FULL METHODS

Materials - Fura-2/AM was from Molecular Probes Inc. (Invitrogen, Paisley, Scotland, UK). Horse radish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were from DakoCytomation (Ely, Cambridgeshire, UK). Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK).

Cell Culture - HEK-293 cells, stably transfected with human parathyroid CaR, were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Ltd., Paisley, Scotland, UK.) and 200μg/ml hygromycin B (Boehringer-Mannheim, Lewes, Sussex, UK).

Intracellular calcium assay - CaR-HEK cells cultured on glass coverslips were loaded with Fura-2/AM (1μM for 1 hour) at room temperature in the dark in Experimental Buffer [20mM HEPES, pH 7.4, 125mM NaCl, 4mM KCl, 1.2mM $CaCl_2$, 0.5mM $MgCl_2$, 5.5mM glucose] supplemented with 0.1% bovine serum albumin. Non-absorbed Fura-2/AM was removed by washing and the cells were equilibrated for 10min in Experimental Buffer (BSA-free) containing the baseline $[Ca^{2+}]_o$ appropriate for the ensuing experiment. The cells were mounted in a perfusion chamber (Warner Instruments, Hamden, CT, USA) and observed through a 40X oil-immersion objective. Dual-excitation wavelength microfluorometry was then performed using a Nikon Diaphot inverted microscope (Cairn Research Ltd., Kent, UK). Experiments were performed at room temperature in Experimental Buffer containing various concentrations of $CaCl_2$ (0.5mM unless otherwise stated). The pH of the HEPES-containing solutions was adjusted using HCl and NaOH as determined using a Denver Instruments UB-10 pH meter (freshly calibrated using pH standard solutions (Hanna Instruments)). Alternatively, a bicarbonate/CO_2 buffer was used [4mM KCl, 0.5mM $MgCl_2$, 2.5mM $CaCl_2$, 5.5mM glucose supplemented with either i) 23.4mM $NaHCO_3$, 114mM NaCl to give pH 7.2 at 20^{o}C, ii) 37.2mM $NaHCO_3$, 100mM NaCl to give pH 7.4, or, iii) 58.9mM $NaHCO_3$, 78mM NaCl to give pH 7.6] and gassed continuously with 5%CO_2/95%O_2 at 20^{o}C and used immediately.

ERK phosphorylation and actin polymerization assays - Cells were grown to 80-90% confluence in 35 mm culture dishes and ERK assayed as described previously (9, 10). Experiments were performed at 37^{o}C prior to lysis on ice in RIPA buffer supplemented with protease and phosphatase inhibitors and then phospho-ERK quantified by semi-quantitative immunoblotting using a phospho-specific polyclonal antibody (Promega) (9, 10). To assess actin stress fiber assembly, cells were grown on glass coverslips, treated as before and then fixed with paraformaldehyde and stained with Phalloidin-TRITC prior to imaging using a Zeiss Axioplan 2 fluorescence microscope with images acquired using a Hamamatsu digital camera. Cytosolic TRITC fluorescence intensity per unit area was quantified using ImageJ software and corrected for background levels (10).

Immunoblotting - Immunoblotting was performed as described previously (9, 10). Anti-CaR mouse monoclonal antibody, raised to amino acids 214-235 (ADD) of the extracellular domain of the human parathyroid CaR was from Affinity Bioreagents (Golden, CO).

Site-directed mutagenesis – Histidine and cysteine mutations were introduced into the wild-type human parathyroid CaR by site-directed mutagenesis using the QuikChange (Stratagene) method. HEK-293 cells were then transiently transfected with wild-type or mutant receptors using FuGENE-6 (Roche Diagnostics).

Parathyroid gland preparation and PTH secretion assay - Bovine superior parathyroid glands, collected from a local abattoir, were processed as described previously (9). The resulting parathyroid cells were grown on collagen-coated coverslips/dishes and used within 24hr to minimise the consequence of CaR downregulation. PTH levels were quantified using a Bovine Intact PTH ELISA kit (Immunodiagnostic Systems Limited, Boldon, Tyne & Wear, UK).

Intracellular pH assay – Intracellular pH was quantified using the pH-sensitive fluorescent dye BCECF, AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester). Cells on glass

coverslips were loaded with 2μM BCECF in 0.5mM $CaCl_2$ experimental buffer for 30min, at room temperature in the dark. Dual-excitation wavelength microfluorometry was then performed using a Nikon TMD inverted microscope with fluorometry attachments (Cairn Research Ltd, Faversham, Kent). The cells were mounted in a perfusion chamber (Warner Instruments, Hamden, CT, USA) and observed through a 40X oil-immersion objective. Positive controls for intracellular acidification and alkalinisation were 20mM ammonium chloride and 20mM sodium acetate respectively. Calibration experiments were conducted in buffer containing 20mM HEPES, 4mM NaCl, 125mM KCl, 0.5mM $CaCl_2$, 0.5mM $MgCl_2$, 5.5mM glucose and 5μM nigericin at pH 6.8, 7.2 and 7.6. Fluorescence data were acquired using DASYLab software (Measurement Computing Corp., Naton, MA, USA).

Human Parathyroid Cell Preparation and PTH analysis - Samples from normal parathyroid cell transplants were obtained at neck surgery at the Royal North Shore Hospital and North Shore Private Hospital (St Leonards, NSW, Australia) and the Mater Misericordiae Hospital (North Sydney, NSW) and collagenase digested in bovine serum albumin-containing MEM medium as described previously (9 and other papers referenced therein). All procedures were performed under guidelines established by the relevant institutional ethics committees, and all patients provided written informed consent for the use of the tissue for experimental purposes. Secretion of PTH from normal human parathyroid cells was performed as described previously (9). Briefly, cells were perifused by gel filtration (with PTH detected in the void volume) using physiological saline [(mM) 125 NaCl, 4.0 KCl, 1.25 $CaCl_2$, 1.0 $MgCl_2$, 0.8 Na_2HPO_4, 20 HEPES (NaOH) pH 7.4] supplemented with 0.1% D-glucose, 1x basal amino acid mixture (total concentration 2.8 mM) as defined previously (9) and 1 mg/ml bovine serum albumin. 4-10 x 10^4 cells were incubated in a small perifusion column above Bio-gel P-4 and held in place using Sephadex G-25 and perifused (1.5 mL/min) at 37 °C. Samples were collected, chilled and then frozen (–80 °C) until analysis for intact human PTH (1-84) using a third generation, two-site chemiluminescence assay on an Immulite 2000 autoanalyzer.

Statistical analysis - Data are presented as means ± S.E. and statistical significance was determined by one-way or Repeated Measures ANOVA (Tukey, Dunnett's or Bonferroni's post-hoc test), Kruskal Wallis (Dunn's) or by paired or unpaired t-test as appropriate (GraphPad Prism).