O53

Rapid effects of 17β-oestradiol on ACh-induced Ca^{2+} signals might be mediated by cGMP in GT1–7 cells

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Hypothalamic LHRH neurons form the final pathway for the central control of reproduction. Release of LHRH from hypothalamic neurons is regulated by the interaction of several neurotransmitter systems with gonadal steroids, which act at different levels of their synaptic network. We previously found that LHRH-producing GT1–7 cells respond to ACh with an increase in $[Ca^{2+}]_i$ through activation of muscarinic receptors. This effect is acutely modulated by 17β-oestradiol (E2) in a manner compatible with specific membrane binding sites (Morales *et al.* 2002). Increasing evidence suggests that second messengers are involved in the rapid action of oestrogen by interacting with membrane receptors (Nadal *et al.* 2000). The aim of this study was to characterize the effect of E2 on ACh-induced Ca^{2+} signals in GT1–7 cells and to identify the potential second messenger systems that might be involved.

$[Ca^{2+}]_i$ measurements were achieved in cells loaded with fluo-3 AM by confocal microscopy as described elsewhere (Nadal *et al.* 2000). Control cells were exposed to two 5 s pulses of 100 μM of ACh separated by 10 min. In oestrogen-treated cells, 2 min prior to the second application of ACh, the cells were exposed to different E2 concentrations from 10 pM to 10 μM. Peak parameters analysed were maximal amplitude (maximal $[Ca^{2+}]_i$), area under the curve (total $[Ca^{2+}]_i$), and $t_{½}$ (time at half-maximal height).

Doses of E2 as low as 10 pM reduced total $[Ca^{2+}]_i$ and $t_{½}$ of the rising phase, while doses over 1 μM were ineffective. Maximal $[Ca^{2+}]_i$ was also reduced by 10, 100 nM or 1 μM. To investigate the involvement of second messengers, dibutyryl cAMP (dB-cAMP, 100 μM), forskolin (FK, 100 nM), 8-bromoguanosine 3´,5´-cyclic monophosphate (8-Br-cGMP, 10 μM) and sodium nitroprusside (SNP, 10 μM) were tested. Application of 8-Br-cGMP also reduced ACh-induced maximal $[Ca^{2+}]_i$, while neither dB-cAMP, nor FK, nor SNP exerted any effect (Fig. 1).

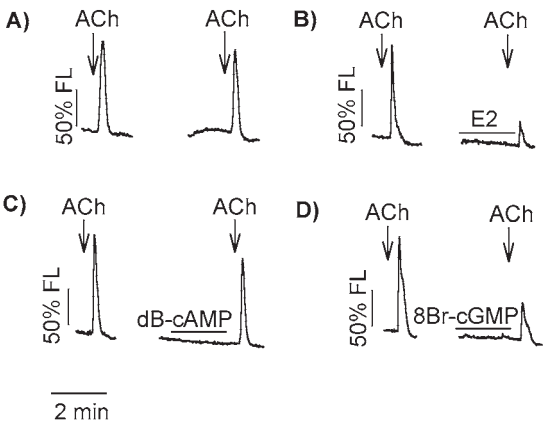

Figure 1. 8-Br-cGMP reproduced the effects of E2 on ACh-induced Ca^{2+} signals in GT1–7. Exposure of cells to two 5 s pulses of ACh (100 μM) separated by 10 min resulted in rapid increases in $[Ca^{2+}]_i$ (A). Two minutes prior to the second application of ACh, the cells were exposed to E2 (10 nM) and calcium signal was reduced (B). Treatment with dB-cAMP (100 μM) (C) had no effect, but 8-Br-cGMP (10 μM) had the same effects as E2 (D).

We conclude that physiological concentrations of E2 affect different mechanisms of ACh-induced Ca^{2+} transients in GT1–7 cells in a rapid manner, which might be related to cGMP production by a membrane-associated guanylyl cyclase.

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O54

The endocrine disruptor bisphenol-A and the native hormone 17β-oestradiol rapidly activate transcription factor CREB in mouse pancreatic β-cells

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O55

Dependence of *de novo* protein synthesis in the temporal potentiation of sensitivity in pancreatic β-cells in mice

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The electrical responsiveness of pancreatic β-cells can be temporally potentiated after the exposure to certain agents. An interesting case, due to its potential physiological relevance, is the temporal potentiation of sensitivity (TPS) to glucose after exposure to carbachol that we have previously described. Due to its temporal nature, a molecule activation after glucose and carbachol exposure can be supposed to be present once the stimulus has disappeared. To elucidate whether this molecule is present before carbachol application or has to be produced by the muscarinic stimulation (*de novo* synthesis) we have applied our stimulation protocol in the presence of an inhibitor of protein synthesis, cycloheximide. The islets of Langerhans were previously incubated for 2 h with the protein inhibitor. We found that with inhibition of protein synthesis, TPS remains unchanged. The quantitative analysis shows a mean increase of 70% in the first pulse after carbachol application, a mean increase of 60% in the second pulse and a mean increase of 30% in the third pulse; these increases are not significantly different from that obtained with control stimulation. These results show the independence of TPS on *de novo* protein synthesis.

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O56

Involvement of endogenous reactive oxygen species in Ca^{2+} oscillations in pancreatic acinar cells

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In pancreatic acinar cells, postprandial levels of cholecystokinin (CCK) evoke oscillations in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) by releasing calcium from IP_3-operated stores. In addition to IP_3, other factors can modulate Ca^{2+} release through this channel. In several models, including pancreatic acinar cells, the mild oxidant thimerosal can increase the sensitivity of IP_3 receptors (Thorn *et al.* 1992). It has also been proposed that mitochondria modulate IP_3-induced signals due to the ability to uptake and release Ca^{2+} in the immediate vicinity of the IP_3 receptors (Rizzuto *et al.* 1993). We have previously shown that mitochondrial inhibitors impair CCK-evoked Ca^{2+} oscillations in pancreatic acinar cells (Camello-Almaraz *et al.* 2002). Since pretreatment with the antioxidant *N*-acetyl-cysteine reduced the percentage of cells with an oscillatory response to CCK, we proposed that ROS generated by mitochondria collaborated in Ca^{2+} release by sensitization of IP_3 receptors (Camello *et al.* 2000). We tested this hypothesis by conventional digital microfluorimetry in fura-2-loaded pancreatic acinar cells freshly isolated from adult mice killed by cervical dislocation. In another sets of experiments we used the patch-clamp technique in the whole-cell configuration to record Ca^{2+}-activated Cl^- currents as an indirect method to monitor $[Ca^{2+}]_i$ changes.

Electrophysiological experiments showed the mitochondrial inhibitor rotenone does not disrupt oscillations by decreasing cytosolic ATP concentration, given that the internal pipette solution contained 2 mM ATP. To assess the role of mitochondrial reactive oxygen species (ROS), we used 200 μM MnTBAP, a mimetic of superoxide dismutase (SOD) and 1–10 μM MitoQ, an antioxidant ubiquinone targeted to mitochondria by a chemical modification (Kelso *et al.* 2001). When applied on top of 20 μM CCK-induced $[Ca^{2+}]_i$ oscillations, MnTBAP induced partial inhibition of the signal. In the case of MitoQ treatment, most of the cells lost Ca^{2+} oscillations, similar to previous observations for rotenone or FCCP. To assess whether mitochondrial inhibitors disrupted oscillations by cessation of Ca^{2+} uptake into mitochondria, we used the inhibitor of the Ca^{2+} uniporter Ru360. However, this compound had no effects on CCK-evoked oscillations.

Our results indicate that, irrespective of the role of mitochondrial Ca^{2+} uptake, the oxidants generated by mitochondria could play a role in the maintenance of Ca^{2+} oscillations.

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This work was supported by SAF-2001-0295. MitoQ was kindly provided by M. Murphy.

All procedures accord with current National guidelines.

O57

Ionic exchange properties of the intravesicular matrix of cholinergic synaptic vesicles from *Torpedo* electric organ

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Since synaptic vesicles in the brain and in the neuromuscular junction have an electrolucent interior when viewed under the electron microscope, it is generally supposed they are filled with an aqueous solution containing neurotransmitters and cotransmitters at a high concentration. However, other secretory granules from mast cells and chromaffin cells are filled with a matrix which adsorbs bioamines by electrostatic forces (Álvarez de Toledo *et al.* 1993; Marszalek *et al.* 1997). The existence of such a matrix has not been considered in synaptic vesicles. Of interest here is the fact that ATP is stored in all synaptic vesicles independently of neurotransmitter type, making this molecule a marker for synaptic vesicular content. We chose the electric organ of *Torpedo* for the present study because the synaptic vesicles fraction is easily isolated. Cholinergic synaptic vesicles of *Torpedo* electric organ store acetylcholine (ACh) and ATP at a concentration near the molar range. These molecules are co-secreted during synaptic transmission, and the release of both can be monitored via specific luminescence assays. Here we study the release of ACh or ATP from permeabilized synaptic vesicles in conditions that mimic the interior of the synaptic vesicle and in the absence of small ions.

Electric organs were removed from *Torpedo* sp. specimens anaesthetized with tricaine (3 g l^{-1} sea water, MS-222, Sigma,). Synaptic vesicle suspensions were extensively dialysed in isotonic media free of small ions (Cl^-, Na^+, K^+, Ca^{2+}). When dialysed synaptic vesicles were permeabilized with Triton X-100 (Fig. 1A), a small release of ACh was detected (6.9 ± 1.5 nmol ACh (mg protein)$^{-1}$, $n = 3$; 0.45 ± 0.06 nmol ATP (mg protein)$^{-1}$, $n = 4$). By comparison, when these permeabilized vesicles were further tested with an additional pulse of NaCl (8 mM), a 10-fold increase of ACh was detected (62 ± 12 nmol ACh (mg protein)$^{-1}$, $n = 3$ and 6.95 ± 0.56 nmol ATP (mg protein)$^{-1}$, $n = 10$).

This result suggests that neurotransmitters are not in a free solution in the vesicles, but are electrostatically adherent to some support. The calculated EC_{50} for NaCl was 2.7 mM. Other salts containing other ions were also tested, and similar results were obtained. Interestingly, the EC_{50} for $CaCl_2$ was 0.3 mM. We ruled out the possibility that the detergent may have interfered with the ionic environment or with the luminescent reaction by bursting synaptic vesicles in ultrapure water. The amount of ATP released (Fig. 1*B*) was 0.54 ± 0.07 nmol ATP (mg protein)$^{-1}$ ($n = 3$), which corresponded to the free ATP inside the synaptic vesicles. An additional bolus of NaCl (8 mM) evoked a second peak of ATP release (9.9 ± 0.93 nmol ATP (mg protein)$^{-1}$, $n = 3$), which emptied the synaptic vesicles. The ratio of free ATP/total ATP in synaptic vesicles burst by ultrapure water was similar (5.45 %, $n = 3$) to that measured in detergent-permeabilized vesicles. We examined whether the synaptic vesicles lysed by ultrapure water could be refilled after a run of release of ACh and ATP. The water-lysed synaptic vesicle fraction was emptied of ACh and

ATP by increasing the NaCl concentration and was refilled for a second time with ACh or ATP by dialysis combination. When maintained in ultrapure water, the refilled matrix kept up a high content of ATP, at least for a few days. However, the addition of a low concentration of NaCl induced the release of ATP. As in the case of intact vesicles, the release of ATP was also salt-concentration dependent. The pretreatment of lysed vesicles with EGTA (10 mM) completely prevents the process of ATP refilling. With respect to ACh refilling, a lysed and emptied synaptic vesicle fraction was refilled with ACh, and the addition of NaCl induced the release of ACh.

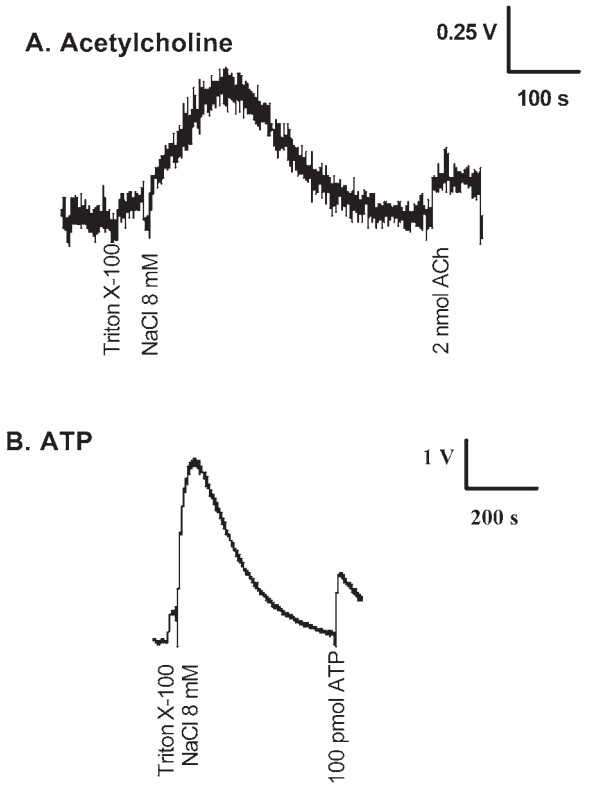

Figure 1. Ionic strength induced release of acetylcholine (*A*) and ATP (*B*) from detergent-permeabilized synaptic vesicles. Isolated synaptic vesicles were suspended in an isotonic medium free of small ions like Na^+, K^+, Ca^{2+} and Cl^-. The pH of the suspension was maintained at 5.5. The release of acetylcholine and ATP was measured using chemi-luminescent reactions. As indicated, the detergent Triton X-100, at a concentration of 0.01%, was added, and a small release of either acetylcholine or ATP was detected. A further increase of release was obtained by increasing the ionic strength (NaCl, 8 mM). When the light baseline was achieved, an external dose of acetylcholine or ATP was added to estimate the amount of acetylcholine or ATP released.

Álvarez de Toledo *et al.* (1993). *Nature* **363**, 554–558.
Marszalek *et al.* (197). *Biophys J* **73**, 1160–1168.

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All procedures accord with current National and local guidelines.

O58

Effect of cholecystokinin on mitochondrial activity in isolated mouse pancreatic acinar cells

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In the present study we have investigated the effect that stimulation of mouse pancreatic acinar cells with the secretagogue cholecystokinin (CCK) has on mitochondrial activity employing confocal laser scanning microscopy. The consequences of changes in $[Ca^{2+}]_i$ in response to the hormone on mitochondrial function were assessed.

Male swiss mice were used throughout the studies. After killing by rapid cervical dislocation the pancreas was removed and acinar cells were isolated by collagenase digestion followed by pipetting through tips of decreasing diameters. Cytosolic ($[Ca^{2+}]_i$) as well as mitochondrial Ca^{2+} concentrations ($[Ca^{2+}]_m$), mitochondrial inner membrane potential (Ψ_m) and FAD autofluorescence were determined. Cells were loaded with fluo-3, rhod-2 or JC-1, for $[Ca^{2+}]_i$, $[Ca^{2+}]_m$ and Ψ_m determinations, respectively (Gonzalez *et al.* 2000). Following dye loading of the cells, bright fluorescent spots could be detected, being in principle spread all through the cytosolic area, although concentration near the zymogen granule area, surrounding the nucleus and beneath the basolateral plasma membrane could be observed too (Voronina *et al.* 2002). FAD autofluorescence depicted similar distribution inside the cells compared to that of the fluorescent dyes. Distribution of fluorescence well correlated with that of MitoTracker Green FM, which selectively accumulates in mitochondria.

Our results show that stimulation of cells with 10 nM CCK led to a transient increase in $[Ca^{2+}]_i$ (3 exp/12 cell). The hormone induced as well an increase in $[Ca^{2+}]_m$ that remained elevated for a longer time compared to cytosolic responses (4 exp/8 cell/46 mit. area). A depolarization of Ψ_m was observed following stimulation of cells with CCK that partially recovered (6 exp/13 cell/61 mit. area); in addition an increase in FAD autofluorescence was observed following stimulation of cells with the Ca^{2+}-mobilizing agonist (7 exp/30 cell). Perfusion of cells with thapsigargin (1 μM) led to an increase in $[Ca^{2+}]_m$ (2 exp/5 cell/32 mit. area) and FAD autofluorescence (3 exp/32 cell), and depolarized Ψ_m (2 exp/3 cell/28 mit. area). Pretretament of cells with thapsigargin blocked CCK-evoked changes in these parameters (100% of cells tested). Preincubation of cells in the presence of 10 μM rotenone, which blocks the mitochondrial electron transport chain, depolarized mitochondria and inhibited the response induced by CCK stimulation on Ψ_m (4 exp/6 cell/17 mit. area). The mitochondrial inhibitor induced as well a decrease in FAD autofluorescence and blocked the CCK-induced increase in FAD autofluoresence (6 exp/19 cell). Monitoring of changes in $[Ca^{2+}]_m$, Ψ_m and FAD autofluorescence were performed in the absence of Ca^{2+} in the perifusion medium to avoid contribution of extracellular Ca^{2+} to cytosolic and mitochondrial signals.

In conclusion, the results presented in this work are consistent with changes in mitochondrial activity in response to stimulation of pancreatic acinar cells with CCK, which might then match the energy supply neccessary for the cell function during secretion. The changes in $[Ca^{2+}]_m$, Ψ_m and FAD autofluorescence depend on mobilization of Ca^{2+} from intracellular stores and are independent of extracellular Ca^{2+}.

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All procedures accord with current local guidelines.

O60

Relation between cell proliferation and capacitative calcium entry amplitude in Hep G2 and HEK 293 cells

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Changes in cytosolic free Ca^{2+} concentration are observed during the cell cycle and the extent of Ca^{2+} entry is related to cell proliferation (Golovina *et al.* 2001; Bodding, 2001). We have compared the intracellular Ca^{2+} stores contents and the amplitudes of capacitative Ca^{2+} entry (CCE) and non-capacitative Ca^{2+} entry (NCCE) in Hep G2 and HEK 293 cells in control cells, under conditions known to block cell-cycle progression, and after recovery.

Cells were kept in serum-free medium for 72 h and recovery measured 1, 4, 8 and 24 h after readmission of serum, or in complete culture medium for the same time period. Thapsigargin (1–3 μM) was used to estimate the intracellular Ca^{2+} store contents in Ca^{2+}-free medium, and CCE amplitude was measured after the addition of 2 mM Ca^{2+}.

In Hep G2 cells, a 72 h incubation in the absence of serum led to a 40 % decrease in CCE from 332 ± 23 nM (mean ± S.E.M., $n = 6$) to 206 ± 10 nM ($n = 6$). A clear recovery was observed after 1 h (224 ± 11 nM, $n = 6$) and 4 h (284 ± 11 nM, $n = 7$) readmission of 10 % FCS. The thapsigargin-sensitive Ca^{2+} store contents were not modified under the same experimental conditions. In HEK 293 cells, a 72 h incubation in the absence of serum led to a 70 ± 2 % ($n = 14$) decrease in CCE. CCE amplitudes were reduced only by 60 ± 6, 32 ± 4 and 28 ± 6 % after 1, 4 and 8 h readmission of 10 % FCS and a full recovery was observed after 24 h. The thapsigargin-sensitive Ca^{2+} store contents showed a similar pattern in these cells. In HEK 293 cells, a 72 h incubation in the absence of serum led to a 48 ± 2 % ($n = 14$) decrease in the thapsigargin-sensitive Ca^{2+} store contents. These contents were reduced only by 37 ± 2 % ($n = 12$), 28 ± 2 % ($n = 12$) and 17 ± 3 % ($n = 8$) after 1, 4 and 8 h readmission of 10 % FCS and a full recovery was observed after 24 h. No change in the amplitude of arachidonic acid-induced non-capacitative Ca^{2+} entry (NCCE; Mignen & Shuttleworth, 2000) was observed in HEK 293 cells in the same conditions. We propose that the amplitude of CCE is tightly related to cell-cycle progression and that the level of expression of plasma membrane Ca^{2+} channels might be under the control of growth and transforming factors in Hep G2 and HEK 293 cells.

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O61

Quantification of the platelet-generating demarcation membrane system in rat megakaryocytes using whole-cell capacitance

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The demarcation membrane system (DMS) develops in megakaryocytes (MKs) to provide additional membrane for platelet production. We recently demonstrated that whole cell capacitance measurements and confocal imaging of impermeant styryl dyes represent novel approaches to studying the DMS in living tissue (Mahaut-Smith *et al.* 2001). This study has now examined whether whole cell capacitance measurements reflect the entire DMS.

Male Wistar rats were humanely killed by CO_2 inhalation and cervical dislocation. Whole-cell patch clamp and confocal fluorescence recordings from MKs were conducted as described previously (Thomas *et al.* 2001). Plasma membranes were stained with di-8-ANEPPS (10 μM, < 30 min), excited at 488 nm (emission > 505 nm). Double stranded DNA was stained with Hoescht 33258, excited at 364 nm (emission > 385 nm). For measurement of membrane potential (E_m), di-8-ANEPPS signals were background-subtracted, corrected for photobleach and expressed as f/f_0 ratios to normalise fluorescence levels (f) to starting fluorescence (f_0). Membrane capacitance was measured by integration or electronic compensation of the current transient following a small (−10 mV) voltage step.

Despite the morphological complexity of the demarcation membranes, the capacitative transient in > 95 % of MKs decayed with a single exponential and a typical initial series resistance (R_s) only 2–3 times (2.4 ± 1.0 MΩ; mean ± S.D., $n = 68$) higher than the pipette resistance (1.8 ± 0.5 MΩ). Di-8-ANEPPS, a styryl indicator with low membrane permeability extensively stained the volume of the MK, even between lobes of the polyploidic nucleus, as judged by co-staining with Hoescht 33258. In combined measurements of E_m and whole-cell voltage clamp with 70–75 % R_s compensation, voltage steps from −75 to 0 mV generated a uniform decrease in corrected f/f_0 488 nm signals throughout the cell. This uniform voltage control, the single exponential decay of capacitative transients and the low series resistance in whole-cell recordings suggest that capacitance measurements represent a quantitative measurement of the entire DMS. MK capacitances covered more than a 10-fold range (64–694 pF) with an average of 236 ± 106 pF ($n = 827$). Based upon the capacitance of a single platelet of 128 fF (Maruyama, 1987), these MKs can generate 500–5000 platelets (average approx. 1850) if all surface-connected membrane in the precursor cell is used in the process of thrombopoiesis.

Mahaut-Smith MP *et al.* (2001). *J Physiol* **536.P**, 102*P*.

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This work was funded by the British Heart Foundation and Medical Research Council.

All procedures accord with current UK legislation.

O62

Effect of sarcoplasmic reticulum Ca^{2+} content on action-potential release of Ca^{2+} in rat skinned muscle fibres

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Increasing the Ca^{2+} content of the sarcoplasmic reticulum (SR) in mammalian skeletal muscle fibres enhances sensitivity to caffeine-induced and Ca^{2+}-induced Ca^{2+} release (Lamb *et al.* 2001). Here we examine what effect SR Ca^{2+} content has on the physiological release mechanism which involves an action-potential in the transverse-tubular (T-) system activating the voltage-sensors and triggering Ca^{2+} release.

Hooded rats were painlessly killed by halothane overdose and the extensor digitorum longus (EDL) muscle dissected. Single fibres were mechanically skinned under paraffin oil, attached to a force tranducer and bathed in a solution that mimicked the normal intracellular milieu (mM: K^+, 125; Na^+, 37; hexamethylene-diamine-tetraacetate, 50; total ATP, 8; total Mg^{2+}, 8.6; free Mg^{2+}, 1; creatine phosphate, 10; total EGTA, 0.05; Hepes, 90; NaN_3, 1; pH 7.10 ± 0.01 and free [Ca^{2+}], 0.0001) at 24 ± 1°C. Electric field stimulation was used to elicit an action-potential in the sealed T-system, which induced a twitch response in the skinned fibre, as previously described (Posterino *et al.* 2000). The amount of Ca^{2+} released by an action potential could be quantified from the force response when Ca^{2+} reaccumulation by the SR was blocked (with 50 μM 2-5-di-tert-butyl-1,4-hydroquinone; TBQ) and Ca^{2+} release was rapidly buffered with a known quantity of the fast Ca^{2+} buffer, BAPTA (250 μM). The SR of the skinned EDL fibres initially contained only the amount of Ca^{2+} present endogenously, and the first twitch response elicited in TBQ/BAPTA reached ~40 % of the maximum Ca^{2+}-activated force, indicating ~300 μM Ca^{2+} was released. Thereafter, two to four more twitch responses could be elicited before the SR was almost fully depleted. The SR of fibres was then near-maximally loaded with Ca^{2+} (~3–4 times endogenous level; Fryer & Stephenson, 1996), and it was found that the first response in TBQ/BAPTA was very similar to that with the endogenous SR Ca^{2+} content (~40 % of maximum force) and the response to successive stimuli remained at approximately the same level for up to 12 repetitions before declining over the last three to four twitches as the SR became depleted as above.

These data indicate that the amount of Ca^{2+} released by an action potential is virtually unchanged when the SR Ca^{2+} content is increased from the endogenous level to near maximum capacity.

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P16

Slow and fast kiss-and-run modes of exocytosis are differentially controlled by extracellular and cytosolic calcium levels

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Kiss-and-run exocytosis is a rapid and efficient method of releasing secretory products to the extracellular medium through the transient formation of a fusion pore. During kiss and run, the vesicle membrane does not collapse into the plasma membrane, maintaining the secretory vesicle's position, identity and potentiality to perform a second round of exocytosis. In a previous report, we found that the incidence of kiss-and-run increases as the extracellular calcium concentration rises in the millimolar range (Ales *et al.* 1999). These kiss-and-run events lasted only for milliseconds (average of 50 ms, called 'fast kiss-and-run') and released the same amount of transmitter as a conventional exocytosis. However, we did not demonstrate whether calcium mediated this effect extracellularly or by an indirect cytosolic calcium increase. To answer this question, we have done whole cell membrane capacitance measurements in peritoneal mast cells of rats that had been humanely killed in which we could experimentally control both the extracellular and the intracellular calcium concentrations. This preparation also allows resolution of individual fusion events. To monitor release, we have made simultaneous measurements of serotonin release with amperometry by positioning a carbon fibre electrode over the cell surface.

We analysed well-defined single exocytotic fusion events from capacitance and amperometric measurements. The incidence of kiss-and-run increased when the rate of degranulation was decreased either by lowering the amount of secretagogue (GTPγS) or by dialysing the cells with lower calcium concentrations. At similar amounts of GTPγS, the number of kiss-and-run events decreased as the cytosolic calcium concentration was raised from 100 nM to 1 μM. Simultaneous amperometric measurements revealed that these kiss-and-run events were slow (lasting on average 500 ms), and release only a small fraction of the vesicle contents. By contrast, when the extracellular calcium concentration was raised from 2 mM up to 90 mM, the incidence of kiss-and-run increased and the amount of serotonin was similar to the quantal contents of a rat mast cell granule, indicating the occurrence of fast kiss-and-run events.

These data reveal that fast kiss-and-run events are evoked by calcium acting from the extracellular medium and that these events represent a different kinetic process from the slow kiss-and-run events. We propose a kinetic model of exocytosis that accounts for slow and fast kiss-and-run exocytosis.

Ales E. *et al.* (1999). *Nat Cell Biol* **1**, 40–44.

All procedures accord with current National and local guidelines.

P17

Hydrogen peroxide induces release of calcium from agonist-sensitive and mitochondrial calcium stores in human platelets

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In non-excitable cells, such as platelets, activation of phospholipase C-coupled receptors results in a transient increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) due to the release of Ca^{2+} from agonist-sensitive stores. In addition, mitochondria, whose redox state control is important for cellular energy conversion, have been reported to be involved in the regulation of Ca^{2+} homeostasis (Rizzuto *et al.* 1999). Ca^{2+} mobilisation plays an important role in the regulation of several cellular events, such as secretion or platelet aggregation. The oxidative balance has been reported to play a crucial role in regulating platelet activity. Hence, we have investigated the effect of oxygen free radicals such as H_2O_2 on Ca^{2+} homeostasis in human platelets.

Blood was drawn from volunteers with local ethical committee approval according to the Declaration of Helsinki. Fura-2-loaded human platelets were prepared as previously described (Rosado *et al.* 2000) and fluorescence was measured using a Shimadzu spectrophotometer.

In a Ca^{2+}-free medium, treatment of platelets with 1 mM H_2O_2 caused a sustained increase in $[Ca^{2+}]_i$ due to the release of Ca^{2+} from internal stores. Addition of H_2O_2 to platelet suspensions whose agonist-releasable Ca^{2+} pools had been previously depleted by thrombin (1 U ml^{-1}) or simultaneous treatment with thapsigargin (TG; 1 μM) and a low concentration of ionomycin (50 nM) was still able to induce a sustained increase in $[Ca^{2+}]_i$ ($n = 7$). Similar results were observed when human platelets were treated with the mitochondrial uncoupler FCCP (1 μM), to dissipate the membrane potential across the inner mitochondrial membrane that sustains the activity of the Ca^{2+} uniporter (Medler & Gleason, 2002), prior to the treatment with H_2O_2 ($n = 8$). In contrast, treatment of platelets with a combination of either FCCP plus thrombin or FCCP plus TG and ionomycin abolished the H_2O_2-induced calcium release ($n = 5$). Finally, addition of H_2O_2 to platelet suspensions reduced calcium mobilisation induced by the physiological agonist thrombin or with TG plus ionomycin by 78 ± 6 and 90 ± 6 %, respectively (S.E.M.; $n = 4$–8, $P < 0.01$, Student's paired t test).

Our findings suggest that H_2O_2 releases Ca^{2+} from mitochondrial and agonist-releasable Ca^{2+} pools which might be involved in the reported effects of the oxygen free radicals in human platelets.

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All procedures accord with current local guidelines and the Declaration of Helsinki.

P18

Evidence for the activation of store-mediated Ca^{2+} entry by a secretion-like coupling mechanism in mouse pancreatic acinar cells

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In non-excitable cells depletion of the intracellular Ca^{2+} stores induces Ca^{2+} influx across the plasma membrane (PM), but the mechanism underlying store-mediated Ca^{2+} entry (SMCE) remains unclear. Hypotheses have considered both direct and indirect coupling mechanisms. Recently, a secretion-like coupling model, which shares properties with the direct coupling, has been proposed (Patterson *et al.* 1999; Rosado *et al.* 2000). This mechanism involves a physical and reversible interaction between the endoplasmic reticulum (ER) and the PM where the actin cytoskeleton plays an important role (Rosado *et al.* 2000; Rosado & Sage, 2001). In the present study, the nature of the mechanism underlying SMCE in pancreatic acinar cells has been investigated through a combination of cytoskeletal modifications.

Donor mice were humanely killed by rapid cervical dislocation. Pancreatic acinar cells were isolated and fluorescence measured as previously described (Pariente *et al.* 2001). Thapsigargin (TG)-induced SMCE was estimated as the integral of the rise in $[Ca^{2+}]_i$ for 2.5 min after addition of Ca^{2+}. In a Ca^{2+}-free medium, TG induced a transient increase in $[Ca^{2+}]_i$ due to Ca^{2+} release from internal stores. The subsequent addition of Ca^{2+} (2 mM) to the medium resulted in a rapid increase in $[Ca^{2+}]_i$ indicative of SMCE. Treatment of pancreatic acinar cells with the cytoskeletal disrupters cytochalasin D (CD; 10 μM) and latrunculin A (Lat A; 3 μM) significantly reduced the activation of SMCE by 78.8 ± 8.8 and 46.6 ± 9.8 %, respectively (S.E.M.; $n = 5$–10, $P < 0.01$, Student's paired t test). In addition, when CD or Lat A was added once SMCE had been activated, Ca^{2+} entry was reduced by 50.5 ± 4.4 and 48.2 ± 1.0 %, respectively ($n = 5$, $P < 0.01$). To investigate the role of the apical cytoskeleton in SMCE we induced stabilisation of a cortical actin barrier using jasplakinolide (JP). Treatment of pancreatic acinar cells with 10 μM JP reduced the activation of SMCE by 52.5 ± 3.8 % ($n = 5$–10, $P < 0.01$) without having any effect on the maintenance. JP also reduced Ca^{2+} entry stimulated by 1 nM CCK-8, a physiological agonist, without having any effect on the release of Ca^{2+} from the stores, suggesting that it is unlikely that a diffusible molecule mediates SMCE in these cells. Such a factor could reach the PM after cytoskeletal modifications since InsP_3, generated by CCK-8, was able to stimulate Ca^{2+} release. These findings suggest that, as for secretion, the actin cytoskeleton plays a double role in SMCE, acting as a negative clamp, preventing the interaction between the ER and PM, but also is required for this mechanism since the cytoskeleton disrupters impaired Ca^{2+} entry.

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All procedures accord with current local guidelines.

P19

Hydrogen peroxide reduces store-mediated Ca^{2+} entry and the plasma membrane calcium ATPase activity in human platelets

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Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is an important factor involved in the modulation of many physiological processes. In non-excitable cells, such as platelets, agonists induce a transient release of Ca^{2+} from internal stores, which activates a sustained store-mediated Ca^{2+} entry (SMCE). In contrast, removal of Ca^{2+} from the cytosol and maintenance of a low $[Ca^{2+}]_i$ is mainly mediated by the plasma membrane Ca^{2+} ATPase (PMCA; Rosado *et al.* 2000). The redox state has been reported to modulate $[Ca^{2+}]_i$ in different cell types (Pariente *et al.* 2001; Törnquist *et al.* 2000) suggesting that oxygen radicals, such as H_2O_2, might act as second messengers. The aim of the present study was to investigate the effect of H_2O_2 in both SMCE and the PMCA activity in human platelets.

Fura-2-loaded human platelets, from volunteers with local ethical committee approval according to the Declaration of Helsinki, were prepared and fluorescence measured as previously described (Rosado *et al.* 2000). SMCE induced by treatment with

thapsigargin (Tg) plus ionomycin (IONO) or thrombin was estimated as the integral of the rise in $[Ca^{2+}]_i$ for 2.5 min after addition of Ca^{2+}. To study the effect of H_2O_2 in the PMCA activity, we compared the rate of decay of $[Ca^{2+}]_i$ to basal values, after treatment in a Ca^{2+}-free medium with Tg plus IONO, in the absence and presence of H_2O_2, by using the constant of the exponential decay. Since H_2O_2 has been shown to release Ca^{2+} from mitochondrial as well as agonist-releasable pools (Pariente *et al.* 2001) a buffer supplemented with oligomycin (10 μM) and rotenone (10 μM) was used to avoid interference with the analysis of Ca^{2+} release from mitochondrial stores.

In a Ca^{2+}-free medium, treatment of platelets with 1 μM Tg plus 50 nM IONO resulted in a transient increase in $[Ca^{2+}]_i$ due to release of Ca^{2+} from intracellular stores; subsequent addition of Ca^{2+} (1.5 mM) to the medium resulted in a sustained increase in $[Ca^{2+}]_i$ indicative of SMCE. Addition of 1 mM H_2O_2 significantly reduced Ca^{2+} entry by 65.1 ± 5.3 % (S.E.M.; $n = 7$; $P < 0.01$, Student's paired t test). Similar results were found when Ca^{2+} entry was activated by the agonist thrombin (0.5 U ml^{-1}). Treatment of human platelets with H_2O_2 (1 mM) reduced thrombin-induced Ca^{2+} entry by 50.8 ± 8.6 % ($n = 5$; $P < 0.01$). In addition, H_2O_2 decreased the rate of decay of $[Ca^{2+}]_i$ to basal levels after activation with 1 μM Tg plus 50 nM IONO. The decay constants were 0.0006 ± 0.0003 in H_2O_2-treated platelets and 0.0069 ± 0.0011 in controls ($n = 5$; $P < 0.001$). These results indicate that H_2O_2 inhibits both SMCE and Ca^{2+} efflux across the plasma membrane in human platelets.

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All procedures accord with current local guidelines and the Declaration of Helsinki.

P20

Hydralazine reduces the quantal size of secretory events by displacement of catecholamines from bovine adrenomedullary chromaffin secretory vesicles

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We have studied the effects of the anti-hypertensive agent hydralazine (1–100 nM) on the exocytotic process of single adrenal chromaffin cells. Hydralazine rapidly slows the rate of catecholamine release from individual exocytotic events and reduces the quantal size of catecholamine exocytosis but it does not change the frequency of exocytotic spikes. Hydralazine rapidly accumulates within secretory vesicles as shown by confocal and standard epifluorescence microscopy studies. Cell incubation with bafilomycin A_1, a compound that blocks the vesicular H^+ pump, inhibits hydralazine uptake. Experiments with permeabilized cells show that hydralazine displaces catecholamines from secretory vesicles. The drug also displaces vesicular Ca^{2+}, as shown by fura-2 microfluorimetry. These data suggest that hydralazine acts at least partially, by interfering with the storage of catecholamines. These effects of hydralazine occurred within seconds and at the tissue concentrations presumably reached in antihypertensive therapy; these concentrations are a thousand times lower than those described for relaxing vascular tissues '*in vitro*'. We proposed that these novel effects could explain many of the therapeutic and side effects of this drug that are likely exerted in sympathetic nerve terminals.

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P21

Cyclooxygenase and cytochrome P-450 pathways induced by fetal calf serum regulate wound closure in 3T6 fibroblast cultures. Role of calcium and cAMP

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The wound healing process involves the movement of neighbouring cells to cover the injury area and the subsequent cell proliferation. These steps may be regulated by growth factors, extracellular components and eicosanoids. In this way, arachidonic acid release and prostaglandin E_2 (PGE_2) production are enhanced in wounded fibroblast cultures and may be involved in the regulation of wound repair process (Lloret & Moreno, 1996; Moreno, 1997). The objective of this study was to determine the role of prostanoids, lipoxygenase and cytochrome P-450 pathway metabolites in the regulation of wound closure of confluent 3T6 fibroblast cultures.

Our results show that the inhibition of the cyclooxygenase and/or cytochrome P-450 pathways significantly decreases the wound closure, whereas that of the lipoxygenase pathway does not modify the wound repair process.

PGE_2 is one of the major eicosanoids synthesized by fibroblasts. PGE_2 effects are initiated on plasma membrane localized GTP-binding protein-coupled receptors. There are four types of PGE_2 receptors (EP1–EP4) (Narumiya *et al.* 1999). EP1 and EP4 are present in 3T6 fibroblasts. Both EP1 and EP4 receptors mediate PGE_2-stimulated 3T6 fibroblast wound closure. Each receptor exerts its effects through changes in a specific cellular pathway. Thus the EP1 receptor increases intracellular calcium concentration, whereas PGE_2 interaction with the EP4 receptor raises intracellular cAMP levels. Both second messengers are involved in the control of the cell cycle machinery of 3T6 fibroblasts (Sánchez & Moreno, 2002) and, thus in the wound repair process. On the other hand, we show that ketoconazole, a cytochrome P-450 inhibitor, hinders the wound closure induced by fetal calf serum in wounded 3T6 cell cultures. 12- and 20-Hydroxyeicosatetraenoic acids, which are key arachidonic acid metabolites synthesised by cytochrome P-450, partially, reverse the effects of ketoconazole on the wound repair process. Moreover, our findings suggest that these cytochrome P-450 pathway metabolites participate in the control of calcium. These events may be involved in the control of DNA synthesis, cell growth and the subsequent wound repair process in wounded 3T6 fibroblast cultures.

In summary, the cyclooxygenase and cytochrome P-450 pathway metabolites of arachidonic acid, PGE_2 and/or 12- and 20-hydroxyeicosatetraenoic acids respectively, play a pivotal role in 3T6 fibroblast wound closure, and calcium and cAMP are involved in the signalling events induced by these eicosanoids in the wound repair process.

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P22

Involvement of p60^{src} in the activation of store-mediated Ca^{2+} entry in mouse pancreatic acinar cells

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Store-mediated calcium entry (SMCE) is one of the main pathways for calcium influx in non-excitable cells, such as pancreatic acinar cells; however the nature of the mechanism involved in this process remains unclear. Recently, a secretion-like coupling model has been proposed (Patterson *et al.* 1999; Rosado *et al.* 2000*a*), which is based on a physical and reversible interaction between the endoplasmic reticulum (ER) and the PM modulated by the actin cytoskeleton (Rosado *et al.* 2000*a*). In pancreatic acinar cells tyrosine kinases, such as p60^{src}, are involved in many cellular events. p60^{src} activity has been suggested to be important for the activation of SMCE in human platelets (Rosado *et al.* 2000*b*). In the present study, the involvement of p60^{src} in the activation of SMCE in pancreatic acini has been investigated.

Donor mice were humanely killed by rapid cervical dislocation. Pancreatic acinar cells were isolated and fluorescence measurement and Western blotting performed as previously described (Rosado *et al.* 2000*b*; Pariente *et al.* 2001). Thapsigargin (TG)-induced SMCE was estimated as the integral of the rise in $[Ca^{2+}]_i$ for 2.5 min after addition of Ca^{2+}. In a Ca^{2+}-free medium, treatment of pancreatic acinar cells with 1 μM TG induced release of Ca^{2+} from internal stores. The subsequent addition of Ca^{2+} (2 mM) to the medium resulted in a rapid increase in $[Ca^{2+}]_i$ indicative of SMCE. Treatment of pancreatic acinar cells with PP1, a specific inhibitor of p60^{src}, reduced the activation of SMCE by 16 ± 4, 48 ± 6 and 71 ± 7 % at 3, 10 and 50 μM, respectively (means ± S.E.M.; $n = 4$–7, $P < 0.05$, ANOVA), without having any effect on its maintenance. In addition, we have found that depletion of the intracellular calcium stores, either using TG or the physiological agonist CCK-8 (1 nM), induced activation and cytoskeletal association of p60^{src}, an event that occurs independently of Ca^{2+} entry. Store depletion-induced activation of p60^{src} was prevented by treatment with cytochalasin D, suggesting that this process requires the integrity of the actin cytoskeleton.

These findings provide evidence for the activation and cytoskeletal association of p60^{src} upon Ca^{2+} store depletion in pancreatic acinar cells, a process that might be important for the activation of SMCE in these cells.

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Rosado JA *et al.* (2000*a*). *J Biol Chem* **275**, 7527–7533.
Rosado JA *et al.* (2000*b*). *Biochem J* **351**, 429–437.

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All procedures accord with current National guidelines.

P23

Role of ovarian steroids on insulin receptor in food restricted (50 %) rats

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Glucose homeostasis is altered by a reduction in calorie intake and this relationship has important implications because calorie restriction is a common treatment for obesity and diabetes II (American Diabetes Association, 1998). Calorie restriction alters insulin action and glucose transport in skeletal muscle. Previous work has shown that ovarian steroids are able to modulate insulin sensitivity (González *et al.* 2000; Livingstone *et al.* 2002). However, the relation between food restriction, insulin signalling and ovarian hormones is partially unknown.

In female food-restricted (50 %) rats (FR), we investigated the relation among ovarian hormones and insulin sensitivity, focusing on muscle insulin receptor (IR) and the possible influence of the food restriction time (5, 10 and 15 days). Analysis of variance and Student-Newman-Keuls tests were employed. $P \leqslant 0.05$ was considered significant.

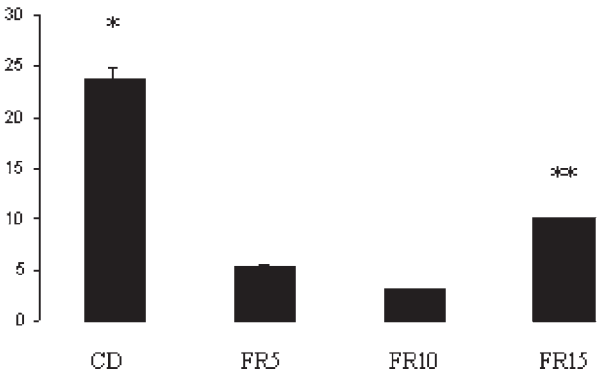

Figure 1. Glucose infusion rates of control (CD) and food restricted (FR) rats. Values are in mg min^{-1} kg^{-1} and are means ± S.E.M. (five animals). Only significant differences are shown. *CD *vs.* FR; **FR15 *vs.* FR5, FR10.

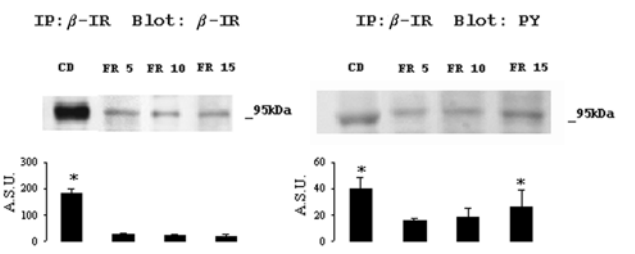

Figure 2. IR levels and phosphorylation in skeletal muscle of control (CD) and food restricted (FR) rats. Scanning densitometry was performed in five experiments. Data are

expressed as means ± S.E.M. Only significant differences are shown. *CD *vs.* FR; **FR15 *vs.* FR5, FR10. A.S.U, arbitrary scanning units.

Table 1. General characteristics of animals. Values are mean ± S.E.M. (five animals). Only significant differences are shown. * = CD vs FR.

		5 days	10 days	15 days
Food intake	FR	9,87±0,15*	9,56±0,16*	9,84±0,05*
g	CD	18,27±0,61	19,27±0,77	20,25±0,89
Body weight	FR	229,50±4,11*	225,17±4,65*	206,92±3,57*
g	CD	261,53±4,27	264,4±4,17	268,71±11,2
Glucose	FR	124,67±5,49*	109,83±3,33*	108,75±5,93*
mg/dl	CD	141,73±6,4	142,34±5,94	143,02±5,57
Insulin	FR	1,27±0,21*	1,49±0,5*	1,17±0,23*
ng/ml	CD	8,02±1,4	7,94±0,89	8,01±1,3
17-B-estradiol	FR	68,43±10,11*	69,10±10,7*	68,95±7,04*
pg/ml	CD	111,37±6,98	112,05±7,02	110,26±6,23
Progesterone	FR	34,74±3,58	31,97±3,83	31,80±2,99
ng/ml	CD	38,3±2,95	37,82±3,05	38,10±3,63

Rats were anaesthetized (pentobarbital 45 mg kg^{-1} intraperitoneal) and euglycaemic–hyperinsulinaemic clamp was performed (González *et al.* 2000). Insulin sensitivity was significantly higher in control (CD) than FR rats. The time of restriction determines the increase in insulin sensitivity (FR15 rats were more sensitivity than FR5 and FR10 rats). The amount and phosphorylation of IR in CD were significantly higher than FR. However, the time of food restriction determines only an increase in IR phosphorylation according to insulin sensitivity.

On the other hand, our food restriction model determinates a decrease in 17β-oestradiol plasma levels, while the progesterone levels does not change and we have shown that ovarian steroids are able to modulate insulin sensitivity involving IR (González *et al.* 2000, 2002*a, b*). Taken together these data show that a special ovarian hormonal millieu associated with food restriction could be related to changes in insulin sensitivity through a specific regulation of the muscle IR.

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All procedures accord with current National and local guidelines.

P24

H_2O_2 induces changes in mitochondrial activity in isolated mouse pancreatic acinar cells

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In the present study we have employed confocal laser scaning microscopy to monitor the effect of reactive oxygen species (ROS) on mitochondrial activity in mouse pancreatic acinar cells. Previous work has shown that the ROS H_2O_2 induced an increase in $[Ca^{2+}]_i$ both in the presence and absence of extracellular Ca^{2+}, i.e. H_2O_2 released Ca^{2+} from intracellular stores (Pariente *et al.* 2001).

Male Swiss mice were used throughout the studies. Animals were killed by rapid cervical dislocation. In our investigations cytosolic ($[Ca^{2+}]_i$) as well as mitochondrial ($[Ca^{2+}]_m$) Ca^{2+} concentrations, mitochondrial inner membrane potential (Ψ_m) and FAD autofluorescence were determined. Following loading of the cells with MitoTracker Green FM, a dye that selectively accummulates in the mitochondria, bright fluorescent spots could be detected, being in principle spread all through the cytosolic area, although the concentration near the zymogen granule area, surrounding the nucleus and beneath the basolateral plasma membrane could be observed too. The same distribution of fluorescence could be observed after loading of cells with rhod-2 and JC-1, for $[Ca^{2+}]_m$ and Ψ_m determinations respectively. FAD autofluorescence depicted similar distribution inside the cells compared to that of the fluorescent dyes. ROS used throughout the studies was H_2O_2 (1 mM).

The results show that perfusion of pancreatic acinar cells with H_2O_2 (1 mM) led to an increase in $[Ca^{2+}]_m$ that slowly returned to prestimulation level, but remained elevated for at least 10 min. In some experiments step increases in rhod-2 fluorescence were observed, which we have related to the oscillatory pattern of changes in $[Ca^{2+}]_i$ induced by H_2O_2. Following H_2O_2 tretament inclusion of CCK (10 nM) in the perfusion medium failed to induce further changes in $[Ca^{2+}]_m$. However, previous stimulation of pancreatic acinar cells with 10 nM CCK, which induced as well an increase in $[Ca^{2+}]_m$, did not block the H_2O_2-induced response.

Increases in $[Ca^{2+}]_m$ induced by H_2O_2 correlated with depolarizations of Ψ_m and increases in FAD autofluorescence. Pre-incubation of cells in the presence of 10 μM rotenone, which blocks the mitochondrial electron transport chain, depolarized mitochondria and inhibited the response induced by perfusion of cells with 1 mM H_2O_2 on Ψ_m. Uncoupling of mitochondria led to a decrease in FAD autofluorescence that, however, did not inhibit the effect of H_2O_2. FCCP (100 nM), another mitochondrial uncoupler, which inhibits accummulation of Ca^{2+} by mitochondria, did not block the effect of H_2O_2 on FAD autofluorescence.

Perfusion of cells with thapsigargin (1 μM) led to an increase in $[Ca^{2+}]_m$ and FAD autofluorescence, and depolarized Ψ_m.

These changes in $[Ca^{2+}]_m$, Ψ_m and FAD autofluorescence were observed in the absence of extracellular Ca^{2+} as well.

In summary our results show that, in the presence of H_2O_2, changes in mitochondrial activity are observed that might not entirely depend on Ca^{2+} mobilization, in opposition to what has been shown for CCK. This might be due to the oxidant nature of H_2O_2 and, therefore, could represent the mechanism of action of ROS to induce cellular damage leading to cell dysfunction and generation of pathologies.

Pariente *et al.* (2001). *J Membr Biol* **179**, 27–35.

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All procedures accord with current local guidelines.

P25

Ca^{2+} stores and Ca^{2+} influx in cerebellar granule cells

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Activation of phospholipase C (PLC) by neurotransmitters and hormones leads to the generation of $InsP_3$, which in turn releases Ca^{2+} from intracellular stores. Depletion of the stores then activates entry of extracellular Ca^{2+} ions, a process termed store-operated or capacitative calcium entry (CCE). This mechanism has been extensively studied in non-excitable cells, although the signal linking depletion of the pools with opening of plasma membrane Ca^{2+} channels is still a matter of speculation. In the case of excitable cells, reports of CCE are scarce, specially for neurones. Though cerebellar granule cells are a model for the study of the PLC/$InsP_3$ pathway, and in spite of being one of the most common models in neurobiology, there is no evidence for the presence of CCE in this cell type. The aim of our study was to test the presence of CCE in granule cells prepared from 4- to 7-day-old rats killed by decapitation after anaesthesia.

We loaded cells cultured on poly-D-Lys-coated coverslips with 10 μM fura-2 AM for 30 min, followed by other 30 min. To study Ca^{2+} entry we determined Ca^{2+} concentration ($[Ca^{2+}]_i$) as a ratio of fura-2 fluorescence using a microscopic digital imaging system.

After depletion of intracellular Ca^{2+} stores with 1 μM thapsigargin (TPS) in a Ca^{2+}-free medium reintroduction of external Ca^{2+} induced a sustained $[Ca^{2+}]_i$ increase (0.062 ± 0.007, $n = 9$, mean ± S.E.M., ratio units). This plateau was due to Ca^{2+} influx from extracellular medium, as shown by the repetitive pattern following removal and readdition of external Ca^{2+}, so that it can be used as index of Ca^{2+} entry. Another approach to induce CCE was coapplication of TPS and 100 nM ionomycin in Ca^{2+}-free medium, a treatment which induced a CCE response significantly higher than TPS alone (0.09 ± 0.009, mean ± S.E.M., $n = 16$, $P \leqslant 0.05$ with respect TPS alone, Student's unpaired t test). Inclusion of the ionotropic glutamate receptor anatagonists DAP5 and NBQX did not modify the pattern of influx, showing that Ca^{2+} entry is independent of receptor-operated channel activation.

To characterise the pathway for Ca^{2+} influx we used several compounds. 2-APB is accepted as a selective inhibitor for capacitative Ca^{2+} channels (Braun *et al.* 2001). Application of this drug resulted in inhibition of the CCE-evoked plateau. Consistent with previous reports in other systems, Zn^{2+}, Cd^{2+} and Gd^{3+} also reduced the Ca^{2+} entry.

In summary, our data show that depletion of Ca^{2+} stores in cerebellar granule cells evokes a component of capacitative calcium entry with some of the features of the typical capacitative entry system.

Braun FJ *et al.* (2001). *J Biol Chem* **276**, 1063–1070.

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All procedures accord with current National guidelines.

P26

Regulation of FSH induced PTTG and HIF-1α expression in ovarian granulosa cells

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Follicular maturation is a complex process controlled by a wide array of molecules that includes pituitary gonadotropins (FSH and LH) and steroid hormones as well as many other locally produced factors. Angiogenesis is initiated early in follicular development and contributes to folliculogenesis because increased vascularization of individual follicles results in the preferential delivery of gonadotrophins. Thus it is believed that blood flow plays an instrumental role in the selective maturation of preovulatory follicles and eventually ovulation. The pituitary hormones FSH and LH are major regulators of angiogenesis in the ovary, as they increase bFGF (basic fibroblast growth factor) and VEGF (vascular epithelial growth factor), important members of the vascular-specific angiogenic factors. Pituitary tumour transforming gene (PTTG) and hypoxia-inducible factor-1α (HIF-1α) are known as transcriptional activators for βFGF and VEGF in several cell lines and tissues. Using oligonucleotides to prime specific sequences in the PTTG and HIF-1α genes we amplified cDNA obtained from ovarian granulosa cells, from Holtsman Sprague-Dawley rats killed by cervical dislocation, treated with 0.2 mIU of FSH. Both PTTG and HIF-1α, transcriptional activators, are expressed in granulosa cells and FSH increased in a time-dependent manner, reaching the maximum levels (4-fold) at 48 h of stimulation, when granulosa cells acquired a pre-ovulatory phenotype. Since forskolin also induced the mRNA levels of both factors we concluded that this is a cAMP-mediated effect. Using specific inhibitors (KT 5720, PD 98059, SB 203580, GF 109203X, LY 2940021) of different pathways we found that the FSH induction of PTTG and HIF-1α is mediated by PKA, but also by MAPKs. Co-treatment of granulosa cells with GnRH, a well known ovarian atretic factor, prevents FSH mediated increases in PTTG mRNA levels.

All procedures accord with current National guidelines.

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Effects of conjugated linoleic acid on leptin gene expression and secretion in cultured rat adipocytes

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Obesity is a complex disease effected by both genetic and environmental factors. The type of fat ingested seems to be an important determinant in the development or prevention of obesity. Several studies have suggested there are antiobesity properties of conjugated linoleic acid (CLA), a group of positional and geometric fatty acid isomers derived from linoleic acid (18:2 (n−6)). Leptin is a hormone secreted by the adipocytes that is implicated in the regulation of body weight via its central actions on food intake and energy expenditure. Leptin has been shown to be regulated by dietary macronutrients, and therefore the aim of the present study was to determine the direct effects of CLA on leptin secretion and expression in cultured primary

adipocytes. Because glucose metabolism plays an important role in the regulation of leptin, the effects of CLA on glucose uptake and lactate production were also studied.

Epididymal fat pads were obtained from Wistar rats. All procedures were conducted according to the University Ethics Committee for the use of laboratory animals and the animals were humanely killed. Adipocytes were isolated by collagenase digestion and cultured with DMEM in the absence or presence of insulin plus several concentrations of CLA (1–200 μM). Leptin release into the media was determined by RIA, while leptin gene expression was analysed by Northern blot. Glucose and lactate were measured with a Cobas Autoanalyser.

Basal leptin secretion (3.9 ± 1.0 ng ml^{-1}, mean ± S.E.M.) was inhibited (30–50 %) by all CLA concentrations tested. As expected, insulin caused a significant increase in leptin secretion (5.4 ± 0.9, $P < 0.001$). When adipocytes were co-treated with CLA in the presence of insulin, a significant dose–response decrease in the insulin-stimulated leptin production was observed (4.3 ± 1.0 ($P < 0.05$), 4.1 ± 1.0, 3.4 ± 0.7, 3.1 ± 0.7, 2.9 ± 0.8 ($P < 0.001$), at concentrations of 1, 10, 50, 100 and 200 μM, respectively). A similar inhibitory action of CLA was observed on leptin gene expression.

Basal glucose uptake (4.2 ± 0.8 μmol) was decreased (20–32%) by all CLA concentrations tested. Insulin induced a significant increase in glucose uptake (81%, $P < 0.001$), and CLA induced a slight but not statistically significant decrease in the insulin-stimulated glucose utilisation. Lactate production was not significantly affected by CLA, although the percentage of glucose released as lactate, which has been shown to be inversely correlated to leptin secretion, was significantly increased by CLA treatment, both in the absence ($P < 0.01$ for all doses tested) or presence of insulin ($P < 0.01$ for 50–200 μM CLA).

In summary, this study shows that the type of dietary fat can affect the regulation of genes implicated in the regulation of the body weight such as leptin. The inhibitory action of CLA on leptin secretion and expression may be mediated at least in part by the alterations induced in glucose uptake and metabolism, but further studies are needed to better understand the molecular mechanisms implicated in CLA actions on leptin.

All procedures accord with current local guidelines.

P28

Role of ATP and adenosine in the modulation of NOS-2 expression in cerebellar astrocytes

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NOS-2, the inducible enzyme isoform in the nitric oxide-generating pathway, is a key regulator of inflammatory processes. In the brain, increased expression of NOS-2 is associated with lesions in demyelinating and neurodegenerative processes (multiple sclerosis, Alzheimer's). In addition, nitric oxide has been implicated in neural damage mechanisms during hypoxia/ischaemia and viral infection. Purine compounds can modulate NOS-2 induction. It has been claimed that ATP down-regulates the induction of NOS-2 expression elicited by IL-1β and γ-interferon. The inhibitory effect was revealed by pre-treatment with ATP for 30–120 min before induction of NOS-2 with IL-1β and IFNγ. However, ATP is very susceptible to hydrolysis by (a) serum esterases or (b) cellular ecto-nucleotidases. The action on NOS-2 induction may be mediated by the degradation products. We have investigated the modulatory role of ATP and adenosine on the expression of NOS-2 in cultures of rat cerebellar astrocytes stimulated with IL-1β (0.5 ng ml^{-1}) and IFNγ (50 U ml^{-1}). Animals (7-day-old rats) were maintained according to international standards and humanely killed by decapitation. The induction of the enzyme was determined after 48 h by measuring nitrite production with the Griess reaction. ATP concentrations were measured by luminescence using luciferase assay.

We have measured the lifetime of ATP in culture medium. There is a rapid hydrolysis of ATP added to DMEM + 10 % FCS medium. ATP was degraded at a rate of 2.5 % min^{-1}. The $t_{½}$ was 40 ± 2 min (mean ± S.D., $n = 3$). The hydrolysis was even faster when ATP (100 μM) was added to astrocyte cultures. The concentration of ATP dropped to levels below 10 nM within the treatment period (< 30 min). The EC_{50} values for ATP acting on P2Y and P2X receptors are typically higher (> 1 μM). In astrocytes, the nitrite production after a 48 h treatment with IL-1β and IFNγ was partially inhibited by ATP. However, the P2 antagonist PPADS (25 μM) and Reactive Blue (25 μM) did not prevent the action of ATP. Furthermore, the addition to the extracellular medium of the enzyme apyrase, to assure the complete hydrolysis of added ATP, failed to block the effect of ATP. Thus, the ATP effect may be mediated by a metabolite, rather than by the nucleotide itself. The end product of extracellular ATP hydrolysis is adenosine. Exogenous adenosine did inhibit NOS-2 induction by IL-1β and IFNγ as ATP did. In addition, the antagonist DPCPX (10 nM–1 μM) completely reversed the action of adenosine and, also, of ATP, indicating that the action of both compounds is mediated by adenosine receptors. The treatment with DPCPX resulted in an overshoot effect, suggesting that there is a tonic inhibition by adenosine in astrocyte cultures. These results indicate that the reported action of ATP in NOS-2 expression is mediated by adenosine receptors, activated by adenosine generated from ATP hydrolysis. This process should be checked whenever the effects of ATP treatments of more than a few minutes are investigated.

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All procedures accord with current National and local guidelines.

P29

Influence of the *in vitro* assay conditions on the spermine and espermidine immunomodulatory effect

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In previous studies carried out on NR8383 cell line cultures (alveolar rat macrophages) enriched with 15 % fetal bovine serum (FBS) we demonstrated that the trophic components spermine and spermidine, added to cell cultures during 18 h, produced a dose-dependent decrease of lipopolysaccharide (LPS)-induced tumour necrosis factor (TNFα) secretion.

We have determined here the effect of these polyamines on the cytokine secretion of NR8383 macrophages under different experimental conditions. We have quantified the secretion of TNFα and monocyte chemoattractant protein-1 (MCP1) after LPS stimulation in macrophages treated with either spermine or spermidine at different times and under different growing conditions.

Cells were plated (10^6 cells per well) and incubated with several dilutions of spermine (0–1000 μM) and spermidine (0–2000 μM) from 18 h pre- to 1.5 h post-stimulation with 10 ng ml^{-1} LPS in 0, 1 and 15 % FBS supplemented cultures. Cells and supernatants were collected after 3 h of LPS stimulation. Cells were used to determine cell viability by flow cytometry and to examine relative levels of TNFα gene expression by RT-PCR after RNA extraction. TNFα and MCP1 secretion in supernatants were measured by a sandwich enzyme immunosorbent assay (ELISA) technique.

Inhibitory concentrations 50 (IC_{50}) for TNFα secretion were about 20–250 μM and 100–1000 μM for spermine and spermidine respectively depending on the different conditions assayed. Maximal inhibition was found when polyamines were added at the same time as LPS and without FBS enrichment. However, both polyamines failed to decrease mRNA levels for TNFα showing a post-transcriptional effect on protein production. When MCP1 secretion was studied, similar results were observed and spermine also exercised the maximal inhibition in cultures without FBS.

In conclusion, spermine and spermidine inhibited the expression of the pro-inflamatory cytokines TNFα and MCP1 under different *in vitro* assay conditions. The immunomodulatory effect of these polyamines was shown to be inversely related to the incubation time of polyamines and FBS concentration in the medium.

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P30

The possible role of IGF-I and oestrogens in the development of canine inflammatory mammary carcinoma

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Inflammatory mammary carcinoma (IMC) is the most aggressive spontaneous type of mammary cancer in both women and dogs. Previous findings indicate that canine IMC could be a source of oestrogens. It has been shown that oestrogens produced in the tumour could act as mitogens and promote tumour growth. A positive correlation between IGF-I and breast cancer risk is reported in women but to date this point is unknown for canine mammary tumours. In order to investigate the possible role of IGF-I and oestrogens in the development of canine IMC, we determined the oestrogen and IGF-I profiles in tissue homogenates of IMC, and then they were compared with mammary dysplasias, benign mammary tumours and other non-IMC malignant mammary tumours.

Eighty-six mammary samples (10 normal mammary tissue (NMG), 21 dysplasias (DYSP), 26 benign (BMT), 22 malignant (MMT) and 7 IMC) from 35 female dogs were used. All tissues were obtained from the Veterinary Teaching Hospital of Madrid and the experimental design was approved by the Ethics Committee. 17β-Oestradiol, SO4E1 and IGF-I were measured by EIA techniques validated for this species. Statistical analysis was done by using Student's paired *t* test.

IMC displayed the following oestrogen and IGF-I levels (mean ± S.E.M.): E2, 675.19 ± 33.00 ng g^{-1}; E1SO4, 2.84 ± 0.32 mg g^{-1}; IGF-I, 194.40 ± 29.15 ng g^{-1}. All the values were significantly higher ($P < 0.001$) when compared with the levels determined for malignant, benign, dysplasias, and normal mammary tissue.

These results point to a role of IGF-I and oestrogens in the suggested autocrine mechanism involved in the development of canine inflammatory carcinoma.

All procedures accord with current National and local guidelines.

P31

Inhibition of serum CD13/aminopeptidase activity by antimalarial drugs

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CD13 is a membrane-bound cell surface glycoprotein that was originally recognized as a marker for subsets of normal and malignant myeloid cells, but later found on other cells and tissues. Sequence comparisons of the cloned cDNA have shown that CD13 is identical to aminopeptidase N (APN; EC 3.4.11.2), a type II membrane-spanning protein belonging to the M1 family of zinc-metallopeptidase which cleaves N-terminal neutral and basic amino acids from peptides and proteins. This ectoenzyme can cleave bioactive proteins on the cell surface, including cytokines, to either activate or inactivate them, and it has been involved in down-regulation of signal peptides, angiogenesis, inflammation, cell signalling and even as a receptor for certain viruses (e.g. coronavirus 229E) enabling host cell infection. Soluble aminopeptidase activity found in serum appears to procede from native membranous APN which is cleaved off by limited proteolysis and is characterized by the absence of the hydrophobic N-terminal polypeptide chain, responsible for anchoring the protein to the membrane.

Since antimalarial drugs are used to treat milder cases of rheumatoid arthritis and systemic lupus erythematosus due to their antiinflammatory properties and since aminopeptidases seem to be involved in the terminal stages of globin degradation in *Plasmodium falciparum* we have investigated the effects of these substances on aminopeptidase activity of human serum in a fluorometric assay that use amino acyl β-naphthylamide (βNA) substrates.

Serum aminopeptidase activities from human healthy donnors varied depending on the substrate used (alanine-β-naphthylamide, arginine-β-naphthylamide or leucine-β-naphthylamide), ala-βNA being the substrate more actively hydrolysed (17.7 ± 1.0 nm min^{-1} ml^{-1}; mean ± S.E.M.); arg-βNA (5.4 ± 0.2 nm min^{-1} ml^{-1}) and leu-βNA (5.9 ± 0.6 nm min^{-1} ml^{-1}) were hydrolysed to a lesser degree.

Quinine (hydrochloride) and chloroquine (diphosphate salt) inhibited AP activities in a dose-related manner independently of the substrate used. In general, chloroquine (IC_{50}: 71.7 mM; substrate: ala-βNA) was more effective inhibiting AP activity than quinine (IC_{50}: 149.6 mM; substrate: ala-βNA). Nevertheless, the lowest IC_{50} was obtained when the substrate was arg-βNA. In all the cases data can be adapted to the negative exponential curve $y = a_0 + a_1 e^{(-x/a2)}$ with a coefficient of determination > 0.99 in every case.

A wide variety of mechanisms of antiinflammatory action have been proposed for antimalarial agents. Reduced T lymphocyte mitogen responsiveness, reduced leucocyte chemotaxis, stabilization of lysosomal membranes or trapping free radicals are the most commonly proposed. The inhibition of aminopeptidase activities by these agents suggests that these enzymes may play a role in the mechanism by which antimalarial drugs have therapeutic effects.