Epac activation, altered calcium homeostasis and ventricular arrhythmogenesis in Langendorff-perfused mouse hearts

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The recently described cAMP sensor, Exchange protein directly activated by cAMP (Epac), has been implicated in distinct cAMP-dependent, protein kinase A-independent cellular signalling pathways (Bos JL, 2006). We investigated effects of Epac activation in catecholamine-induced ventricular arrhythmogenesis. In contrast to control findings (n = 20), monophasic action potentials showed spontaneous triggered activity in 2 out of 10 intrinsically beating and 5 out of 20 extrinsically-paced Langendorff-perfused murine hearts perfused with the specific Epac activator 8-pCPT-2’-O-Me-cAMP (8-CPT, 1 μM) (Christensen AE et al. 2003). During steady extrinsic pacing at 8 Hz, 3 out of 20 such hearts showed spontaneous ventricular tachycardia (VT). Programmed electrical stimulation provoked VT in 10 of 20 similarly treated hearts (P < 0.001; n = 20, Fisher’s Exact Test). However, no statistically significant changes (P > 0.05, ANOVA) in left ventricular epicardial (40.7 ± 1.2 versus 44.0 ± 1.7 ms; n = 10), or endocardial action potential durations (APD90) (51.8 ± 2.3 versus 51.9 ± 2.2 ms; n = 10), transmural (ΔAPD90) (11.1 ± 2.6 versus 7.9 ± 2.8 ms; n = 10) or apico-basal gradients of repolarization, ventricular effective refractory periods (29.1 ± 1.7 versus 31.2 ± 2.4 ms in control and 8-CPT-treated hearts, respectively; n = 10) and APD90 restitution characteristics accompanied these arrhythmogenic effects. However, fluo-3 fluorescence imaging of cytosolic Ca2+ demonstrated alterations in Ca2+ homeostasis in the form of increased Ca2+ wave generation in both paced and resting isolated 8-CPT-treated ventricular myocytes. An independent method of Epac activation that applied 100 nM isoproterenol to stimulate beta-adrenoreceptors in parallel with protein kinase A inhibition by 2 μM H-89, was also arrhythmogenic in the whole heart and similarly altered cytosolic Ca2+ homeostasis. The Epac-dependent effects on both the whole heart and cellular levels were reduced by inhibition of Ca2+/calmodulin-dependent protein kinase II (CaMKII) with 1 μM KN-93. These findings associate VT in an intact cardiac preparation with altered cellular Ca2+ homeostasis and Epac activation through a CaMKII-dependent mechanism for the first time, in the absence of the altered repolarization gradients previously implicated in re-entrant arrhythmogenesis (Killeen MJ et al. 2007; Thomas G et al. 2007).

Christensen AE et al. (2003). J Biol Chem 278, 35394-402
Killeen MJ et al. (2007). Acta Physiol (Oxf) 189, 33-46

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PC118

Is nitric oxide (NO) important in the adenosine A2A-receptor-mediated vasodilatation of skeletal muscle contraction?

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During systemic hypoxia the contribution of adenosine to skeletal muscle vasodilatation is dependent on the presence of NO; NO is required for the release of adenosine from the endothelium (Edmunds et al. 2003) and mediates dilatation via endothelial A1-receptors (Ray & Marshall, 2005). By contrast, skeletal muscle vasodilatation accompanying muscle contraction (exercise hyperaemia) is mediated by adenosine acting at A2A receptors, but not A1 receptors (Ray & Marshall, 2008). Adenosine can release NO from endothelium by acting at A2A receptors (Ray et al. 2002). Thus, we investigated the role of NO in exercise hyperaemia.

In three groups of rats, anaesthetized with Saffan (7-12 mgkg-1 hr-1 I.V.), we recorded arterial blood pressure (ABP), femoral blood flow (FBF) and tension in the extensor digitorum longus. Isometric twitch contractions were evoked by stimulation of the sciatic nerve at 4Hz. Integral femoral vascular conductance (IntFVC) was calculated off-line. Group 1 (n=7) was the time control for, Group 2 (n=10), which received NOS inhibitor L-NAME before the third, and A2A-receptor antagonist ZM241385, before the fourth contraction. Group 3 (n=12) received L-NAME before the third, the NO-donor SNAP to restore baseline FVC during the fourth and fifth contraction and ZM241385 before the fifth.

Time controls showed consistent tension and hyperaemic responses. In Group 2, baseline IntFVC was reduced by L-NAME (0.555 ± 0.04 (mean ± SEM) to 0.297 ± 0.02CU*, ANOVA for repeated measures, p<0.001) but not by ZM241385. L-NAME reduced exercise hyperaemia (13.91 ± 1.31 to 9.52 ± 1.09CU*), and it was further attenuated by ZM241385 (5.467 ± 1.12CU*).

In Group 3, SNAP after L-NAME restored baseline IntFVC to control levels (Control: 0.702 ± 0.10CU, L-NAME: 0.377 ± 0.09CU*, L-NAME + SNAP: 0.616 ± 0.09CU); ZM241385 had no further effect. Exercise hyperaemia was also restored to control levels after L-NAME by SNAP (Control: 16.99 ± 0.98CU*, L-NAME + SNAP: 19.70 ± 1.38CU*, and this response was further attenuated by ZM241385 (12.75 ± 0.98CU*).

These results confirm that adenosine acting via A2A-receptors contributes to exercise hyperaemia. However, they indicate
level. Overall our data suggest that either the sparse labeling of PSD does not reflect the presence of functional receptors, or the level of synaptic NMDAR expression is below that which can be resolved during our recordings. Alternatively, NMDARs are present but not activated during the brief glutamate transient arising from the release of single quanta. Future modeling studies will address this question.


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showed that KChIP2 mRNA was more abundant in the epicardium than the endocardium both in the neonate and adult. However, in the neonate, whereas Nav1.5 mRNA was more abundant in the endocardium than the epicardium, it was uniformly distributed across the ventricle in adult. Cav1.2 mRNA was uniformly distributed across the neonatal ventricle, while in the adult ventricle, Cav1.2 mRNA was significantly more abundant in the epicardium than the endocardium. Immunohistochemistry confirmed that NCX1, SERCA2a and RyR2 proteins were more abundant in the epicardium than the endocardium in the adult, but not in the neonate. To conclude, there are complex developmental changes in ion channel and Ca\(^{2+}\) handling protein expression across the ventricle that may have implications for the treatment of arrhythmias.

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**PC83**

**Regulation of plasma membrane expression of P2X4 receptors in immune cells**

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P2X4 receptors are one of the predominant subtypes of purinergic receptors expressed in macrophages and microglia and their up-regulation has been shown to contribute to neuropathic pain. P2X4 receptors are prominently localized to lysosomes and resist degradation by virtue of N-linked glycans decorating the intra-luminal loop of the receptor. In order to understand how the expression of these receptors at the plasma membrane is regulated, we compared the proportion of receptors expressed at the cell surface in cultured microglia and macrophages following exposure to modulators of microglial/macrophage activation. Surface expression was analysed by biotinylation of exposed proteins and by cross-linking proteins with membrane impermeant cross-linkers, followed by SDS-PAGE and western blotting. The modulators included lipopolysaccharide (LPS), ATP and phorbol esters. A 24h incubation with LPS (500ng/ml) resulted in an up-regulation of P2X4 surface expression in cultured microglia without an evident increase in total expression of the receptor, indicating redistribution from intracellular compartments to the plasma membrane. In contrast, similar exposure of cultured astrocytes to LPS had no effect on the surface expression of P2X4 receptors. Exposure of microglia to LPS was sufficient to inhibit proliferation and we have compared the involvement of the P2X7 receptor in both the anti-proliferative effects of LPS and the up-regulation of P2X4 receptors. Brief incubations with phorbol esters produced a similar up-regulation of surface P2X4 receptors in bone marrow derived macrophages (BMDMs) and we are examining the underlying mechanisms involved.

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**PC84**

**Channel blocking properties of a partial agonist at the human muscle acetylcholine receptor**

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Agonists at the muscle acetylcholine receptor (AChR) can all block the channel as well as activate it. For many partial agonists e.g. choline or tetramethylammonium (TMA), concentrations for activation and block are similar. We recorded cell-attached TMA-activated single-channel currents from HEK293 cells transfected with human nicotinic AChRs (\(\alpha\beta\delta\epsilon\), transfection ratio 2:1:1:1). In single-channel records at -80 mV, the amplitude of the openings appears to decrease progressively with agonist concentration because of fast channel block. Several records obtained at different TMA concentrations were fitted simultaneously with HJCFIT\(^1\). For TMA the equilibrium constant, \(K_p\), for open channel block was 8.9 ± 0.6 mM, as estimated from the reduction of apparent single-channel amplitude, cf \(EC_{50}\) of 2.2 ± 0.5 mM. If essentially no blockages are detected, the open state and the open-blocked state can be treated as a single compound open state for the purpose of analysing kinetics. Such compound states are indicated by the boxes in Fig 1a and 1b. During fitting, the exit from the compound open state in Fig 1a is given by a transition rate that is not \(\alpha_2\) but rather \(\alpha_2/[1+c_B]\), where \(c_B = [B]/K_B\) and [B] is the blocker (agonist) concentration. This reflects the fact that the compound state spends only a fraction of time \(1/(1 + c_B)\) in the state from which exit can occur. Similarly, for the mechanism in Fig 1b, the transition rate for leaving the compound open state via the blocked state \((A2^* B)\) is taken not as \(\alpha_B\) but rather \(\alpha_B/[1+c_B]\), i.e. \(c_B\) is multiplied by the fraction of time which the compound open state spends in the blocked state. The left column shows a fit with a mechanism that allows block of channels only when they are open. The predicted distribution of apparent open times at the lower concentration (1 mM, Fig 1c) of TMA superimposes on the observations quite well, but at the higher concentration (10 mM, Fig 1e) the prediction is poor. The predominant mean apparent open time is about 1.5 times smaller than is predicted.

The right column in Fig 1 shows fit of a mechanism (Fig 1b) in which the block is not selective for the open state, but can occur from any state. In this case the distribution of apparent open times is predicted accurately at both low and high concentrations of TMA. The mean values were \(\alpha_2 = 2370 s^{-1} ± 12\% (CVM, n = 4 fits)\) and \(\alpha_B = 1550 s^{-1} ± 19\%\), so the channel shuts almost as fast when it is blocked as when it is not. The mean opening transition rate that for the unblocked channel was \(\beta_2 = 71300 s^{-1} ± 9\%\), similar to that for acetylcholine\(^2\), and for the blocked channel, the mean opening rate was almost as fast, \(\beta_B = 49000 s^{-1} ± 9\%\). Thus TMA seems not act as a pure open channel blocker, but AChRs blocked by TMA can close and return to their resting state without re-opening.
Backround: Inhibition of the apical Na+/H+ exchanger isoform 1Medizinische Hochschule Hannover, Hannover, Germany, M. Chen 1, A. Singh 1, U. Dringenberg 1, R. Engelhardt 1, B. Riederer 1, M. Manns 1, I. Rubio 2, M. Soleimani 3, G. Shull 3, 3University of Cincinnati, Cincinnati, OH, USA

Aim and Methods: In this study the effects of phenylephrine on spontaneous electrical activity in the guinea-pig prostate were investigated using intracellular microelectrodes to record changes in membrane potentials. Paired Student’s t-test was used for tests of significance, values are expressed as mean ± SEM and p<0.05 was considered to be significant.

Results: Phenylephrine (1μM) increased the frequency of slow wave activity from 3.9 ± 0.9 min⁻¹ to 9.4 ± 2.2 min⁻¹ (n=6, p<0.05) and pacemaker activity from 6.8 ± 1.0 min⁻¹ to 9.0 ± 0.9 min⁻¹ (n=3, p<0.05) without affecting other measured parameters. In the presence of nifedipine (1μM), phenylephrine also increased the frequency of both waveforms and in addition caused a membrane depolarisation from -53.4 ± 2.8 mV to -51.0 ± 1.9 mV in slow waves (n=8, p<0.05) and -51.4 ± 2.8 mV to -47.6 ± 2.5 mV in pacemaker potentials (n=3, p<0.05). In cells where nifedipine abolished the spontaneous electrical activity, phenylephrine was able to restore activity which was associated with a resting membrane depolarisation of 2-4mV. (n=7, p<0.05). In the presence of nifedipine, cyclopiazonic acid (CPA, 10μM), carbonyl cyanide 3-chloropropionylhydrazone (CCCP, 1-10μM) or niflumic acid (10-100μM) abolished electrical activity. In the presence of CPA or CCCP, phenylephrine...
that NO is not required for adenosine to be released during contraction, as adenosine acting on A2A receptors still contributed to exercise hyperaemia when NOS was inhibited. Further, as the contribution of adenosine acting via A2A receptors to exercise hyperaemia was fully restored when tested against a background of NOS inhibition and tonic NO-induced dilatation, it seems that adenosine released during contraction does not depend on new synthesis of NO to produce vasodilatation. We therefore propose that, during muscle contraction adenosine is released from the skeletal muscle fibres independently of NO and acts directly on A2A receptors on the vascular smooth muscle to cause vasodilatation.


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PC119

Hypotensive effect of the nitrosyl ruthenium complex nitric oxide donor in renal hypertensive rats

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Nitrosyl ruthenium complexes have been studied as a new class of NO donors. We have described a new compound (trans-[RuCl([15]aneN4)NO]2+), which in vitro releases NO by the action of a reducing agent such as catecholamines. The NO released from this compound induces vasorelaxation and cytosolic calcium decrease in the vascular smooth muscle cells. The renal hypertension induced by one kidney clipping (2K-1C; performed under tribromoethanol (0.25g/kg i.p.) anaesthesia) presents high sympathetic activity. Therefore, the present study aimed to investigate the effect of this NO donor on the arterial pressure in severe and moderate renal hypertensive 2K-1C rats. We also evaluated the toxicity of the complex in the vascular smooth muscle cells in the concentration used to induce the maximum vasodilatation. The mean arterial pressure (MAP) was measured before and up to six hours after intravenous in bolus injection of trans-[RuCl([15]aneN4)NO]2+ (10 mM/kg) in conscious hypertensive and normotensive (2K) rats. In the hypertensive rats (basal 196.70 ± 8.70 mmHg, n=5), the MAP was reduced in -34.25 ± 13.50 mmHg (P<0.05) 6 hours after the compound injection. In 2K rats the compound had no hypotensive effect. We have also studied the effect of injection of 0.1 mM/kg in normotensive (basal 118.20 ± 11.25 mmHg, n=4), moderate (basal 160.90 ± 2.30 mmHg, n=6), and severe hypertensive rats (basal 202.46 ± 16.74 mmHg, n=6). The compound at the dose of 0.1 mmol/L/kg did not have effect (P>0.05) on MAP of normotensive and moderate hypertensive rats. However, in the severe hypertensive rats there was a significant reduction on the MAP of -28.64 ± 12.45 mmHg, that corresponds to 13.72 ± 5.04% of reduction of the basal MAP. The cell viability after the incubation of vascular smooth muscle cells with 0.1 mM trans-[RuCl([15]aneN4)NO]2+ for 3 h was not toxic as revealed by the MTT assay. The cells viability was 100% in the absence (control) and 97.0 ± 0.5% in the presence of the NO donor. In conclusion, the NO donor reduced the MAP of all hypertensive rats in the dose of 10mm/kg and in the severe hypertensive rats at the dose of 0.1mM/kg. The present investigation showed the hypotensive effect of the new NO donor reinforcing the idea that the use of this compound could be useful in different degrees of hypertension.

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PC120

Respiratory sinus arrhythmia and the distribution of heartbeats throughout the respiratory cycle

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It has been suggested that respiratory sinus arrhythmia (RSA) contributes to the optimisation of pulmonary gas exchange efficiency by clustering heartbeats in the inspiratory phase of the respiratory cycle (Hayano’s hypothesis). This hypothesis is supported by animal and human studies, which show a significant correlation between RSA magnitude and indices of gas exchange efficiency (1, 2). However, recent studies indicate that RSA may play a limited role in clustering heartbeats (3, 4) and that changes in the proportion of heartbeats in inspiration (HBinsp) are more closely related to changes in the inspiratory period to breath period ratio (IE/II ratio) than RSA magnitude per se. In this study we sought to examine the pattern of heartbeat distribution throughout the respiratory cycle across a range of RSA magnitudes likely to be observed physiologically in man.

In 12 healthy male volunteers (aged 20-25) we recorded ECG, respiratory flow and continuous BP measurements in the supine position and modified RSA magnitude by fixed paced breathing at 6, 9 and 12 breaths per minute. RSA pattern and magnitude were obtained by cubic spline interpolation of cardiac cycle intervals as a function of the respiratory cycle. One-way repeated measures ANOVA showed that reductions in breathing frequency resulted in a significant increase in RSA magnitude were obtained by cubic spline interpolation of cardiac cycle intervals as a function of the respiratory cycle. One-way repeated measures ANOVA showed that reductions in breathing frequency resulted in a significant increase in RSA magnitude. However, in the severe hypertensive rats there was a significant reduction on the MAP of -28.64 ± 12.45 mmHg, that corresponds to 13.72 ± 5.04% of reduction of the basal MAP. The cell viability after the incubation of vascular smooth muscle cells with 0.1 mM trans-[RuCl([15]aneN4)NO]2+ for 3 h was not toxic as revealed by the MTT assay. The cells viability was 100% in the absence (control) and 97.0 ± 0.5% in the presence of the NO donor. In conclusion, the NO donor reduced the MAP of all hypertensive rats in the dose of 10mm/kg and in the severe hypertensive rats at the dose of 0.1mM/kg. The present investigation showed the hypotensive effect of the new NO donor reinforcing the idea that the use of this compound could be useful in different degrees of hypertension.

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showed a strong relationship between HB_{insp} and changes in IE/II ratio ($r = 0.85$, $p < 0.01$) but not with changes in RSA magnitude ($r = 0.19$, $p = 0.55$).

These results suggest that, contrary to the common view, RSA magnitude does not cause significant heartbeat clustering into inspiration in humans. The mechanism behind associations between RSA and indices of gas exchange efficiency and the underlying function of RSA remain unclear.


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### PC121

**Virally-mediated expression of ATP degrading enzymes as a new tool to study ATP-mediated signalling in vivo and in vitro**

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Recent data suggest that ATP is released in the medulla in response to hypoxia and hypercapnia and may mediate excitation of presympathetic rostral ventro-lateral medulla (RVLM) neurones. Thus, during hypoxia or hypercapnia ATP is released within the areas where these neurones are located (Gourine et al. 2005b). Activation of P2 receptors in the RVLM evokes marked increases in blood pressure and renal sympathetic nerve activity (Horiiuchi et al. 1999; Ralevic et al. 1999; Thomas et al. 2001) while exogenous ATP excites bulbospinal presympathetic RVLM neurones (Ralevic et al., 1999). However, further studies into the role of ATP in central respiratory and sympathetic chemosensitivity are hampered by the lack of selective pharmacological tools. The existing P2 antagonists (e.g. PPADS and/or local ethical requirements.

Expressed PLAP under control of a non-specific EL1α promoter leading to the gene expression in neurones, glia and other cells. In rats (n=8) four unilateral injections of LVV-EL1α-hPLAP were made unilaterally into the right RVLM (under ketamine (60 mg/kg) and medetomidine (250 μg/kg) i.m. anaesthesia). Seven days later horizontal slices containing the ventral medullary surface were prepared and used for in vitro experiments as described in (Gourine et al. 2005a; Gourine et al. 2005b). ATP microelectrode biosensors were used to determine release of ATP from the ventral surface chemosensitive areas in response to isohydric hypercapnia using aCSF solution in which NaHCO3 was increased to 50 mM (isosmotically replacing NaCl) and equilibrated with 10% CO2/90% O2 (pH ~ 7.45, pCO2 ~ 65 mmHg at 37°C). It was found that the amount of ATP released in response to isohydric hypercapnia was significantly (by ~50%; P=0.008) smaller on the transduced side of the medulla. Thus, expression of PLAP in the RVLM can be used as a highly effective approach for rapid degradation of ATP released into the extracellular space during chemosensory stimulation. We conclude that virally-mediated expression of ATP degrading ecto-enzymes can be used as a novel tool to study the multiple functional roles of ATP-mediated signalling in the central nervous system.


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### PC122

**Effects of chronic hypoxia on diaphragm function in juvenile and adult rats**

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Chronic hypoxia occurs in normal individuals at altitude and also in patients suffering from respiratory disease. Skeletal muscle structure and oxidative capacity are age dependent and are known to be affected by chronic hypoxia. The aim of this study was to examine the age-dependent effects of chronic hypoxia on diaphragm muscle contractile and endurance properties. Adult (12 week old) and juvenile (3 week old) Wistar rats were exposed to either hypobaric hypoxia (barometric pressure 380mmHg) (n=12) or normobaric normoxia (n=12) for 6 weeks. At the end of the treatment periods, isometric contractile and endurance properties of isolated strips of diaphragm muscle were measured in tissue baths under hypoxic conditions.

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