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Stimulation of TRPC5 cationic channels by low micromolar concentrations of lead ions (Pb^{2+})

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ABSTRACT

Lead toxicity is long-recognised but continues to be a major public health problem. Its effects are wide-ranging and include induction of hyper-anxiety states. In general it is thought to act by interfering with Ca^{2+} signalling but specific targets are not clearly identified. Transient receptor potential canonical 5 (TRPC5) is a Ca^{2+}-permeable ion channel that is linked positively to innate fear responses and unusual amongst ion channels in being stimulated by trivalent lanthanides, which include gadolinium. Here we show investigation of the effect of lead, which is a divalent ion (Pb^{2+}). Intracellular Ca^{2+} and whole-cell patch-clamp recordings were performed on HEK 293 cells conditionally over-expressing TRPC5 or other TRP channels. Extracellular application of Pb^{2+} stimulated TRPC5 at concentrations greater than 1 μM. Control cells without TRPC5 showed little or no response to Pb^{2+} and expression of other TRP channels (TRPM2 or TRPM3) revealed partial inhibition by 10 μM Pb^{2+}. The stimulatory effect on TRPC5 depended on an extracellular residue (E543) near the ion pore: similar to gadolinium action, E543Q TRPC5 was resistant to Pb^{2+} but showed normal stimulation by the receptor agonist sphingosine-1-phosphate. The study shows that Pb^{2+} is a relatively potent stimulator of the TRPC5 channel, generating the hypothesis that a function of the channel is to sense metal ion poisoning.

Introduction

Lead (Pb^{2+}) is a naturally occurring divalent cationic heavy metal with high electronegativity and flexible coordination number that facilitates its interactions with oxygen and sulphur atoms of proteins to form stable complexes [1]. It has no known physiological function but is widely used in many commercial products. The commonest sources of environmental Pb^{2+} are leaded gasoline, food containers, toys, smelters, recycled batteries, paints, electronics, water pipes and traditional medicines. The main route of entry to the body is ingestion, particularly in children, and inhalation through occupational exposure [2]. The toxic effects of Pb^{2+} depend on the duration and magnitude of its exposure and also age and nutritional status [3,4]. About 75–90% of absorbed Pb^{2+} is stored in bones and teeth with the remainder in red blood cells and soft tissues including liver [2].

Central nervous system defects presenting as developmental abnormalities are the most common symptom in children. Although Pb^{2+} can influence any part of the brain, it preferentially affects prefrontal cerebral cortex, cerebellum and hippocampus leading to cognitive defects, motor function and memory disturbances [3,5]. Anxiety states have been noted and animals exposed to Pb^{2+} exhibited increased fear of aversive stimuli [6]. There are also other effects on the body, including on the cardiovascular, renal and endocrine systems [3]. Suggested mechanisms of action include induction of oxidative stress and interference with Ca^{2+} and other signalling pathways but the exact molecular targets for Pb^{2+} have not been clearly established [3,7,8].

The mammalian transient receptor potential (TRP) family of cationic channels are widely expressed across many cell types [9–11]. One of the members of the Canonical subfamily is TRPC5, which is a Ca^{2+} permeable channel that is stimulated by a range of factors [12], which include lanthanide ions and protons [13,14]. Lanthanides appear to act relatively directly via a common mechanism that depends on amino acid residues (notably E543) in the outer pore region of the channel [13,14].

An area of prominent TRPC5 expression is the mammalian brain where specific neuronal functions are starting to emerge. TRPC5 inhibits or potentiates neuronal growth cone formation depending on the conditions [15–17] and mice lacking TRPC5 exhibit diminished innate fear in response to aversive stimuli [18]. TRPC5 is also detected elsewhere, including in the cardiovascular system where it contributes to heteromultimeric channel assemblies and has putative roles in development and remodelling [19,20].

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Here we report on the effect of Pb^{2+} on TRPC5 compared with two other TRP channels (TRPM2 and TRPM3) which are also broadly expressed, including in neurones [21,22].

Methods

Cell culture, plasmids, mutagenesis and transfection. Wild-type and T-rex HEK 293 cells (Invitrogen) stably expressing human TRPC5 [23], TRPM2 [24] or TRPM3 [25] under a tetracycline inducible promoter were cultured in DMEM containing 10% foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. TRP channel expression in T-rex cells was induced by 1 μg/ml tetracycline (tet+; Sigma) for 24–72 h before experimentation [23,25,26]. Non-induced cells cultured without addition of tetracycline (tet−) were controls. For transient transfection, human TRPC5 cDNAs was cloned into pEGFPN1 and point mutations were introduced using QuikChange® site-directed mutagenesis (Stratagene) and appropriate primer sets (forward (5′–3′): GCTTTACTTCTATTATCAAACCAG AGCTATCGATG; reverse (5′–3′): CATCGATAGCTCTGGTTTGATAATA GAAGTAAAGC). The mutation was confirmed by direct sequencing (University of Leeds DNA sequencing facility). cDNAs were transiently transfected into wild-type HEK 293 cells with FuGENE 6 transfection reagent (Roche) 48 h prior to recording.

Intracellular Ca^{2+} measurement. HEK 293 cells were plated in 96-well biocoat plates (Corning) at 60–70% confluence 24 h before experiments. Prior to recordings, cells were incubated for 1 h at 37 °C in 2 μM fura-2AM dispersed in standard bath solution (SBS) containing (mM): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 8 glucose and 10 HEPES titrated to pH 7.4 using NaOH. The cells were washed for 0.5 h in SBS and measurements were made on a 96-well bench-top scanning fluorimeter (FlexStation II) with SoftMax Pro 4.7.1 (Molecular Devices, Sunnyvale, CA, USA) at room temperature (21 ± 2 °C). Fura-2 was excited with 340 and 380 nm light and emission was collected at 510 nm. Intracellular Ca^{2+} was indicated by the ratio of emission intensities for the two excitation wavelengths.

Electrophysiology. Whole-cell patch-clamp recordings were performed at room temperature as previously described [27]. Patch pipette solution contained (mM): 115 CsCl, 2 $MgCl_2$, 5 Na_2ATP, 0.1 NaGTP, 10 HEPES, 10 EGTA and 5.7 $CaCl_2$; final pH was adjusted to 7.2 with CsOH. The extracellular solution was SBS. Signals were amplified with an Axopatch 200B patch-clamp amplifier and software was Signal 3.05 (CED, UK). A ramp voltage protocol from −100 mV to +100 mV of 1 s in duration was applied every 10 s from a holding potential of 0 mV. Current signals were filtered at 1 kHz and digitally sampled at 3 kHz. Patch pipettes had resistances of 3–5 MΩ.

Chemicals and reagents. All chemicals were from Sigma (UK) except for fura-2AM (Invitrogen). One hundred millimolar stock solution of lead (II) nitrate ($Pb(NO_3)_2$) salt was prepared in water freshly for each experiment.

Data analysis. Data sets were compared using Student's *t*-tests and expressed as mean ± SEM. Probability <0.05 (*) was considered statistically significant difference. All data are from at least three independent experiments and single example results are representative of at least three independent experiments. ORIGIN software was used for data analysis and presentation.

Results

Pb^{2+} stimulates TRPC5 channels

The effect of Pb^{2+} was studied using whole-cell patch-clamp applied to TRPC5-expressing (tet+) HEK 293 cells. As shown in the representative time-series plot, external application of 10 μM Pb^{2+} stimulated robust current in TRPC5 (tet+) cells that was inhibited by the common TRPC channel blocker 2-aminoethoxydiphenyl borate (2-APB; 75 μM; Fig. 1A). The current–voltage relationship (*I–V*) of the current evoked by Pb^{2+} had the signature shape expected for TRPC5 channels (Fig. 1B cf. [13,23]). No current was evoked by Pb^{2+} in control (tet−) cells that were not over-expressing TRPC5 (Fig. 1C).

Pb^{2+} evoked Ca^{2+}-entry was also detected in HEK 293 cells over-expressing TRPC5 (Fig. 1D). Construction of a concentration–response curve revealed a robust response at 2 μM Pb^{2+} (Fig. 1D and E) and an EC_{50} of ~5 μM (Fig. 1E). In control (tet−) cells, 10 μM Pb^{2+} failed to evoke a Ca^{2+} signal but there were small responses to high concentrations of Pb^{2+} (Fig. 1E).

The data suggest that Pb^{2+} is a reasonably potent stimulator of TRPC5 channels.

Stimulation depends on glutamate 543 (E543)

Conservative mutation of a glutamate residue (E543) to glutamine (Q) in the predicted outer pore loop has been shown to abolish lanthanide stimulation of TRPC5 channels [13]. This result was confirmed in HEK 293 cells transiently transfected with wild type TRPC5 or E543Q mutant TRPC5 in whole-cell patch-clamp experiments (Fig. 2A and B). The E543Q TRPC5 channels were successfully expressed and functional because they could be stimulated by sphingosine-1-phosphate (S1P) (Fig. 2B), which acts via a different mechanism [19].

We therefore investigated whether stimulation of TRPC5 by Pb^{2+} might also depend on E543. Cells expressing E543Q TRPC5 were strikingly insensitive to Pb^{2+} yet responded robustly to S1P (Fig. 2C and D) with the characteristic TRPC5 I-V (Fig. 2E).

The data suggest that stimulation of TRPC5 by Pb^{2+} depends on the negative charge of glutamate 543 and therefore has a mechanism of action that is shared by lanthanides.

Inhibition of other types of TRP channel

For comparison, Pb^{2+} was tested against other types of TRP channel for which we have generated the same type of conditional expression system.

Hydrogen peroxide (H_2O_2) is a stimulator of TRPM2 channels [24] and was used to evoke TRPM2 activity in Ca^{2+} measurement assays. H_2O_2 but not Pb^{2+} (10 μM) evoked Ca^{2+} influx in induced TRPM2 (tet+) cells (Fig. 3A). The potential for an inhibitory effect of Pb^{2+} was evaluated by pre-incubating TRPM2 cells (tet+) with 10 μM Pb^{2+} and then measuring Ca^{2+} influx in response to H_2O_2. There was an inhibitory effect of Pb^{2+} (Fig. 3B and C).

Pregnenolone sulphate (PregS) is a stimulator of TRPM3 channels [28] and was used to evoke TRPM3 activity in Ca^{2+} measurement assays. PregS but not Pb^{2+} (10 μM) evoked Ca^{2+} influx in induced TRPM3 (tet+) cells (Fig. 3D). The potential for an inhibitory effect of Pb^{2+} was evaluated by pre-incubating TRPM3 cells (tet+) with 10 μM Pb^{2+} and then measuring Ca^{2+} influx in response to PregS. There was an inhibitory effect of Pb^{2+} (Fig. 3E and F).

The data suggest that Pb^{2+} is not specific for TRPC5 because it also affected other TRP channels (i.e. TRPM2 and TRPM3). However, the other TRP channels were inhibited rather than stimulated by Pb^{2+}.

Discussion

The study shows that low micromolar concentrations of Pb^{2+} stimulate TRPC5 channels via a mechanism that depends on a negatively-charged amino acid in the third extracellular loop of TRPC5. To our knowledge it is the first report of the molecular identity of a