Experimental and theoretical investigation of Ca^{2+} signalling upon ryanodine receptor stimulation in airway myocytes

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Ryanodine receptors (RyR) have been shown to be involved in the Ca^{2+} response in airway myocytes from various species (Kannan *et al.* 1997). The aim of this study was to develop a theoretical model of $[Ca^{2+}]_i$ variations upon RyR stimulation and to compare its predictions with experimental observations in airway myocytes.

Experimental studies were performed on myocytes freshly isolated from rat trachea. Rats were humanely killed according to national guidelines. $[Ca^{2+}]_i$ responses were measured by microspectrofluorimetry using the Ca^{2+}-sensitive fluorescent dye indo-1. Statistical comparisons were done by paired or unpaired Student's t tests as appropriate, and considered as significant when $P < 0.05$. Values are means ± S.E.M.

The mathematical model describing the mechanism of Ca^{2+} handling upon RyR stimulation included the sarcoplasmic reticulum (SR) and also cytosolic proteins and mitochondria as additional Ca^{2+} buffers. Our model predicts that after Ca^{2+} release from the SR Ca^{2+} is first sequestrated into mitochondria and binds to protein fast binding sites. Ca^{2+} is then shifted to the protein slow binding sites, and finally pumped back into the SR. RyR stimulation by 5 mM caffeine (CAF) for 30 s induced a first transient peak followed by a progressive decay to a plateau phase. When CAF exposure was stopped during the decay phase, $[Ca^{2+}]_i$ decay was increased and returned to baseline, indicating an immediate washout of CAF and closure of RyR. Stimulation by CAF for 1 s induced a Ca^{2+} peak (mean value 714 ± 120.8 nM, $n = 8$) followed by a quick decay, the RyR being closed. A second stimulation 15 s after the first one induced a second Ca^{2+} peak (amplitude 144 ± 93 nM). The baseline value after 30 s exposure to 10 μM cyclopiazonic acid (CPA), a reversible inhibitor of the sarco-endoplasmic Ca^{2+}-ATPase (SERCA) was not significantly modified (127 ± 6.7 nM; $n = 8$) *vs.* control (118 ± 6 nM; $n = 8$). When cells were stimulated by CAF 30 s after the beginning of exposure to CPA, the first peak was not modified *vs.* control (713 ± 124.4 nM), but the Ca^{2+} response to the second stimulation was abolished (25 ± 8.2 nM), indicating that the SERCA was blocked by CPA. However, the exponential decay in $[Ca^{2+}]_i$ following Ca^{2+} increase was similar in the absence and in the presence of CPA.

These experimental results indicate in accordance with the predictions of our theoretical model that though Ca^{2+} pumping back by SERCA is active after Ca^{2+} release from SR upon RyR stimulation, it is not primarily involved in $[Ca^{2+}]_i$ decrease that may be due to other buffering processes.

Kannan, M.S. *et al.* (1997). *Am. J. Physiol.* **272**, L659–664.

All procedures accord with current National guidelines.

Delivery of thyroid hormone from plasma membrane to nucleus: a role for the cytoskeleton and/or vesicular trafficking?

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Thyroid hormones (TH; T_4 and T_3) promote normal growth and development and also play a key role in regulation of many metabolic processes. TH exert their effects predominantly at a transcriptional level through binding to thyroid receptors (TR) in the cell nucleus (Yen, 2001). There is evidence (Larsen *et al.* 1981; Henneman *et al.* 2001) to suggest that TH transported into cells are targeted preferentially if not directly to the nucleus, rather than entering a cytoplasmic pool. Zhu *et al.* (1998) demonstrated that TR in the cytosol may translocate to the nucleus after TH stimulation, providing a mechanism for both selective delivery of TH to the nucleus and regulation of TR function by T_3-induced nuclear entry. We hypothesised that such a translocation mechanism might involve trafficking of TR along cytoskeletal elements and have therefore investigated the effect of disrupting the cytoskeleton on nuclear appearance of T_3 from the extracellular medium in human cell lines.

Initial experiments were performed using monolayer cultures of SHSY5Y human neuroblastoma cells. Cells were pre-incubated with cytoskeleton-disrupting drugs cytochalasin D (2 μM) or colchicine (10 μM) for 20 min. Cells were then incubated with 1 nM ^{125}I-T_3 for 30 min (initial-rate uptake conditions) followed by rapid processing into nuclear and cytosolic fractions using a Nuclei EZ-Prep kit (Sigma). Total exposure of cells to drug was therefore 50 min. Cellular uptake of T_3 (0.28 ± 0.04 pmol (mg protein)$^{-1}$ (30 min)$^{-1}$; mean ± S.D.) was unaffected by drugs and the ratio nuclear ^{125}I-T_3/cytoplasmic ^{125}I-T_3 remained at 0.045 ± 0.006 under all experimental conditions ($n > 3$ separate experiments). Similar results were obtained with BeWo human choriocarcinoma cells. We also investigated the effects of 20 min pre-incubation (50 min total exposure) with vesicular trafficking inhibitors on nuclear delivery of ^{125}I-T_3. Neither the endocytosis inhibitor monodansylcadaverine (50 μM) nor the vesicle recycling inhibitor chloroquine (100 μM) significantly reduced nuclear/cytoplasmic ^{125}I-T_3 ratio in either SHSY5Y or BeWo cells, although chloroquine treatment did significantly reduce total cellular ^{125}I-T_3 uptake to 50 % of control value in both cell types.

These results do not support the idea that TH delivery to cell nuclei involves trafficking along an intact cytoskeleton. The effect of chloroquine on cellular T_3 uptake may reflect inhibition of TH transporter recycling and/or recruitment at the plasma membrane.

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Pulmonary and systemic vascular smooth muscle demonstrate differential sensitivity to hypoxia in terms of the rise in mitochondrial NAD(P)H

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We have recently demonstrated that moderate hypoxia causes a substantial increase in NAD(P)H-related fluorescence in pulmonary arteries (Leach *et al.* 2001), and others have suggested that ROS generation is increased. It is, however, unclear whether this phenomenon also occurs in systemic arteries (which do not constrict to hypoxia), and the subcellular origins are not known. We therefore examined NAD(P)H-related autofluorescence in isolated rat pulmonary and mesenteric arteries (taken from humanely killed rats) using a 340/380 nm ratiometric technique, and in freshly isolated VSM cells using imaging of autofluorescence at an excitation wavelength of 340 nm. ROS generation was estimated using the rate of oxidation of the fluorescent dye dichlorodihydro-fluorescein diacetate (DCF). Preparations were subjected to moderate (1 % oxygen, ~20 mmHg) or severe hypoxia (0 %, < 5 mmHg); addition of rotenone (100 nM) at the end of each experiment was used to estimate complete reduction to NAD(P)H. Localisation of the hypoxia-induced rise in NAD(P)H fluorescence was performed using imaging and the mitochondrial marker Mitotracker green. Changes in NAD(P)H-related fluorescence are expressed as a percentage of the rotenone-induced response. In pulmonary arteries 1 % oxygen caused a rapid rise in fluorescence to 69.7 ± 6.1 % (mean ± S.E.M.; $n = 8$) of that induced by rotenone; as would be predicted, this reached 99.5 ± 1.0 % ($n = 9$) with 0 % oxygen. In contrast, 1 % oxygen caused a significantly smaller rise in NAD(P)H in mesenteric arteries than in pulmonary (17.1 ± 4.6 %; $n = 6$; $P < 0.001$, paired t test), although 0 % oxygen was similar (101.3 ± 5.5 %; $n = 5$). Imaging of pulmonary and mesenteric VSM cells demonstrated a reticular pattern of autofluorescence that strongly co-localised with MitoTracker Green. During hypoxia the increase in autofluorescence was strongly correlated with the mitochondria (> 85 % co-localisation, $n = 9$), whereas in cytosolic areas the increase was weak or absent. Hypoxia (1 %) caused a ~500 % increase in DCF oxidation rate in isolated pulmonary arteries and VSM cells ($n = 3$ and 10). A similar increase was observed in mesenteric VSM cells ($n = 8$), but relative quantification was difficult due to differences in loading. Our results suggest that mitochondria from pulmonary VSM are more sensitive to hypoxia than those from mesenteric VSM in terms of reducing NAD(P)H consumption by the electron transport chain.

Leach, R.M. *et al.* (2001). *J. Physiol.* **536**, 211–224.

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All procedures accord with current UK legislation.