines inside boxes indicate the corresponding medians. *p < 0.05 (Student's *t*-test for paired samples) represent significant differences when compared to baseline.

3.6. Localization of P2X4, NCX1 and HCN4 proteins in the rat heart

Confocal micrographs shown in Figure 27 demonstrate that P2X4 receptor protein is expressed in the plasma membrane of cardiomyocytes of all assayed regions of the rat heart; in these experiments we used a knockout validated antibody targeting the amino acid residues 370-388 of the C-terminus of the rat P2X4 receptor (Apr-002 from Alomone). Using tissues prepared in identical conditions and visualized with the same acquisition settings, one may conclude that the P2X4 receptor expression is higher in the SAN followed by the RV and RA. This regional difference was confirmed using a distinct antibody targeting amino acid residues 301-313 of the extracellular loop of the rat P2X4 receptor (Apr-024 from Alomone) (Figure 28).

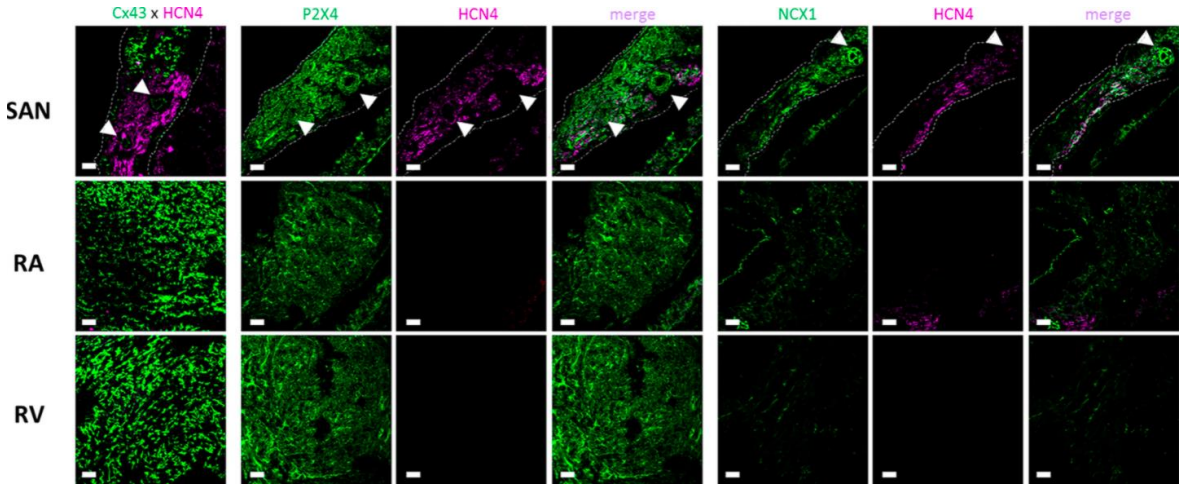

Figure 27. Representative confocal micrographs showing the immunolocalization of the P2X4 receptor (Apr-002, C-terminus, Alomone) and NCX1 (Anx-011, Alomone) protein in the sinoatrial node (SAN), right atria (RA) and right ventricle (RV). The SAN was identified based on its low Cx43 (green) and high HCN4 (magenta) protein expression (left hand-side images). Images were taken

from whole-mount heart preparations including the three analyzed regions, SAN, RA and RV. Dashed lines represent boundaries of the SAN. The pulmonary parenchyma was used as a structural support to facilitate immunostaining of myocardial sections and it is visible in the bottom right quadrant of each SAN image. White arrows indicate blood vessels including the SAN artery. Scale bar 30 µm. Images are representative of three different individuals.

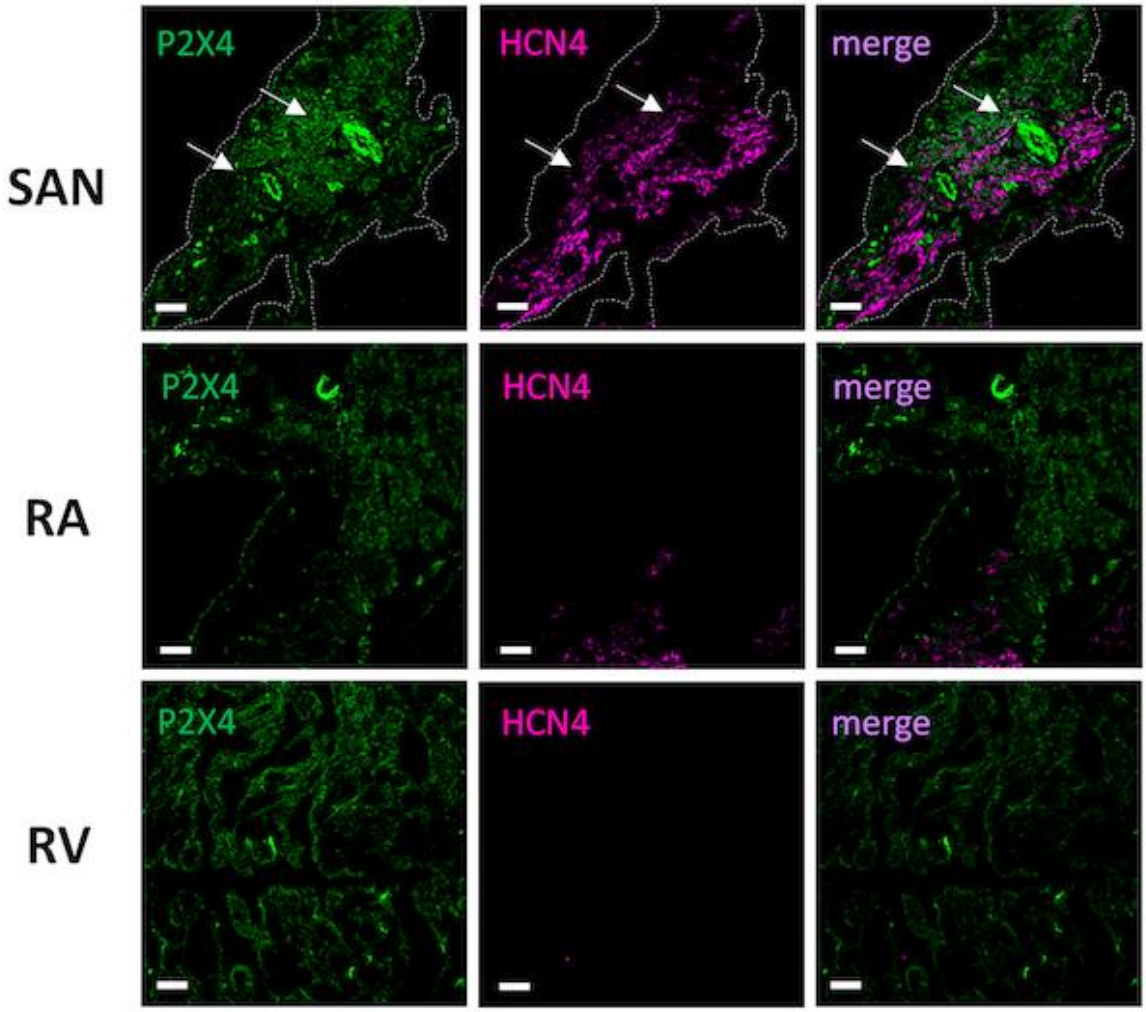

Figure 28. Representative confocal micrographs showing the immunolocalization of the P2X4 receptor (Apr-024, extracellular loop, Alomone) and of the HCN4 channel (Agp-004, Alomone) in the rat sinoatrial node (SAN); images obtained in right atria (RA) and right ventricle (RV) are also shown for comparison. It is worth noting that while the SAN was positive for both markers, RA and RV were positive for the P2X4 receptor (green) but negative for the HCN4 (magenta). Images were taken from one whole-mount preparation of the rat heart including the three regions, SAN, RA and RV. Dashed lines represent boundaries of the SAN region. White arrows indicate blood vessels including the SAN artery. Scale bar 30 µm.

The immunoreactivity against NCX1 protein followed the same staining pattern to that found for the P2X4 receptor; the strongest immunofluorescence signal was also found in the SAN followed by other regions of the rat heart (Figure 27). Cardiomyocytes of the SAN region staining positively against NCX1 also exhibit immunoreactivity against the HCN4 protein. The same occurred regarding co-localization of P2X4 and HCN4. Taking this into consideration, even though double immunolabelling against P2X4 and NCX1 was not

possible because available antibodies were raised in the same species (rabbit), it looks like that the staining pattern obtained with both P2X4 and NCX1 antibodies indicates that they may co-localize in HCN4 positive cardiomyocytes of the SAN (Figure 27). Please note that the smooth muscular layer of SAN blood vessels also exhibits strong immunoreactivity against P2X4 and NCX proteins (Figure 27, arrow heads). Likewise, these two proteins also co-localize with neurofilament 160 (NF160) in neuronal fibers of the SAN region (Figure 29).

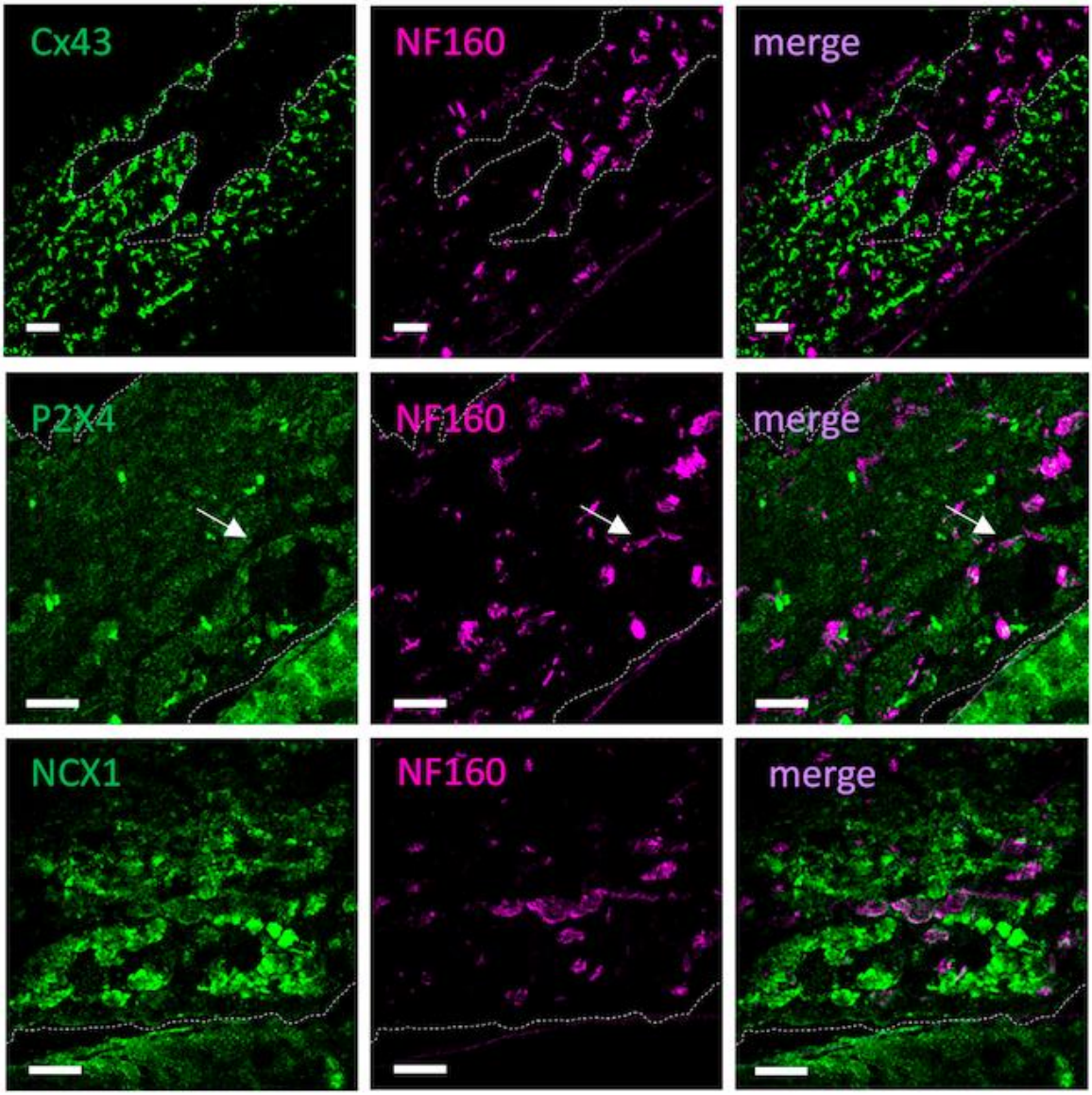

Figure 29. Representative confocal micrographs of SAN showing positive immunoreactivity against P2X4 (Apr-002, Alomone), NCX1 (Anx-011, Alomone), and NF160 (Ab7794, Abcam) proteins. The upper three panels show lower magnification confocal images of the SAN identified by the enrichment in NF160-positive nerve fibers; images also show surrounding atrial cardiomyocytes characterized as being NF160-negative and Cx43-positive. Images were taken from only one atrial preparation. Dashed lines represent boundaries of the SAN region. White arrows indicate blood vessels including the SAN artery. Scale bar 30 µm.

4. Discussion

Data suggest that activation of ATP-sensitive P2X4 receptors plays a major contribution in decreasing the spontaneous activity of the SAN while partially offsetting the negative inotropic effect of the nucleotide by downstream reversing the electrogenic NCX mode of function (Figure 30).

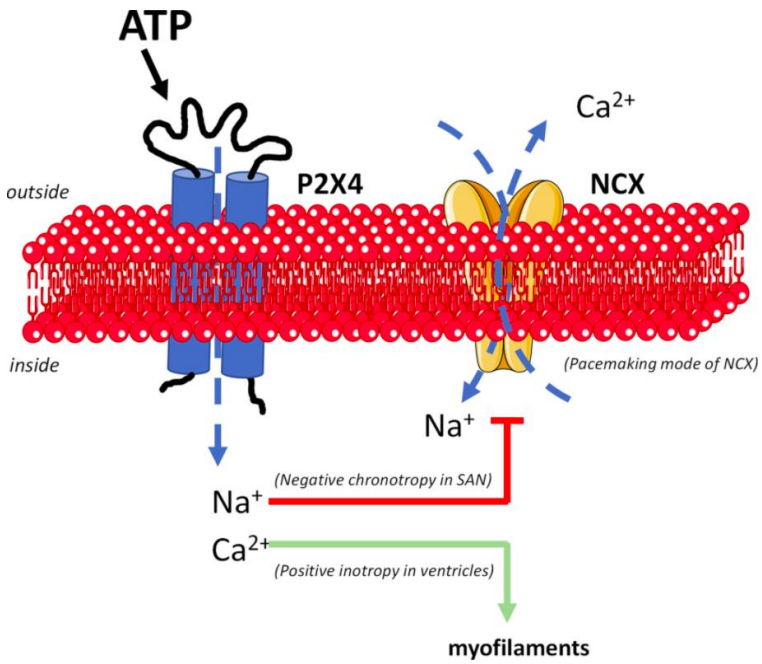

Figure 30. The mechanism underlying the dual P2X4 receptor-mediated effects on cardiac chronotropy and inotropy implicates downstream modulation of NCX activity (digitalis-like phenomenon). Besides ion fluxes carried by pacemaker HCN channels (not represented), the unstable resting membrane potential and the spontaneous firing of SAN cardiomyocytes are attributed mainly to the electrogenic NCX transport operating in the forward Ca2+-extrusion mode. Na+ influx through the P2X4 receptor pore dissipates the electrochemical gradient of this ion across the plasma membrane leading to inhibition and/or reversion of the NCX pacemaker current. This may justify slowing down of SAN cells depolarizations and the negative chronotropic effect of ATP. Likewise, intracellular Ca2+ accumulation due both (1) to Ca2+ influx through the P2X4 receptor pore, and (2) to reversal of NCX activity may explain the positive inotropic effect of the P2X4 receptor in paced ventricular cardiomyocytes. Figure composition used elements from Servier Medical Art.

4.1. ATP-sensitive P2X4 receptors decrease sinoatrial pacemaker activity

Extracellular ATP is an endogenous regulator of the cardiovascular function by acting either directly on P2 receptors or indirectly on P1 receptors after its breakdown to adenosine by NTPDases (Yegutkin, 2008; Headrick et al., 2013; Burnstock and Pelleg, 2015). Although the mammalian myocardium expresses multiple purinoceptors (Musa et al., 2009), the adenosine A_1 receptor has received most attention due to its relative abundance and well characterized functional role in the acute regulation of the heart (Musa et al., 2009; Chandrasekera et al., 2010; Headrick et al., 2013). Adenosine A_1 receptors activation decreases cardiac chronotropy, dromotropy, inotropy and counteracts adrenergic stimulation by a dual mechanism involving inhibition of AC and opening of potassium

channels (Belardinelli and Lerman, 1991; Burnstock and Pelleg, 2015; Braganca et al., 2016). Despite ATP effects may be mediated by breakdown to adenosine, the negative chronotropic action of ATP was insensitive to blockage of adenosine A_1 receptors with DPCPX used in a 6-fold higher concentration (3 nM) than that required to block this receptor (K_i~0.45 nM) (Lohse et al., 1987). However, one cannot exclude ATP conversion into adenosine during incubation with the nucleotide, yet even if this had occurred in our experimental conditions the amount of adenosine falls below the threshold to activate A_1 receptors in the SAN. These findings contrast with those obtained by Camara et al. (2015); these authors concluded that the negative chronotropic effect of ATP was dependent on A_1 receptors activation by using DPCPX in a concentration (1 μM) that is more than 2,000-fold higher than the K_i value for this antagonist to block the A_1 receptor (Camara et al., 2015). Under such conditions, off-target effects of DPCPX may appear, which include inhibition of phosphodiesterases that may explain reversal of the negative chronotropic effect of ATP (Camara et al., 2018).

Our theory that the negative chronotropic effect of ATP in spontaneously beating atria strips is mediated primarily via the activation of nucleotide-sensitive P2 purinoceptors is further supported by the fact that (1) it was reproduced by the enzymatically stable ATP analogue, ATPγS, (2) it was blocked by PPADS, a non-selective P2 purinoceptors antagonist exhibiting no affinity for adenosine receptors, and (3) prevention of ATP breakdown into adenosine with the NTPDase inhibitor, POM-1, increased rather than decreased ATP-induced negative chronotropism. Our findings agree with previous reports in the literature about the role of ATP and related adenine nucleotides on cardiac function (Versprille and van Duyn, 1966; Lundberg et al., 1984; Camara et al., 2015)) and questions the most accepted hypothesis that the negative chronotropic action of ATP is most likely due to A_1 receptors activation after its rapid conversion into adenosine (Pelleg and Belhassen, 2010). While this hypothesis neglected the pivotal role of P2 purinoceptors in the control of spontaneous activity of the SAN, it has been demonstrated that ATP was more potent than adenosine in reducing heart rate (Pelleg et al., 1985; Sharma and Klein, 1988), which was interpreted as being due to an additional vagal reflex of ATP via sensory P2X2 and/or P2X3 receptors (Pelleg et al., 1987; Xu et al., 2005). This idea is difficult to admit in the present experimental conditions due to the fact that blockage of muscarinic acetylcholine receptors with atropine (1μM) failed to affect the rate and tension of spontaneous atrial contractions and did not modify ATP-induced effects, thus indicating that the cholinergic vagal tone is irrelevant for the P2-mediated effects of the nucleotide.

To the best of our knowledge, this is the first study demonstrating a role for the P2X4 receptor in the regulation of sinoatrial node automatism. Despite limited availability of selective drugs acting on the P2X4 receptor, it may be pharmacologically characterized by

comparing agonists rank order of potency: ATP>2-methylthioATP>CTP>α,β-methyleneATP (Soto et al., 1996), as well as by the use of selective antagonists and allosteric modulators (reviewed in (Stokes et al., 2017). The potent and selective P2X4 receptor antagonist, 5-BDBD, with an IC_{50} value of about 1 μM, attenuated the negative chronotropic effect of ATP on spontaneously beating atria, while the positive allosteric modulator of the P2X4 receptor, ivermectin, potentiated the nucleotide response. Furthermore, we show here that besides ATP and its stable analogue, ATPγS, also the P2X4 agonist, CTP, whose hydrolysis does not directly yield adenosine, decreased the spontaneous atrial rate in a 5-BDBD-sensitive manner, but with a weaker potency comparing with adenine nucleotides (Soto et al., 1996; Kasuya et al., 2017). In this study we used 5-BDBD at a concentration (10 μM) that might also interfere with P2X1- and P2X3-mediated actions (Coddou et al., 2019), but we are confident that this is irrelevant in this case because very low amounts of these receptors are expressed in the SAN (Musa et al., 2009).

The P2X4 receptor shares structural and functional properties with other P2X receptors. For instance, it is known that the rat P2X4 receptor is relatively insensitive to PPADS (IC_{50}~100 μM), in contrast to mouse and human P2X4 orthologs (IC_{50}~10 μM; (Jones et al., 2000). Although exhibiting a weaker potency for the rat P2X4 receptor, we almost prevented the negative chronotropic effect of ATP using 100 μM PPADS. One cannot, however, exclude a minor participation of PPADS-sensitive metabotropic P2Y receptors in the bradycardic effect of ATP. The ionotropic P2X4 receptor is slowly desensitized by ATP (Jarvis and Khakh, 2009). This feature might explain the relatively sustained negative chronotropic effect of ATP and its analogue, ATPγS, during the time (at least for 5 min) of incubation with these compounds. However, the sustained negative chronotropic effect of ATP does not explain the potentiating action the NTPDase inhibitor, POM-1, unless one hypothesizes that extracellular ATP accumulation also contributes to reduce adenosine formation by feed-forwardly inhibiting ecto-5'-nucleotidase/CD73, as demonstrated in other studies (Magalhães-Cardoso et al., 2003; Duarte-Araújo et al., 2009; Vieira et al., 2014).

Notwithstanding our observations, other studies failed to demonstrate the involvement of P2X receptors in the control of heart rate. For instance, infusion of 2-methylthioATP did not change heart rate in the Langendorff-perfused heart (Mei and Liang, 2001), most probably because the used concentration (100 nM) of the ATP analogue falls below the threshold (1 μM) required to activate the P2X4 in the SAN (Jarvis and Khakh, 2009). The same group also failed to find any difference in the spontaneous heart rate when comparing wild-type with mice overexpressing or missing the P2X4 receptor (Hu et al., 2001; Yang et al., 2014). One must, however, emphasize that these studies were designed

to evaluate the P2X4 receptor tone under basal conditions, *i.e.* in the absence of any P2X4 agonist, which is a different situation from the present report. Thus, future studies are required to elucidate the role of the P2X4 receptor in the *in vivo* control of heart rate.

In the rat heart, the P2X4 receptor is the third most abundant P2X receptor after P2X7 and P2X5 receptors, while in the human heart it is considered the most expressed P2X receptor subtype (Musa et al., 2009). Also, the regional distribution of the P2X4 receptor in the heart displays some differences among species. Using immunofluorescence confocal microscopy, we show here that the P2X4 protein is slightly more expressed in the plasma membrane of SAN cells (mostly cardiomyocytes, but also blood vessels and nerve fibers) followed by the RV and RA of the rat. This is slightly different from data obtained in humans where the P2X4 receptor mRNA seems to be evenly expressed through the myocardium (Musa et al., 2009).

4.2. P2X4-induced negative chronotropism requires reversal of the NCX activity mode

Interestingly, the distribution of the P2X4 receptor in SAN cardiomyocytes matches the immunofluorescence staining pattern of NCX1 and HCN4 in the rat. This led us to hypothesize that the P2X4 receptor-mediated negative chronotropic effect of ATP could involve downstream modulation of NCX and/or HCN pacemaker activities. Crosstalk between P2X4- and NCX-mediated effects has been demonstrated (Shen et al., 2014). Opening of the P2X4 ion pore mediates the influx of positive charges, mainly Na^+ and Ca^{2+} in a 1:4 ratio (Jarvis and Khakh, 2009), in the proximity of NCX carriers, which might affect their operation mode. Indeed, the ATP analogue, 2-methylthioATP (3 μM), inhibited the electrogenic forward mode of NCX in ventricular myocytes via an increase (by about 1 mM) in the intracellular Na^+ concentration (Shen et al., 2014), which represents a net increase of 7-25% considering the resting intracellular Na^+ concentration (Despa and Bers, 2013). Increases in intracellular Na^+ may be even more relevant in cells with limited pathways for Na^+ entry due to low expression levels of voltage-sensitive Na^+ channels, like the SAN cardiomyocytes (Remme and Bezzina, 2010). Likewise, it has been demonstrated that persistent Na^+ currents evoked by veratridine triggers intracellular calcium transients by reversing the operation mode of NCX in CA1 pyramidal cells (Fekete et al., 2009). Although speculative, reversal of the NCX function mode by the influx of Na^+ represents an alternative mechanism for dysrhythmias (including bradycardia) in some inherited cardiac sodium channelopathies, such as the type 3 long QT syndrome associated with SCN5A mutations and persistent sodium currents (Remme and Bezzina, 2010). In agreement with our theory that Na^+ influx via the P2X4 receptor pore might affect the NCX mode of function to decrease heart rate (Figure 30), we showed here for the first time that partial blockage of

NCX, but not HCN channel, with two distinct inhibitors, KR-R7943 or ORM-10103, turned the spontaneously beating atria less sensitive to the negative chronotropic effect of ATP. Although beyond the scope of the present work, the interplay between P2X4 and NCX deserves further investigations using highly demanding electrophysiology patch-clamp techniques in acutely isolated SAN cardiomyocytes from both rats and humans (ongoing research project).

Co-localization of P2X4 and NCX1 immunoreactivity in NF160 positive neuronal fibers was also detected. The presence of the P2X4 receptor in neuronal structures is widely accepted, but its function remains to be explored (Stokes et al., 2017). The SAN and the surrounding myocardium are regulated by a dense network of autonomic fibers, which are mainly parasympathetic followed by a sympathetic origin (Crick et al., 1994; Crick et al., 1999; Pauza et al., 2013; Zarzoso et al., 2013; Rajendran et al., 2019). Interestingly, some intracardiac neurons within atria contain ATP stored in vesicles (Crowe and Burnstock, 1982), which upon activation may represent an important source of extracellular ATP (Burnstock, 1972; Fredholm et al., 1982; Tokunaga et al., 1995). Reversion of NCX forward activity during ischemic conditions contributes to increase the magnitude of Ca^{2+} transients and, thus, the release of neurotransmitters from presynaptic nerve terminals (Lee and Kim, 2015). Given the co-localization and putative interplay between the P2X4 receptor and NCX in NF160-positive nerve fibers, one may speculate that these players may also interact to control the activity of cardiac neurons (Griffioen et al., 2007). This is even more relevant taking into consideration that cardiac ischemia is accompanied by P2X4 overexpression, particularly in the SAN (Musa et al., 2009). Thus, ATP released from autonomic cardiac nerves may trigger a positive feedback loop involving the NCX leading to an increase in the purinergic control of atrial cardiomyocyte function at both pre- and post-junctional levels.

4.3. ATP-induced negative inotropism is partially offset by P2X4 activation and NCX transport reversal

The inotropic effect of ATP was investigated in paced RV strips; the nucleotide decreased ventricular inotropy in a concentration-dependent manner, yet changes in paced ventricular tension were less potent than the recorded ATP-induced negative chronotropic actions in spontaneously beating atria. This raised the possibility for the existence of a yet unraveled ATP-induced negative inotropic offsetting mechanism. Although we did not fully characterized the receptors involved in the negative inotropic effect of ATP, previous studies agree that P2 purinoceptors activation may be necessary, also taking into consideration that adenosine plays a minor (if any) role on ventricular inotropy (Burnstock and Meghji, 1983; Belardinelli et al., 1995; Balogh et al., 2005). There is, however, a contention regarding to whether ATP exerts a positive or a negative inotropic effect on ventricular

contractions. In contrast to our findings, most reports in the literature suggest that ATP exerts a predominant positive inotropic effect in the heart. Nonetheless, it is worth to emphasize that the vast majority of these studies were performed in isolated ventricular myocytes (Danziger et al., 1988; De Young and Scarpa, 1989; Christie et al., 1992; Podrasky et al., 1997; Mei and Liang, 2001; Balogh et al., 2005). These findings attenuate the theory that ATP-induced positive inotropism could be mediated by P2X4 receptors facilitating noradrenaline release from sympathetic nerve terminals in paced ventricular strips. Only three studies were performed in more complex tissue preparations, namely in rat papillary muscles (Legssyer et al., 1988; Scamps et al., 1990) and in the frog ventricle (Flitney and Singh, 1980). Interestingly, the Legssyer's and Flitney's studies reported a dual and opposing role of ATP in cardiac tissues. In support of a negative inotropic role for ATP, a recent study performed in intact isolated hearts, as well as in ventricular fragments and acutely isolated myocytes proposed that diadenosine tetraphosphate decreased ventricular inotropy probably via the activation of P2Y purinoceptors (Pakhomov et al., 2018).

Notwithstanding the conflicting results regarding the nature of the inotropic role of ATP, the use of ATP analogues and more selective P2 receptor modulators, in combination with genetic and other advanced biochemical techniques, provided strong evidence that several P2X and P2Y receptors may be positive ventricular inotropic mediators (reviewed in (Erlinge and Burnstock, 2008; Burnstock and Pelleg, 2015). Regarding the P2Y receptor family, positive inotropy is generally attributed to stimulation of Gs and Gq-protein coupled receptors (Erlinge and Burnstock, 2008). Among them, ATP preferentially activates the $P2Y_{11}$ receptor (Abbracchio et al., 2006). The selective $P2Y_{11}$ agonist, AR-C67085, increased contraction in isolated cardiomyocytes as well as in isolated trabecular preparations. In that study, $P2Y_{12}$ and $P2Y_{13}$ receptors were excluded by the lack of effect of the stable ADP analogue, 2-methylthioADP, in cardiomyocytes contractile activity, which also nearly exclude any involvement of ATP-sensitive Gi-protein coupled P2Y receptor (Balogh et al., 2005). Of note, it is likely that these authors performed their studies in a mixed population of ventricular and atrial cardiomyocytes, as these cells were not separated by the enzymatic digestion of the heart. The putative involvement of P2Y receptors in the negative inotropic effect of ATP in paced ventricular strips was not assessed here, which is a limitation of our study that certainly deserves further investigations along with the corresponding effects in the *in vivo* animal.

Given the involvement of the P2X4 receptor in the negative control of sinoatrial automatism (see above), we focused our interest at investigating the role of this ionotropic receptor on ventricular contractile activity (Erlinge and Burnstock, 2008; Burnstock and Pelleg, 2015). This question was raised because heart rate slowing drugs devoid of effect or with a moderate positive inotropic action on ventricular contraction may be relevant to

treat heart failure. Our findings show that selective blockage of the P2X4 receptor activation with 5-BDBD significantly increased the negative inotropic effect of ATP in paced RV strips, thus suggesting that the P2X4 receptor may exert a counteracting positive inotropic action that is responsible for partially offsetting ATP-induce downsizing of ventricular contractions. As a matter of fact, the ATP analogues, 2-methylthioATP and α,β-methyleneATP, increased contractions of isolated ventricular cells, as well as of ventricular strips and isolated working hearts in rodents (Burnstock and Meghji, 1983; Hu et al., 2001; Mei and Liang, 2001). Overexpression of the P2X4 receptor (1) enhances ATP-induced cardiac contractility in the intact heart, and (2) rescues the systolic function and increase survival of animals with cardiomyopathy (Yang et al., 2004; Shen et al., 2009). The beneficial effects of the P2X4 receptor on cardiac function were attributed to activation of calcium-dependent endothelial-type nitric oxide synthase (Blaustein and Lederer, 1999; Yang et al., 2015). Ca^{2+} influx through the P2X4 receptor pore may itself account for the positive inotropic action of ATP analogues. On the other hand, Na^{+} influx through the P2X4 receptor also contributes to inhibit or, even revert, the electrogenic transport of NCX in the forward mode (Ca^{2+} extrusion mode) leading to an additional increase in the amplitude and duration of Ca^{2+} transients inside cardiomyocytes, which boosts their contractile activity (Shen et al., 2014); see Figure 30).

This concept may also explain the biphasic effect of ATP on atrial inotropy reported in this study and by other authors (Froldi et al., 1994; Gergs et al., 2008b), which consisted of a transient decrease followed by a gradual recovery of the amplitude of atrial contractions while the preparations were still in contact with the nucleotide. Even though atrial inotropy represents an important reserve to maintain cardiac output in demanding conditions and in the setting of ventricular diastolic dysfunction, this phenomenon was not further evaluated in spontaneously beating rat atria due to significant bias introduced by changes in the rate of contractions.

Another relevant aspect of the P2X4 receptor regulation with potential implications for cardiac pathophysiology is its sensitivity to pH; in acidotic conditions, as it occurs in ischemia/hypoxia or renal failure, the P2X4 receptor activity significantly decreases, whereas the opposite occurs alkaline conditions (Wildman et al., 1999). Growing evidence exist demonstrating that the P2X4 receptor is overexpressed in ventricles under stressful conditions, namely in pulmonary hypertension and ischemia-induced heart failure (Sonin et al., 2008; Musa et al., 2009; Ohata et al., 2011). Altogether these findings strengthen the potential involvement of the P2X4 receptor in cardiac normal physiology and diseases progression.

5. Conclusion

Overall, data suggest that ATP-sensitive P2X4 ionotropic receptors play a major role in decreasing the spontaneous activity of the SAN while partially offsetting the negative inotropic effect of the nucleotide in paced rat ventricles. The mechanism underlying the dual P2X4 receptor-mediated effects on cardiac chronotropy and inotropy involves downstream interaction with the activity of NCX. Na^+ influx via the P2X4 receptor pore may inhibit and/or revert the electrogenic forward current of the NCX, thus decreasing chronotropy. Likewise, intracellular Ca^{2+} accumulation due to interference with NCX might explain the positive inotropic effect attributed to the P2X4 receptor activation on paced RV strips (Figure 30). Regional differences observed for the distribution of the P2X4 receptor, along with its biophysical properties, bring new therapeutic opportunities for P2X4 activation with potential to create novel well-tolerated heart-rate lowering drugs with promising benefits in patients with deteriorated ventricular function.

6. Concluding remarks

Another important reflection about inhibitory role of P2X4 on NCX function come from studies that showed that in HF both NCX protein and function are upregulated (Reinecke et al., 1996). In HF progression systolic function becomes severely compromised at some point of its course, and NCX upregulation may represent an adaptive mechanism to counteracts abnormal Ca^{2+} handling and decrease of inotropy (Reinecke et al., 1996; Schillinger et al., 2000). However, in consequence of hyperfunction of Ca^{2+}-extrusion mode of NCX, $[Na^+]_i$ becomes inappropriately elevated (Eisner et al., 2020), with potential to enhance arrhythmogenic phenomena (Reinecke et al., 1996). In fact, targeting NCX inhibition with several pharmacological inhibitors have been demonstrating some beneficial effects in protecting heart from myocardial dysfunction and arrhythmias, at least in animal models (Roe et al., 2015). However, it should be warned that inhibition of Ca^{2+}-extrusion NCX mode might have opposing and conflicting results depending whether the main mechanism for HF is diastolic or systolic dysfunction, with benefits toward systolic dysfunction (Roe et al., 2015).

PAPER 3

Int J Mol Sci. 2020 Jul 15;21(14):5005.
doi: 10.3390/ijms21145005.

Resolving the Ionotropic P2X4 Receptor Mystery Points Towards a New Therapeutic Target for Cardiovascular Diseases

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Abstract

Adenosine triphosphate (ATP) is a primordial versatile autacoid that changes its role from an intracellular energy saver to a signaling molecule once released to the extracellular milieu. Extracellular ATP and its adenosine metabolite are the main activators of the P2 and P1 purinoceptor families, respectively. Mounting evidence suggests that the ionotropic P2X4 receptor (P2X4R) plays pivotal roles in the regulation of the cardiovascular system, yet further therapeutic advances have been hampered by the lack of selective P2X4R agonists. In this review, we provide the state of the art of the P2X4R activity in the cardiovascular system. We also discuss the role of P2X4R activation in kidney and lungs vis a vis their interplay to control cardiovascular functions and dysfunctions, including putative adverse effects emerging from P2X4R activation. Gathering this information may prompt further development of selective P2X4R agonists and its translation to the clinical practice.

1. The "Purinome"

The concept of "purinome" encompasses the molecular machinery necessary for purinergic signaling. The core of this system is adenine nucleotides and nucleosides and their activity over a wide range of transmembrane purinoceptors. Terms like "purinergic nerves" and "purinoceptors" were coined in the early 1970s after Geoffrey Burnstock, one of the most widely cited scientists who recently (2 June 2020) passed away at the age of 91. The purinoceptor family is further subdivided into P1 and P2 receptor subtypes according to their sensitivity to adenosine and adenine and uracil nucleotides (e.g., ATP, ADP, UTP, UDP, UDP-glucose), respectively. Four subtypes of adenosine-sensitive G protein-coupled P1 receptors have been cloned, A_1, A_{2A}, A_{2B}, and A_3. Regarding the P2 receptor family, it is sub-classified into seven ionotropic P2X receptors (P2X1-7) and eight G-protein-coupled metabotropic P2Y receptors ($P2Y_{1,2,4,6,11,12,13,14}$). Each purinoceptor has a specific pharmacological profile and biological fingerprint, which allows its identification overtaking the scarcity of selective pharmacological tools (Burnstock, 2018). Besides lytic mechanisms that occur after cellular damage, the release of ATP and other

purines/pyrimidines may be elicited by multiple stimuli (e.g., hypoxia, inflammation, mechanical stimuli) and occur via highly regulated processes, such as vesicle exocytosis and diffusion through plasma membrane channels (Lazarowski, 2012; Taruno, 2018). Once in the extracellular milieu, ATP is sequentially hydrolyzed to adenosine by a cascade of plasma membrane-bound nucleotidases (e.g., NTPDase1/CD39, ecto-5′-nucleotidase/CD73), shifting cellular signaling from P2- to P1-mediated responses. Then, adenosine follows one of two removal pathways: (1) cellular reuptake by nucleoside transporters or (2) enzymatic deamination to inosine by adenosine deaminase (Yegutkin, 2014).

The relevance of purinergic signaling to control cardiovascular functions is indisputable, spanning from the pioneering work of Drury and Szent-Györgyi in 1929 on the role of adenosine in the mammalian heart (Drury and Szent-Gyorgyi, 1929) to more recent reports leading to the use of $P2Y_{12}$ receptor antagonists to reduce platelet aggregation in thrombotic diseases (e.g., myocardial infarction, stroke) (Roffi et al., 2016; van der Meijden and Heemskerk, 2019). Notwithstanding this, drugs other than adenosine (and its derivatives) and $P2Y_{12}$ receptor antagonists have already entered clinical trials for cardiovascular diseases, with some other drugs still under development and awaiting clearance to enter the clinical trial pipeline (Burnstock, 2017c).

Nowadays, there is an increasing enthusiasm for the modulation of P2X purinoceptors functions in health and disease, with the P2X7R as the most promising candidate confirmed by successfully completed clinical trials. More recently, the P2X4R has emerged as another promising drug target. Despite the lack of selective P2X4R agonists available so far, modulation of the P2X4R has been demonstrated to be beneficial in cardiac diseases, pain sensation, cancer, and inflammatory diseases (Burnstock, 2017c; Suurvali et al., 2017). Due to these putative therapeutic hints, we expect a boost in the development of selective P2X4R agonists in the forthcoming years. This prompted us to review the recent advances in deciphering the pathophysiological relevance of the P2X4R in the cardiovascular system. Considering that impairment of other body systems (e.g., renal and respiratory) might have a huge impact in the management and prognosis of cardiac patients, we thought it was useful to discuss the (beneficial and/or detrimental) repercussions of the P2X4R activation in these territories.

2. The P2X4 Receptor: From Molecular Characteristics to Its Modes of Action

The P2X4R was the first crystal structure of a P2X receptor to be resolved (Kawate et al., 2009). Sequence homology of P2X4R in mammals exceeds 85%, with some genetic variants been described for this receptor (Stokes et al., 2011; Sophocleous et al., 2020). The P2X4R results from the assembly of three subunits organized in a dolphin-like

structure. Each subunit has two membrane-spanning domains, with C- and N-terminals located intracellularly, and a large ectodomain for ATP binding (Kawate et al., 2009). Structural homology among P2X receptors allows different assemblies of P2X subunits. Heterotrimeric P2X receptors have different pharmacological and functional properties from homotrimeric versions (Saul et al., 2013). Heterotrimers have been described for P2X4R, particularly in combination with P2X6R subunits; however, it remains to be clarified whether they exist in native systems (Saul et al., 2013; Antonio et al., 2014).

P2X4R orthosteric binding sites can be found between two adjacent subunits, which means that each P2X4R can harbor up to three ATP molecules (Hattori and Gouaux, 2012; Suurvali et al., 2017); the pEC_{50} for ATP of the Human P2X4R is in the low micromolar range (Table 7) (Jacobson et al., 2002). The P2X4R ATP-binding site lacks some specificity for the purine base allowing binding of other nucleotides including non-adenine compounds, such as cytidine triphosphate (CTP); the P2X4R exhibits low affinity (millimolar range) for this pyrimidine (Kasuya et al., 2017). Similarly to other P2X receptors, the P2X4R has two main conformational states: closed and open. Occupancy of the orthosteric binding pocket by ATP and other agonists induces structural changes that open a permeation pore for Na^+, K^+, and Ca^{2+} ions (Egan et al., 2006; Hattori and Gouaux, 2012). Opening of P2X4R generates a depolarizing inward current, with a fourfold permeability for Ca^{2+} in relation to Na^+ ($P_{Ca}/P_{Na} \approx 4$), contributing to the activation of several Ca^{2+}-dependent intracellular signaling processes (Egan et al., 2006; Samways et al., 2014). The P2X4R reversion potential is around 0 millivolts, which means that P2X4R-mediated currents easily become outward at positive potentials (Soto et al., 1996; Coddou et al., 2011b). Interestingly, two P2X4R permeant conformations seem to exist regarding the dimensions of the channel pore (small vs. large) (Khakh et al., 1999a; Coddou et al., 2011b; Samways et al., 2014). Indeed, the P2X4R may share some permeant characteristics with the P2X7R, including the formation of a high-permeability pore that allows passage of large molecules, including large fluorescent dyes. This high-permeant state of the P2X4R exists on its own, without needing the accessory proteins (e.g., pannexin 1) required to form the high-permeability pore together with the P2X7R (Khakh et al., 1999a; Bernier et al., 2012). Like its P2X7R sibling, the P2X4R large pore formation is favored by low extracellular Ca^{2+} and is regulated by phosphatidylinositol 4,5-bisphosphate (PIP2) content in the plasma membrane (Shinozaki et al., 2009; Bernier et al., 2012).

Table 7. Pharmacology of the P2X4 receptor.

	Compound	EC_{50} or IC_{50} (μM)*			P2X cross-reactivity / Fold selectivity	References
		Human	Rat	Mouse		
Agonist	ATP	0.7 - 5.7	1.7 - 16.2	0.3 - 2.3		(Bo et al., 1995; Soto et al., 1996; Khakh et al., 1999a; Jones et al., 2000; Stokes et al., 2011; Abdelrahman et al., 2017; Kasuya et al., 2017)
	ATPγS	10.9				(Bianchi et al., 1999)
	2-meSATP	0.3 - 2.2	~ 1 - 10	1.4		(Soto et al., 1996; Bianchi et al., 1999; He et al., 2003; Abdelrahman et al., 2017)
	CTP		~0.1 - 1 mM		P2X1,2,3,7	(Soto et al., 1996; Roberts and Evans, 2004; Browne and North, 2013; Yusuf et al., 2016)
	BzATP	0.5 - 9.4	>100	2.9	P2X7 (h)	(Soto et al., 1996; Bianchi et al., 1999; Abdelrahman et al., 2017)
	α,β-meATP	0.8 - 19	≥100	7->100	P2X1,3	(Bianchi et al., 1999; Jones et al., 2000; Abdelrahman et al., 2017)
	β,γ-meATP	>100	>100	>100		(Abdelrahman et al., 2017)
	AP4A[¥]	0.1-3	20 - >100	2.6->100		(Bianchi et al., 1999; Jones et al., 2000; Abdelrahman et al., 2017)
	AR-C67085MX	2.5	>100	>100		(Abdelrahman et al., 2017)
	8-Azido-ATP	>100	>100	>100		(Abdelrahman et al., 2017)
	β,γ-Imido-ATP	6.5	>100	>100		(Abdelrahman et al., 2017)
	MRS-2339					(Shen et al., 2007)
	MRS-2978					(Kumar et al., 2013)
	PSB-0412	2.1				(Abdelrahman et al., 2017)
Antagonist	PPADS	9.6 - >100	>300	>100		(Bianchi et al., 1999; Jones et al., 2000)
	Suramin	>100	>100	>100		(Bianchi et al., 1999; Jones et al., 2000)
	KN-62	>10	>10	>10		(Jones et al., 2000)
	TNP-ATP	1.5	1.3 - 4.71	1.3 - 4.2	P2X1,2,3,7	(Virginio et al., 1998; Bo et al., 2001; Hernandez-Olmos et al., 2012; Balazs et al., 2013; Abdelrahman et al., 2017)
	Brilliant Blue G	3-100			1000-fold more potent at P2X7 (r)	(Tomioka et al., 2000)
	5-BDBD	0.3-1.2	0.75 - 3.5	2.04	10-fold P2X1 (r), 3-fold P2X3 (r)	(Balazs et al., 2013; Abdelrahman et al., 2017; Coddou et al., 2019)
	BX-430	0.54 - 1			10 to 100-fold P2X1,3,5,7	(Ase et al., 2015; Yoshida et al., 2019)
	Carbamazepine der.[†]	3.44	54.6	14.9	2 to 30-fold P2X1,2,3,7 (h)	(Tian et al., 2014)
	PSB-12054	0.19	2.10	1.8	≥50-fold P2X1,2,3,7 (h)	(Hernandez-Olmos et al., 2012)
	PSB-12062	1.4	0.9	1.8	≥35-fold P2X1,2,3,7 (h)	(Hernandez-Olmos et al., 2012)
	PSB-15417	10 [‡]			>5-fold P2X7 (h)	(Schneider et al., 2017)
	NP-1815-PX	0.26			P2X1,2,3,7	(Matsumura et al., 2016)
	NC-2600[℧]					(Inoue and Tsuda, 2018)
	UoS14919	61 nM				(Beswick et al., 2019)
	Paroxetine	1.87 - 4.8	1.64 - 2.45	0.7		(Nagata et al., 2009; Abdelrahman et al., 2017)
	Duloxetine	1.59 - 17				(Masuda et al., 2014; Yamashita et al., 2016)
	BAY-1797	0.2				(Werner et al., 2019)
	IgG#151-LO[‖]	0.7			10,000-fold more potent over other P2X	(Williams et al., 2019)
PAM	Ivermectin	0.1			P2X7 (h)	(Norenberg et al., 2012; Shen et al., 2020)
	Cibacron blue	>300				(Miller et al., 1998)
	Ginsenosides	7.5 – 10.5			P2X7	(Dhuna et al., 2019)
	Testosterone butyrate		30 [‡]		P2X2	(Sivcev et al., 2019)
	Zn^{2+}		1.8			(Coddou et al., 2005)
	Cd^{2+}		7.3			(Coddou et al., 2005)
	Alfaxalone		0.4-1.6			(Codocedo et al., 2009)
	Allopregnanolone		0.4			(Codocedo et al., 2009)
	Propofol	56				(Tomioka et al., 2000)
NM	Ethanol		5-200 mM[‡]		PAM at rat P2X3	(Davies et al., 2005; Popova et al., 2010)
	H^+	pKa ~6.8			inhibits P2X1,3,4; stimulates P2X2	(Stoop et al., 1997)
	Hg^{2+}		9			(Coddou et al., 2005)
	Cu^{2+}		8.6			(Coddou et al., 2005)
	Fluvastatin[¥]	10 for 1h				(Li and Fountain, 2012)
	Filipin III[¥]	10 for 30min				(Li and Fountain, 2012)
	Methyl-β-cyclodextrin[¥]	10mM, 1h				(Li and Fountain, 2012)
	t-DCA[§]	160				(Ilyaskin et al., 2019)

Data are mostly from functional studies (e.g. electrophysiological currents, intracellular calcium oscillations) in heterologous systems expressing human (h), rat (r) or mouse (m) P2X4 receptors. *Potency of agonists and positive allosteric modulators (PAM) is represented as half maximal effective concentration (EC_{50}), whereas for antagonists and negative modulators (NM) indicated is the half maximal inhibitory concentration (IC_{50}). EC_{50} and IC_{50} values are in μM, unless stated otherwise.

¥AP4A, adenosine tetraphosphate. †N,N-diisopropyl-5H-dibenzo[b,f]azepine-5-carboxamide. ℧ NC-2600 is a specific P2X4 antagonist from *Chemifar*®, with unknown structure and selectivity, currently in phase I clinical trial for neuropathic pain. ‡ Potency not calculated, it indicates the concentration of the compound used to produce the functional effect. ¥Activity suppressed by cholesterol depletion. §Tauro-deoxycholic acid (t-DCA). ||Mouse antibody anti-P2X4 IgG#151-LO.

The role of the P2X receptor in several physiological processes has been hindered by the current lack of selective agonists and antagonists. This limitation has been partially overridden by the pharmacological characterization of agonist profiles, gating kinetics, and function in comparison with other P2X receptors (Coddou et al., 2011b). The P2X4R is a fast purinergic excitatory receptor, and, in comparison with other P2X receptors, it exhibits intermediate sensitivity for ATP (≈10μM) and desensitization time course in rodents (Khakh et al., 1999a; Coddou et al., 2011b; Schmid and Evans, 2019). Synthetic ATP derivatives (e.g., αβ-methylene ATP (αβ-meATP); βγ-methyleneATP (βγ-meATP); adenosine 5′-O-(3-thio)triphosphate (ATPγS), 2-MethylthioATP (2-meSATP), 2,3-O-(4-benzoyl-benzoyl)ATP (BzATP)) activate all P2X receptors, yet, contrary to other P2X receptor subtypes, the P2X4R is relatively insensitive to αβ-meATP in rodents (Table 7). The agonist rank potency order for the P2X4R is as follows: ATP > ATPγS > 2-meSATP ≥ CTP > αβ-meATP (Garcia-Guzman et al., 1997; Coddou et al., 2011b). The list of available P2X4R antagonists is gradually expanding with some compounds presenting well-acceptable selectivity over other P2X receptor subtypes, like 5-BDBD (5-(3-bromophenyl)-1,3-dihydro-(1)benzofuro(3,2-e)(1,4)diazepin-2-one; pIC50~5-6) [11,20,27]. Relative insensitivity to pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), and suramin, two broad-spectrum P2 antagonists, is another pharmacological characteristic of the P2X4R (Table 7) (Coddou et al., 2011b).

Regulation of the P2X4R activity is complex and not limited to binding and unbinding of ATP (Coddou et al., 2011a; Coddou et al., 2011b; Suurvali et al., 2017). Trace metals are important modulators of the P2X4R activity, and their role may be used as a strategy for this receptor characterization (Table 1). For instance, zinc ions (Zn^{2+}) bind to the “M1 site” of the P2X4R and increase its affinity for ATP (Garcia-Guzman et al., 1997; Kasuya et al., 2016). Conversely, copper ions (Cu^{2+}) inhibit the P2X4R maximal response without affecting its affinity for ATP (Coddou et al., 2011b). This receptor is also sensitive to pH, a property derived from the surface lining of protonable histidines. In acidic conditions, like those occurring during ischemia or inflammation, protons (H^{+}) inhibit the P2X4R activity (Clarke et al., 2000; Coddou et al., 2011a). Allosteric modulators are other important pharmacological tools. This is headed by the prototypical positive allosteric modulator of the P2X4R, ivermectin, which highly increases the ATP agonist potency (Khakh et al., 1999b; Coddou et al., 2011a). Other allosteric modulators have been described, which include several commonly used drugs like paroxetine, alcohol, and propofol (Coddou et al., 2011b; Ase et al., 2015; Ilyaskin et al., 2019; Sivcev et al., 2019). The C-terminal of the P2X4R is a target for kinase regulation, namely by protein kinase A (PKA), which increases the receptor activity. Rather than a direct regulation by

phosphorylation, PKA stimulation recruits accessory proteins to bind to the C-terminal of the P2X4R (Brown and Yule, 2010). The P2X4R is also sensitive to the composition of plasma membrane; for instance, depletion of membrane PIP2 by stimulation of phospholipase C (PLC) decreases the current induced by the receptor and slows its recovery from desensitization (Bernier et al., 2008).

Gene expression and cellular localization represent another level of regulation of the P2X4R. This receptor is present in multiple cells throughout the body, but its relative expression depends on cell types, development status, and health/disease conditions (Soto et al., 1996; Garcia-Guzman et al., 1997; Coddou et al., 2011b). Expression of the P2X4R is highly sensitive to certain stimuli, including hypoxia (Li et al., 2011; Ohata et al., 2011) and inflammation (Tang et al., 2008), and is controlled by specific transcription factors. STAT1, GATA-2, and IRF-5 transcription factors are known activators of the P2X4R gene promoter (Tang et al., 2008; Gu et al., 2010; Masuda et al., 2014). Trafficking of the P2X4R to the plasma membrane is paramount to sense extracellular ATP, while internalization represents an important negative feedback mechanism to avoid overactivation. The C-terminal tail of the P2X4R is a key element in desensitization and internalization processes; it functions as an endocytic-sorting signal to lysosomes (Fountain and North, 2006). Intracellular localization of the P2X4R has increasingly been demonstrated in certain cell types; localization of this receptor in vesicles and lysosomes supports its role in vesicle turnover, as demonstrated concerning exocytosis (Xu et al., 2014; Murrell-Lagnado, 2018; Sophocleous et al., 2020).

3. Benefits from the P2X4 Receptor Activation in the Heart

Cardiac output is a function of heart rate and stroke volume, with the latter being influenced by pre-load, after-load, and inotropy. ATP and other purines regulate all these features. For decades it was generally accepted that intravenous infusion of ATP decreased cardiac works by means of its breakdown to adenosine via activation of the inhibitory A_1 receptor. With experimental advances, the “true” ATP effects soon became resolved, and now there is plenty of evidence to support involvement of P2 receptors in acute and chronic physiological processes in the heart. P2 receptors are expressed not only in the working myocardium, but also in other cardiac cells (Erlinge and Burnstock, 2008; Burnstock and Pelleg, 2015; Burnstock, 2017a). Among P2X receptors, the P2X4R is the most expressed receptor subtype in the heart, particularly in supraventricular tissues (Bogdanov et al., 1998; Musa et al., 2009). The P2X4R is distributed in various cardiac cells, including cardiomyocytes, endothelial and smooth muscle cells of cardiac blood vessels, cells of the cardiac conduction system (Musa et al., 2009; Braganca et al., 2019), intracardiac neurons (Ma et al., 2005), and cardiac fibroblasts (Chen et al., 2012).

ATP, through activation of P2 purinoceptors, increases cardiac inotropy. Although some conflicting results exist regarding the receptor involved in this effect, accumulating evidence suggests an involvement of P2X receptors, particularly the P2X4R subtype (Burnstock, 2017a). In 2001, a seminal study from B.T. Liang's group demonstrated that cardiac preparations overexpressing the human P2X4R exhibited increased contractile performance when challenged with the P2X receptor agonist, 2-meSATP. Remarkably, baseline cardiac output was also incremented, but no effects were observed regarding chronotropy and/or ventricular remodeling (Hu et al., 2001). Later on, the same group showed that P2X4R overexpression was indeed protective by increasing survival both in ischemic and in non-ischemic models of heart failure. This theory was strengthened by finding that overexpression of the P2X4R favors rescuing of several hallmarks of heart failure (HF), namely ventricular systolic dysfunction, β-adrenergic desensitization, hypertrophy, and maladaptive remodeling (Yang et al., 2004; Sonin et al., 2008; Shen et al., 2009). Interestingly, HF and coronary artery disease are on their own associated with upregulation of the P2X4R expression in the heart (Shen et al., 2007; Musa et al., 2009). In a rodent model of hypoxia-induced pulmonary hypertension, the P2X4R was found to be upregulated in the right ventricle (Ohata et al., 2011). Likewise, a compensatory upregulation of P2X currents was observed in the heart of animals exhibiting cardiomyopathy due to calsequestrin overexpression; in this animal model stimulation of P2X receptors with MRS-2339, a charged methanocarba derivative of 2-Cl-AMP resulted in reversion of maladaptive cardiac remodeling and prolonged survival (Shen et al., 2007). The recently developed diester-masked uncharged phosphonate, MRS-2978, a compound structurally related with MRS-2339 but chemically modified to be orally bioavailable and more resistant to enzymatic hydrolysis, presents benefits in improving cardiac dysfunction in both ischemic and pressure-overload HF animal models (Kumar et al., 2013; Shen et al., 2020). Conversely, knocking down the P2X4R is normally associated with deterioration of cardiac ventricular function and progression to HF (Yang et al., 2014). Data, thus, indicate that stimulation of the P2X4R ameliorates cardiac contractile performance by increasing inotropy and by favoring the recruitment of protective signaling mechanisms.

As aforementioned, the P2X4R activation increases Ca^{2+} influx into the cells (Garcia-Guzman et al., 1997; Egan et al., 2006; Samways et al., 2014). Ca^{2+} transients induced by P2X4R activation further stimulate massive Ca^{2+} release from sarcoplasmic reticulum through a mechanism of calcium-induced calcium release, which might explain the positive inotropic effect of this receptor (Eisner et al., 2017). It has also been demonstrated that the P2X4R modulates the activity of the Na^{+}-Ca^{2+} exchanger (NCX), which contributes to prolonging intracellular Ca^{2+} accumulation and signaling. In ventricular myocytes from both wild-type and P2X4R-overexpressing mice, 2-meSATP caused an

inward Na^+ current which was high enough (about 1 mM) to potentiate Ca^{2+}-entry via the NCX functioning in the reverse mode (Shen et al., 2014). These findings led to the conclusion that Na^+ influx via the P2X4R may putatively interfere with normal function of NCX, thus favoring intracellular Ca^{2+} accumulation (Figure 31).

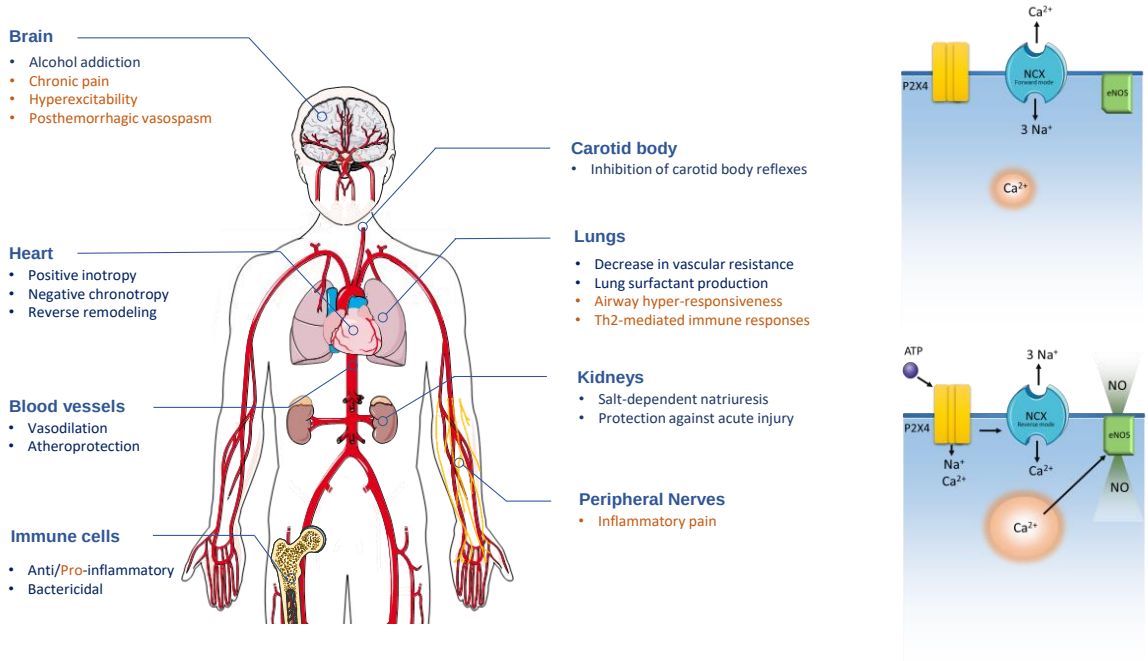

Figure 31. Schematic overview of systemic P2X4R stimulation. An overall cardioprotective effect is observed with P2X4R stimulation. Permeation of Na^+ and Ca^{2+} ions through P2X4R interferes with the forward mode of the Na^+-Ca^{2+} exchanger (NCX) and Ca^{2+}-dependent activation of endothelial nitric oxide synthase (eNOS) (right panels). In the heart, stimulation of P2X4R improves cardiac muscle contraction and optimizes myocardial energy supply vs. demand by reducing chronotropy. The diuretic effect of P2X4R activation together with its vasodilation properties in both systemic and pulmonary arteries optimizes preload and afterload and, thereby, cardiac output in HF. Chronic activation of the P2X4R reverses maladaptive cardiac remodeling and improves the outcome of HF. Future P2X4R agonist therapies must deal with the wide P2X4R distribution throughout the human body resulting in putative unwanted side-effects (marked in orange). Figure composition used elements from Servier Medical Art (https://smart.servier.com).

Recently, our group demonstrated that in the sinoatrial node (SAN), the first element of the cardiac conduction system, the P2X4R interferes with the Ca^{2+}-extrusion (forward) mode of the NCX (Braganca et al., 2019), which constitutes a fundamental pacemaking mechanism to drive spontaneous SAN depolarization and to set sinus rhythm (Lakatta et al., 2010). Our study demonstrated for the first time that rather than requiring its breakdown to adenosine, ATP on its own is able to decrease chronotropy via P2X4R activation. The dual role of the P2X4R in decreasing chronotropy while increasing inotropy strikingly resembles the effect of Na^+/K^+-ATPase inhibition by cardiac glycosides (e.g., digoxin) (Belz et al., 2001; Braganca et al., 2019), expanding the clinical usefulness of P2X4R agonists to the treatment of tachyarrhythmias and congestive HF (Kirchhof et al.,

2016; Ponikowski et al., 2016; Whayne, 2018). In support of this mechanism, istaroxime, a newer intracellular Na^+ enhancer, has been demonstrated to improve both diastolic and systolic function in patients with decompensated HF with reduced ejection fraction (HFrEF), along with increasing systolic blood pressure while reducing heart rate, with no evidence of increases in the probability of cardiac arrhythmias (Shah et al., 2009; Bossu et al., 2018; Carubelli et al., 2020; Chioncel et al., 2020). Notwithstanding this, a note of caution must be taken concerning intracellular Na^+ overload as a consequence of chronic P2X4R activation, since increases in diastolic Ca^{2+} concentration may worsen the diastolic dysfunction in HF conditions (Eisner et al., 2020). Another caveat is related to rhythm disturbances that may arise from perturbations of Na^+ and Ca^{2+}-handling resulting from prolonged activation of the P2X4R. As a matter of fact, some reports ascribed a pro-arrhythmic potential to ATP (Belhassen et al., 1984; Tao et al., 2014), which deserves to be further explored in future studies.

Apart from its key role in cardiac contraction and heartbeat initiation, Ca^{2+} is an important intracellular second messenger (Gilbert et al., 2020). Yang and coworkers demonstrated that cardiac protection triggered by the P2X4R involves stimulation of endothelial synthesis of nitric oxide (NO) (Yang et al., 2014). Synthesis of NO is a downstream target of Ca^{2+} signaling. The beneficial effects of NO in the cardiovascular system are well-known, which include vasodilation, inhibition of platelet aggregation, and regulation Ca^{2+} homeostasis (Farah et al., 2018). Putative reversion of pathological cardiac remodeling may also be owed to NO production (Figure 31), though the P2X4R activation has been linked to proliferation and migration of cultured human cardiac fibroblasts, but this was only reported once several years ago (Chen et al., 2012). Clearly, further studies are also needed to clarify the role of P2X4R in cardiac remodeling.

4. Cardiovascular Risk Factors: Is the P2X4 Receptor Friend or Foe?

Hypertension, diabetes, obesity, and dyslipidemia have a close relationship with heart diseases, major cardiovascular events, and death (Vasan et al., 2005). Strict control of these modifiable cardiovascular risk factors has a major impact on patients' prognosis (Group et al., 2015; Yusuf et al., 2016; Rawshani et al., 2018). Not controlling these risk factors engage the cardiovascular disease continuum driven by atherosclerosis and inflammation (Dzau et al., 2006).

Purinergic receptors have been targeted for the treatment of diabetes, hypertension, and other conditions associated with the metabolic syndrome (Sparks and Chatterjee, 2012; Burnstock and Novak, 2013; Novak and Solini, 2018). Purines regulate multiple functions in blood vessels, ranging from short-lived mechanisms to more sustained processes, including those involved in maladaptive remodeling of hypertension and

atherosclerosis (Burnstock, 2017a). The net vascular response depends on where and when the purinergic transmission occurs. Like acetylcholine (ACh), ATP has a dual response in controlling blood vessel tone, with both vasodilatory or vasoconstrictor responses (Ren et al., 1993; Burnstock, 2017a). One important source of ATP is provided from sympathetic nerves surrounding blood vessels. Stimulation of sympathetic nerves releases norepinephrine and ATP in the smooth muscle layer of blood vessels; these two potent vasoconstrictors are upregulated in hypertension (Brock and Van Helden, 1995). ATP exerts transient vasoconstriction mostly through activation of the P2X1R on vascular smooth muscle cells (VSMCs) (Lewis and Evans, 2001; Vial and Evans, 2002), followed by a smaller yet sustained contraction via P2X4R activation (Harhun et al., 2014). Both P2X1R and P2X4R localized on VSMCs contribute to vasospasm occurring as a consequence of hemorrhagic stroke (Harhun et al., 2014).

When acting at the luminal side of intact blood vessels, circulating ATP causes vasodilation through activation of endothelial metabotropic $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptor subtypes; while the $P2Y_1$ receptor is more sensitive to the ATP metabolite, ADP, the $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptor subtypes are mainly activated by uracil nucleotides, namely UTP and UDP, respectively. Increasing evidence shows that endothelial ATP-sensitive ionotropic P2X4R also participates in vasodilation. These receptors are particularly abundant in coronary and cerebral arteries (Lewis and Evans, 2001). Noteworthy is that endothelial cells release huge amounts of ATP in response to increased blood pressure/flow (Milner et al., 1990), thus creating a “self-regenerative ATP activation wave” spreading along endothelial cells in the vicinity (Burnstock, 2017a). In this sense, endothelial P2X4R may act as immediate mechano-transducers to re-establish local blood pressure/flow by promoting the endothelial release of vasodilation signaling mediators like NO (Yamamoto et al., 2006). This hypothesis is strengthened by findings linking hypertensive and vascular remodeling phenotypes to knockdown or loss-of-function mutations of the P2X4R (Yamamoto et al., 2006; Stokes et al., 2011). For instance, the loss-of-function tyrosine-to-cysteine polymorphism in the 315 position of the human P2X4R ectodomain (Tyr 315 > Cys) was associated with higher blood pressure and reduced arterial compliance as a result of impaired endothelium-dependent vasodilation in large arteries (Stokes et al., 2011). Pathological alterations of renal P2X4R may also contribute to arterial hypertension (see below).

Apart from the local control, vascular tone can be also regulated centrally through cardiovascular reflexes involving carotid bodies (CBs) and aortic bodies (ABs). In CB, a cluster of chemoreceptor cells releases excitatory ATP in response to several chemostimulants such as hypoxia and hypercapnia, which then stimulates P2X2/3R located in sensory afferents of the carotid sinus nerve (CSN), a branch of the petrosal nerve. Firing

of the CSN will then be integrated centrally at the brainstem to increase ventilation and to cause bradycardia and peripheral vasoconstriction (Piskuric and Nurse, 2013). Feedback inhibition of CB-mediated responses involves nitrergic nerves from the glossopharyngeal nerve (GPN). Interestingly, these inhibitory nitrergic nerves can be stimulated by several ATP-sensitive P2X receptors, namely P2X2/3R, P2X4R, and P2X7R, but contrary to the P2X2/3R that is present both in afferent and in efferent nerves, the P2X4R exclusively mediates the inhibitory pathway (Campanucci et al., 2006; Piskuric and Nurse, 2013). Hence, selective stimulation of the CB inhibitory drive with P2X4R agonists or blockage of P2X2/3R-mediated CB excitation may represent putative targets to control cardiorespiratory reflexes, particularly in patients who are refractory to common anti-hypertensive drugs (Pijacka et al., 2016; Conde et al., 2017). Noteworthy, overactivation of CB is also observed in obstructive sleep apnea and type 2 diabetes and may represent a pivotal element in the vicious cycle causing drug-refractory hypertension and type 2 diabetes (Conde et al., 2017).

Atherosclerosis is a low-grade chronic inflammatory process that develops upon persistence of one or more of the aforementioned vascular risk factors (Libby et al., 2019). ATP, acting mainly via metabotropic P2Y receptors, has potent mitogenic and pro-inflammatory effects involved in stepwise formation of atheroma plaques (Burnstock, 2017a). Evidence also exists on direct involvement of P2X receptors in atherosclerosis (Stachon et al., 2013; Burnstock, 2017a; Zhao et al., 2019). For instance, upregulation of endothelial P2X4R and/or P2X7R has been observed in response to cellular damage inflicted by dyslipidemia and hyperglycemia; overactivation of these receptors has been linked to the release of pro-inflammatory cytokines and increases in endothelial permeability (Sathanoori et al., 2015b). Moreover, overexpression of the P2X4R in medial and neointima of rabbit aorta may ascribe the pro-atherosclerotic role of ATP in this vessel (Pulvirenti et al., 2000).

In primary monocyte-derived human macrophages, the P2X4R stimulates the release of CXCL5, a pro-inflammatory chemokine involved in neutrophil recruitment (Layhadi et al., 2018). Nevertheless, caution must be taken in attributing a deleterious role to the P2X4R in atherosclerosis development, because increased production of endothelial CXCL5 was protective against macrophage foam cell accumulation in atherosclerotic plaques by a mechanism that involves cholesterol efflux from macrophages in a rodent model of atherosclerosis (Rousselle et al., 2013). Moreover, the P2X4R was responsible for increases in Krüppel-like factor 2 (KLF2), an atheroprotective transcription factor (Sathanoori et al., 2015a).

As atherosclerotic plaques grow, the lumen of blood vessels becomes narrow, thus limiting blood flow and distal delivery of oxygen and nutrients. At this stage of atherosclerosis development, blood-flow-mediated shear stress dramatically increases

along with overactivation of endothelial P2X4R (see above). In this context, activation of endothelial P2X4R engages protective mechanisms against ischemia in blood vessels where its density is higher, like coronary and cerebral arteries; this results in the increased expression of osteopontin, a well-known neuroprotective factor (Ozaki et al., 2016). In the late stage of atherosclerosis, erosion and plaque rupture occurs leading to endothelial cells damage and inability to prevent thrombotic events, thus prompting acute coronary and stroke events. Endothelial damage exposes not only the procoagulant and platelet-aggregating features of subendothelial matrix proteins (van der Meijden and Heemskerk, 2019) but also the vasoconstrictive P2X1R and P2X4R on VSMCs to surplus amounts of ATP derived from platelet aggregation and damaged cells (Lewis and Evans, 2001; Harhun et al., 2014). Thus, one may hypothesize that the P2X4R exerts a protective role in blood vessels when the endothelium is functional, but its action is detrimental once blood vessels are severely damaged, such as in acute coronary and aortic syndromes, as well as in stroke.

5. Role Played by the P2X4 Receptor in Renal Function and Dysfunction

Renal integrity and function are of utmost importance in patients with cardiovascular diseases. Acute and chronic kidney diseases have a negative impact on the prognosis of patients with HF and coronary artery diseases (Anavekar et al., 2004; McAlister et al., 2012; Legrand and Rossignol, 2020). Multiple factors are involved in the cardiorenal axis regulation. These include neurohormonal mediators and the autonomic nervous system (Rangaswami et al., 2019). Renal purinoceptors control blood flow and glomerular filtration, which are main determinants of the water-electrolyte balance (Burnstock et al., 2014).

In healthy conditions, myogenic and tubule–glomerular feedback (TFG) mechanisms ensure that renal blood flow matches glomerular filtration rate (GFR) for proper water and solute handling. TFG is orchestrated by macula densa cells in distal tubules. These cells react to low NaCl levels in the lumen of distal tubules by favoring dilation of afferent arterioles and renin–angiotensin-dependent constriction of efferent arterioles (reviewed in (Carlstrom et al., 2015)). Infusion of ATP into the renal artery exerts a dual effect in afferent arterioles that is dependent on the vascular tone. Upon increasing renal vascular resistance, ATP effects shift from P2X1R-mediated vasoconstriction to P2Y/P2X4R-induced NO-mediated vasodilation (Fernandez et al., 2000; Menzies et al., 2013; Burnstock et al., 2014). Blood filtration yields the urinary ultrafiltrate, and its composition changes as it moves throughout renal tubules. Multiple P2 receptors are expressed in renal tubules, and their activation promotes natriuresis through inhibition of several sodium transporters (Menzies et al., 2015). Interestingly, after surgical renal

denervation for treatment of drug-refractory hypertension, the P2-mediated natriuresis is significantly favored (Kowalski et al., 2012). Although the P2X4R is abundantly expressed in all renal segments, its functions are better characterized in distal segments of the nephron (Burnstock et al., 2014). Activation of the P2X4R in this region contributes to modulate the activity of epithelial sodium channels (ENaC) (Wildman et al., 2005). However, regulation of ENaC activity by the P2X4R is not linear, and ATP may exert a dual role depending on sodium and body fluid composition. In low-salt conditions, stimulation of the P2X4R increases ENaC activity, while in salt-overload conditions the P2X4R exerts an opposite effect and promotes natriuresis (Wildman et al., 2008; Craigie et al., 2018). As mentioned before, P2X4R^{-}null mice exhibit a hypertensive phenotype (Craigie et al., 2018). Therefore, it is tempting to speculate that in patients with essential hypertension, P2X4R agonists may contribute to promote natriuresis and to lower blood pressure.

Acute kidney injury (AKI) inflicts cardiovascular damage by means of local inflammation, altered mitochondrial function, and the development of local fibrosis (Legrand and Rossignol, 2020). Inflammation-induced P2 X7R receptor activation display an important role in renal disease progression, with some properties being shared by its counterpart P2X4R (Howarth et al., 2015). In a rodent model of ischemic AKI, activation of the P2X4R exacerbates tubular necrosis and NOD-like receptor 3 (NLRP3)-dependent inflammation (Han et al., 2020). In contrast to the cardiac P2X4R, the activation of which putatively favors fibrosis (Chen et al., 2012), the renal P2X4R seems to have an antifibrotic role (Kim et al., 2014).

Chronic elevation of intraglomerular pressure caused by arterial hypertension is accompanied by proteinuria, as a consequence of podocyte damage. Proteinuria is a surrogate marker of cardiovascular diseases (Cohn et al., 2004). Podocytes are sensitive to intraglomerular pressure, and they are important elements of the glomerular filtration barrier. Interestingly, podocytes are endowed with P2X4Rs operating mechanotransduction by interacting with podocin and highly enriched cholesterol domains (Forst et al., 2016). Microalbuminuria is a hallmark of diabetic nephropathy, the progression of which has been associated with P2X4R activation and downstream stimulation of NLP3 inflammasome in podocytes (Chen et al., 2013a). However, it still remains to be clarified whether the mechanosensitive role of the P2X4R in podocytes has a protective or deleterious effect in the progression to glomerulosclerosis secondary to hypertension and diabetes.

6. Is the P2X4 Receptor Activation Beneficial or Detrimental in the Lung?

Heart and lungs operate as a coupled unit, with numerous hemodynamic and neurohormonal interactions between them. Pulmonary diseases increase morbidity and worsen survival in patients with cardiovascular diseases. Pulmonary arterial hypertension

(PAH) is a prototypic disease model of pulmonary and cardiac functions' interdependence. In PAH, there is a disproportionate increase in pulmonary vascular resistance (PVR), resulting in maladaptive remodeling of the right ventricle (RV) and HF (Forfia et al., 2013). Pulmonary vasodilators decrease PVR and improve PAH, particularly in group 1 PAH patients (Galie et al., 2016). ATP and its metabolite, adenosine, have important roles in controlling pulmonary vascular tone. Similar to that occurring in other vascular beds, ATP has both vasodilatory and vasoconstrictive effects in pulmonary arteries. Pulmonary vasoconstriction caused by ATP depends on activation of P2X1, $P2Y_{2,}$ and $P2Y_4$ receptors on VSMCs (Guibert et al., 1996; Neely et al., 1996; Syed et al., 2010; Visovatti et al., 2016), while vasodilation prevails when it activates endothelial-located metabotropic $P2Y_1$, $P2Y_{2,}$ $P2Y_4$, and/or ionotropic P2X4R (Guibert et al., 1996; Neely et al., 1996; Syed et al., 2010; Visovatti et al., 2016). As aforementioned, the pulmonary arteries express high levels of the P2X4R (Qasabian et al., 1997; Yamamoto et al., 2003), where it seems to mediate shear-stress-induced ATP vasodilation (Lewis and Evans, 2001; Syed et al., 2010). Despite the fact that it has been demonstrated that intravenous delivery of P2X4R agonists has the potential to cause pulmonary vasodilation and to decrease RV overload, this warrants further investigations in disease models of PAH.

Respiratory infections and exacerbation of chronic lung diseases are common causes of acute decompensated heart failure (ADHF) (Yamamoto et al., 2003; Yamamoto et al., 2018). Moreover, pulmonary congestion in ADHF often accompanies with ventilation/perfusion mismatch, which intensifies respiratory insufficiency with hypoxemia and hypercapnia, a situation that is already compromised in patients with underlying chronic pulmonary disease. As mentioned above, chronic hypoxemia, as well as respiratory acidosis, stimulate peripheral chemoreceptors and trigger several cardiovascular reflexes, accompanied by deleterious sympathetic overactivation and abnormal handling of water and natriuresis (Forfia et al., 2013; Piskuric and Nurse, 2013). Most patients presenting with ADHF usually require diuretic, vasodilatory, and inotropic therapies to reduce congestion and optimize respiratory function and cardiac output (Ponikowski et al., 2016). Considering the proposed framework for the P2X4R in cardiovascular diseases, development of new and better-tolerated agonists for this receptor may represent an opportunity to improve ADHF patients' symptomatology, as they would reduce pulmonary congestion subsequently to increases in cardiac inotropy, diuresis, and vasodilation.

Apart from endothelial-induced vasodilation of pulmonary vessels and decrease of right ventricle afterload, prolonged P2X4R activation has been associated with inflammation, hyperreactivity, and mucus production in the airways of susceptible individuals (Burnstock et al., 2012; Gao and Jacobson, 2017; Antonioli et al., 2019). Chronic lung diseases usually have in their background an inflammatory response that precipitates

deterioration of both cardiac and respiratory functions (Celutkiene et al., 2017; Le et al., 2019). Extracellular ATP raises dramatically during inflammatory conditions to levels high enough to activate P2X7, $P2Y_2$, and $P2Y_6$ receptors, which normally exhibit low affinity for the nucleotide (see Section 2). Activation of these receptors is often associated with tissue fibrosis, polymorphonuclear leukocytes infiltration, and other immune cell activation, which are hallmarks of maladaptive pulmonary remodeling (Le et al., 2019). Indeed, termination of P2 purinoceptors-mediated signaling by enzymatic conversion of ATP to adenosine revealed to be protective in lung injury (Eltzschig et al., 2004).

Although the P2X4R is present in immune cells from both myeloid and lymphoid lineages (Antonioli et al., 2019), and its expression is upregulated in inflammatory conditions (Winkelmann et al., 2019), there is still no consensus about its role in inflammation given that both pro- and anti-inflammatory effects have been described in the literature (Burnstock, 2016; Zech et al., 2016; Csoka et al., 2018; Zabala et al., 2018; Antonioli et al., 2019). In a murine experimental model of asthma, a positive correlation between P2X4R levels and inflammation severity has been found; blockage of P2X4R activation reversed some asthma remodeling hallmarks, including bronchoalveolar lavage fluid eosinophilia, peri-bronchial inflammation, Th2 cytokine production, and bronchial hyper-responsiveness (Chen et al., 2016; Zech et al., 2016). Recently, it was demonstrated that stimulation of the P2X4R favors T cell migration to the lungs, whereas blockage of this receptor is protective against acute rejection of lung allotransplants (Ledderose et al., 2018). The pro-inflammatory roles of the P2X4R are, in part, due to its expression in leucocytes, namely lymphocytes and eosinophils (Ledderose et al., 2018; Paalme et al., 2019). Moreover, it seems that ATP-induced mast cell degranulation involves the P2X4R activation (Yoshida et al., 2019), and this receptor is also implicated in the contraction of airway smooth muscle cells (Nagaoka et al., 2009). Overall, these features may contribute to favor bronchoconstriction and allergic responses in inflammatory lung diseases.

ATP-induced airway secretion has gained special attention in the last decade. The P2X4R is involved in mucous secretion and in the control of lung surfactant production (Burnstock et al., 2012; Winkelmann et al., 2019). Mechanical stretch of alveoli increases the release of ATP to the extracellular milieu along with increases in lung surfactant production (Patel et al., 2005; Mishra et al., 2011). Alveolar type II (AT II) cells are specialized in the secretion of lung surfactant (Han and Mallampalli, 2015); these cells harbor the P2X4R as the most abundant P2X receptor subtype (Miklavc et al., 2011). The P2X4R has a unique subcellular location in AT II cells, being found in lamellar bodies that contain lung surfactant. The release of lung surfactant is an exocytotic process designated by fusion-activated Ca2+ entry (FACE), which is operated by P2X4 (and possibly $P2Y_2$) receptors activation (Miklavc et al., 2011). The P2X4R inward current also drives water

reabsorption across the alveolar epithelium, which is crucial to maintain alveoli relatively dry to increase gas exchange efficiency (Thompson et al., 2013). Although not completely resolved, it is believed that the main extracellular ATP source for P2X4R activation is alveolar type I (AT I) cells (Patel et al., 2005; Mishra et al., 2011), yet extracellular release of ATP may be further amplified by P2X4R-induced lysosome fusion, resulting in the release of ATP stored inside lamellar bodies (Fois et al., 2018).

Acute lung inflammation causes a disproportionate increase in extracellular ATP, which contributes to dysregulation of surfactant production and initiation of the inflammatory response (Hasan et al., 2018). Although speculative, from a mechanistic point of view, alveolar P2X4Rs may have some role in acute respiratory distress syndrome (ARDS), a life-threatening condition frequently requiring emergent positive-pressure ventilation support, so it is pursued nowadays in the SARS-CoV-2/COVID-19 pandemic (Rajagopal et al., 2020). In this context, selective modulation of P2X4Rs activation may control lung surfactant production and protect patients from barotrauma and alveolar collapse, which are common features in ARDS (Albert, 2012; Matthay et al., 2019). Likewise, patients presenting with ADHF usually require non-invasive positive-pressure ventilation to resolve signs and symptoms of congestion. It is, thus, tempting to hypothesize that activation of alveolar P2X4Rs may also be beneficial to these patients' outcomes.

7. Is there a Dark Side of P2X4 Receptor Activation Outside the Cardiovascular System?

In this section, we will briefly address putative side-effects of P2X4R activation outside the cardiovascular system, which might be relevant when translating the use of P2X4R agonists to the clinical practice.

The P2X4R was first identified in the central nervous system (Soto et al., 1996), with the following reports showing its widespread expression both in central and in peripheral neurons (reviewed in (Stokes et al., 2017; Suurvali et al., 2017; Zhang et al., 2020). Great attention has been given to the modulation of P2X4R activity to control chronic pain. The P2X4R is upregulated in spinal microglia cells following peripheral nerve injury; genetic ablation or pharmacological blockage of overexpressed P2X4Rs can reverse allodynia occurring in this condition (Tsuda et al., 2003). Increasing evidence demonstrates that blockade of the P2X4R alleviates neuropathic and inflammatory pain (Ulmann et al., 2008; Ulmann et al., 2010; Matsumura et al., 2016), as well as other causes of chronic pain, including migraine (Long et al., 2020a). Hyperalgesia caused by P2X4R activation seems to depend on brain-derived neurotrophic factor (BDNF) release (Ulmann et al., 2008; Beggs et al., 2012). In this context, experimental data indicate that the P2X4R may play a role in long-term potentiation and synaptic plasticity (Sim et al., 2006; Baxter et al., 2011), which

may underlie its effect on chronic pain. Notwithstanding the involvement of the P2X4R in neuropathic and inflammatory pain conditions, its activation did not affect acute pain sensation in animals not prone to pain hypersensitivity (Tsuda et al., 2003). Although changes in nociception may be a problem for the clinical use of P2X4R agonists in cardiovascular diseases, this warrants confirmation in clinical trials due to contradictory results using animal models. The same holds true regarding involvement of the P2X4R in neuroinflammatory conditions, as conflicting results, either protective (Ozaki et al., 2016; Zabala et al., 2018) or deleterious (de Rivero Vaccari et al., 2012; Ulmann et al., 2013), have emerged. With this in mind, forthcoming P2X4R agonists targeting the cardiovascular system should not cross the blood–brain barrier; otherwise, prolonged stimulation may have negative impacts in the course of some neurodegenerative diseases, chronic pain conditions, and epilepsy in susceptible individuals (Ulmann et al., 2013; Burnstock, 2017c; Suurvali et al., 2017).

Rather than deleterious, the P2X4R expression and function in the brain reward system have been associated with salutary effects in alcohol abuse. Ethanol is a preferential inhibitor of the P2X4R, a situation that can be overcome by positive allosteric modulators, like ivermectin or derivates, which have been shown to reduce ethanol drinking behavior (reviewed in (Franklin et al., 2014; Khoja et al., 2018)).

Inflammatory flairs may also occur with P2X4Rs activation, particularly in patients with proinflammatory backgrounds, such as rheumatoid arthritis and asthma (Li et al., 2014; Zech et al., 2016; Burnstock, 2017c; Suurvali et al., 2017; Antonioli et al., 2019). Pruritus and hypersensitivity reactions can also be a consequence of stimulation of P2X4R in mast cells and other immunocytes (Antonioli et al., 2019; Yoshida et al., 2019). Cancer is another type of inflammatory condition where the P2X4R might be involved, although its role remains uncertain (Burnstock, 2017c).

In the opposite direction of the inflammatory spectrum, activation of P2X4R in macrophages exhibited both anti-inflammatory properties and bactericidal activity against Gram-negative bacteria in the cecal ligation and puncture-induced sepsis rodent model. This P2X4R-mediated protection improved multi-organ failure and sepsis-related death (Csoka et al., 2018), which supports future therapeutics with P2X4R agonists to treat patients with ADHF secondary to sepsis.

8. Conclusions

The development of novel purinergic-based medications has been struggling with the ubiquitous nature of P1 and P2 purinoceptors and lack of drug selectivity. Pushed by the success of $P2Y_{12}$ receptor antagonists as anti-thrombotic drugs, last decades have provided new experimental and pharmacological tools to dissect the purinergic signaling

cascade, including the purinoceptors function, which have granted a series of promising druggable targets, and some of them were already admitted to clinical trials. Selective drugs targeting the P2X4R are limited to few antagonists and positive allosteric modulators, without any selective agonist being available so far. Despite this, researchers worldwide have been unlocking the potential of the ionotropic P2X4R to treat several diseases. By reviewing recent data in the literature, we have highlighted here the benefits putatively arising from activation of the P2X4R, either by directly ameliorating the cardiac function or by indirectly improving the function of other systems impacting cardiovascular disease conditions. We also provide some hints about problems to be solved regarding unwanted side effects that may emerge upon activation of P2X4R to stimulate the cardiovascular system, such as arrhythmias and worsening of diastolic dysfunction in the heart; water–electrolyte imbalance and remodeling in renal injury; inflammation, mucous secretion, and hyperreactivity in airway diseases; and nociceptive-related changes. Taken together, we provide input for forthcoming development of medications based on selective activation of the P2X4R to reduce the burden of cardiovascular diseases.

VII. FINAL REMARKS

Over the years, purinergic research has been a valuable source of knowledge to understand pleiotropic disease conditions and to develop putative innovative therapies. Last year's advances in the purinergic field have been fueled by the discovery and chemical improvement of drugs targeting this system with recognized clinical efficacy. The cardiovascular field has been collecting most of these advances, being $P2Y_{12}R$ antagonists, for coronary artery diseases, and adenosine, for termination of supraventricular tachyarrhythmias and imaging diagnosis of coronary artery disease, among the most remarkable examples of purinergic-based therapies applied to the clinical practice. It is, however, elementary to recognize that most of these discoveries began with early descriptions and characterization of the purinergic signaling pathways in experimental models of human physiology and pathology. Within the fundamental research scope of this thesis, it aimed at solving unmet today's clinical problems and raise new hypotheses for the improvement of tomorrow's medicine. Data presented here provide new insights about how purines acutely regulate cardiac hemodynamics under physiological conditions and unveil new mechanisms that can be exploited in the future for better control of clinically relevant pathological conditions.

Data show here that adenosine is more potent in reducing chronotropy than atrial inotropy, a property here coined as "chronoselectivity". Both A_1R and M_2R have inhibitory actions in the heart through the opening of GIRK channels and inhibition of AC. Despite this similarity, stimulation of M_2R did not exhibit chronoselectivity, thus suggesting that other signaling mechanism(s) must be involved. Adenosine A_1R-induced chronoselectivity was sensitive to blockage of SK potassium currents and L-type calcium channels, which did not occur regarding the inhibitory effect of the M_2R agonist, oxotremorine. Stimulation of atrial A_1R restrains SK channel opening and repolarization of atrial cadiomyocytes, thus prolonging AP and allowing more time for Ca^{2+} entry via L-type calcium channels. These features may explain the relatively lower probability of adenosine to reduce inotropy *vis a vis* chronotropy compared with other cardiodepressant compounds acting via $G\alpha_{i/o}$-protein-coupled receptors. Data also strengthen the theory that SK and LCC currents play significant roles in EC coupling, as later demonstrated in cardiomyocytes by other authors (Zhang et al., 2018). It remains, however, to be elucidated the molecular mechanism underlying A_1R interaction with SK channels. Preliminary results from our lab also pointed out for the involvement of the A_1R-PLC-PKC axis to explain adenosine-induced chronoselectivity, indicating as being very likely the existence of a downstream interaction at the second messengers' level.

From a clinical point of view, several important insights emerge from these results. One interpretation that comes directly from these data is that adenosine and its analogs are able to reduce heart rate without impacting on atrial inotropy. While atrial inotropy is

negligible in healthy individuals, the use of drugs that preserve the atrial contractile function may be of particular interest in those with severe diastolic impairment, such as patients with HF where atrial contraction deficits are detrimental for cardiac output. As a corollary, this study suggests (1) that adenosine or related compounds should be used as first-line therapy for acute management of supraventricular tachyarrhythmias in patients with HF, and (2) that combination of adenosine with L-type calcium channel blockers should be used with caution in these situations. Although not directly explored in this thesis, the proposed interaction between A_1R and SK/K_{Ca} channels also expands the antiarrhythmic potential of adenosine (Matthews and Grace, 2020; Gupta et al., 2021). Although rare, pro-arrhythmic side effects may occur as a consequence of usage of adenosine agonists (e.g., regadenoson) or dipyridamole in the context of myocardial stress studies to detect coronary artery obstructions (Brink et al., 2015). Despite this concern, the use of adenosine and adenosine-related compounds is relatively safe, with few short-lived arrhythmias (e.g., SAN pauses and AVN blocks) being reported (Subbiah and Patil, 2017). In this regard, unpublished observations using monophasic action potential recordings in atria showed that, contrary to M_2R stimulation, A_1R activation preserves atrial effective refractory period (Bragança et al., 2017, conference presentation at the Portuguese Society of Pharmacology), thus providing a rationale for the low incidence of arrhythmias with adenosine and related compounds.

SK channels have been associated with the development and maintenance of atrial fibrillation, as well other ventricular arrhythmias (Mahida, 2014; Qi et al., 2021). Interestingly, the net effect of SK channel modulation is likely to be disease-dependent, with their inhibition being antiarrhythmic in remodeled hearts and proarrhythmic in normal hearts (Qi et al., 2021; Takahashi et al., 2021). Interaction with SK channels may also help to explain some arrhythmias often observed with adenosine-based treatments (Guieu et al., 2020). Due to its ability to awake dormant arrhythmogenic foci, adenosine has been valuable for electrophysiologists in the identification of pulmonary veins not successfully isolated during ablation of atrial fibrillation, thus reducing the probability of recurrence of and repeated interventions (Gupta et al., 2021). Remarkably, the SK channel blocker apamin also demonstrated to stimulate the electrophysiological activity of pulmonary veins (Chen et al., 2013b), which confirms a shared mechanism with adenosine. Despite these conflicting results regarding the arrhythmogenic effect of SK channels, the identified interaction with A_1R raise new questions about the therapeutic potential, as well as side effects, of adenosine-like compounds in clinical practice.

This thesis also provides solid ground for the involvement of P2 receptors in regulating SAN function and ventricular inotropy. The interaction between P2X4 and NCX also exists in ventricles but, here, rather than having an inhibitory action, stimulation of P2X4 improves the contractile activity. The dual role of P2X4 receptor in stimulating inotropy while

inhibiting chronotropy is unique among cardioactive agents, only matched by digitalis. The bradycardic effect of ATP is well-known. This effect was formerly attributed to ATP breakdown into adenosine and subsequent activation of A_1R. Despite early evidence about the expression of several P2 receptor subtypes in the cardiac conduction system, the hypothesis of ATP being itself a modulator of the conduction system remained unexplored until very recently, mainly because of the close resemblance with adenosine-mediated effects. However, it is hard to accept that mammalian purinergic receptors resisted to evolution if their function were physiological meaningless. The first evidence for the involvement of P2 receptors in the acute regulation of chronotropy was somehow indirect, with a description of P2X2/3 receptors in afferent cardiac nerve endings and their contribution to cardio-cardiac vagal depressor reflex. In view of this, amplification of the bradycardic component helps to explain previous studies indicating that ATP was more potent than adenosine in reducing chronotropy and dromotropy and, therefore, in terminating some tachyarrhythmias (Pelleg and Belhassen, 2010). Data presented in this thesis further expands this concept, as it demonstrates for the very first time that ATP is a potent negative chronotropic agent on its own through the activation of ionotropic P2X4R, which are abundantly expressed in SAN cells. Once activated, the P2X4R allows a large influx of cations interfering with the electrogenic or pacemaking mode of NCX, which decreases the probability of spontaneous generation of APs slowing down the sinus heart rate. Modulation of NCX by P2X4 seems to occur by changing the ion gradients across the plasma membrane rather than at the protein-protein level (Yang et al., 2015). Interestingly, this mechanism closely resembles the action of digitalis in the heart, where inhibition of NKA interferes with NCX causing a dual negative chronotropic and positive inotropic effect (Belz et al., 2001). Therefore, therapies targeting stimulation of P2X4 have also a potential benefit to be used in the treatment of tachyarrhythmias, including rate control of atrial fibrillation and heart failure (Blomstrom-Lundqvist et al., 2003a; Yang et al., 2015; Hindricks et al., 2021).

Noteworthy, this study also demonstrated strong expression of P2X4R in arteries supplying the SAN. The P2X4R is coupled to NO generation and its activation has been associated with vasodilation of some vascular beds (Yamamoto et al., 2006; Stokes et al., 2011), which raises the possibility of drugs targeting P2X4R being also valuable for coronary artery disease (Braganca and Correia-de-Sa, 2020). It is highly likely that other P2 receptors may also have a role in controlling sinus rate and inotropy, since ATP-mediated effects were not fully prevented by P2X4R blockers. The development of purinergic armamentarium will surely help to identify hidden functions of other P2 receptors expressed in the SAN and working myocardium.

Another relevant issue that arises from the discovery of the role of P2X4 in the SAN is its potential involvement in the sick sinus syndrome (SSS). This syndrome is highly prevalent in elderly people, but it can also be present in younger individuals in its primary form, usually associated with dysfunction of ion channels, connexins, and other structural proteins expressed in cardiac cells. The SSS is characterized by an inability of the SAN to generate timely and effective APs, which clinically manifests by longer sinoatrial arrest and symptomatic bradycardia. Current medical therapies rely on positive chronotropic agents, such as anti-muscarinics, β-selective agonists, adenosine antagonists, but often with limited benefit and not avoiding implantation of a pacemaker (Mesirca et al., 2021). In this scenario, selective blockage of P2X4R may represent a new pharmacological approach to manage SSS.

As previously mentioned, both ATP and adenosine induce robust electrophysiological effects at the AVN and conduction blockade that, once again, is thought to be mediated by A_1R. However, based on these results, it is reasonable to speculate that the same mechanism described for P2X4 in the SAN can also take place in the AVN. As a matter of fact, the AVN expresses several proteins that are also present in the SAN, allowing it to function as a secondary pacemaker site (Ye Sheng et al., 2011). Further studies aiming at characterizing P2 receptors in the AVN conduction system, either by non-invasive, such as surface electrocardiography, or other invasive electrophysiological studies, will be valuable to address this hypothesis. Likewise, it will be also important to explore the role of the P2X4 in arrhythmias, particularly those of supraventricular origin where adenosine has demonstrated to be highly effective (Matthews and Grace, 2020; Gupta et al., 2021).

Another interesting finding from this study was the demonstration that the P2X4 is not restricted to SAN cells, as some NF160-positive nerve terminals also showed strong immunoreactivity against P2X4. The heart receives multiple inputs from a dense network of neurons and ganglia of the autonomic nervous system (Durães Campos et al., 2018). The balance between sympathetic and parasympathetic tonus has been associated with the development and maintenance of arrhythmias, hypertension, syncope, heart failure, and even sudden cardiac death (La Rovere et al., 2020). As described throughout this thesis, purines are important players in the autonomic neurotransmission. The presence of P2 receptors both in parasympathetic and sympathetic branches of the autonomic nervous system may fine-tuning modulate cardiac neurotransmission (Ma et al., 2005). There is also evidence about the existence of non-cholinergic and non-adrenergic nerves containing ATP secretory vesicles, which identity and function remains to be resolved (Crowe and Burnstock, 1982; Burnstock, 2017b). This study did not fully characterize the intracardiac P2X4-positive nerves, but this surely deserves to be explored in future studies.

Altogether data unravels new avenues for drugs targeting P2X4 in order to better control arrhythmias, to improve systolic function in HF patients, and to decrease symptoms of coronary artery disease. Accumulating evidence also points for salutary effects of P2X4R in organs other than the heart, such as blood vessels, lungs, and kidneys (reviewed in (Braganca and Correia-de-Sa, 2020)). Regrettably, promising therapies targeting P2X4R face the current lack of selective agonists and antagonists. There are few selective P2X4 antagonists; however, some drawbacks of bioavailability limit their use in “in vivo” experimental models and clinical studies (Matsumura et al., 2016). P2X4R agonists are mostly limited to ATP and CTP, both non-selective P2X agonists, with no selective agonist being currently available, which creates enormous difficulties in assessing the direct effects of P2X4 stimulation. Ivermectin, a selective positive allosteric modulator of P2X4, has been valuable in identifying P2X4-mediated responses, as it was done here. Its effects strengthen the participation of the P2X4 receptor in the regulation of chronotropy. Ivermectin is widely used in clinical practice as an old broad-spectrum anti-parasitic agent rather than a P2X4 modulator. Despite odd at first glance, this creates an opportunity for repurposing ivermectin for identification of new therapeutic applications, such as in cardiovascular diseases. Repurposing ivermectin is now in the spotlight of COVID-19 pandemics, as it has been demonstrated to reduce viral load and symptomatic relief (Chaccour et al., 2021). Forthcoming years are expected to come with the discovery and development of new selective drugs targeting P2X4 that, based on these data, will hopefully help in reducing the global burden of cardiovascular disease.

VIII. REFERENCES

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