CHAPTER 6

The Effect of Calcium on Osteoblastic Cells

6.1 The effect of calcium on OPG and DKK-1 production in osteoblastic cells

Calcium is transcytosed through the osteoclast during bone resorption and slowly released into the local environment. The use of Bradykinin (chapter 3) stimulated DKK-1 production in MG63 cells. Therefore OPG and DKK-1 production in osteoblastic cells in response to calcium in the form of calcium chloride ($CaCl_2$) was investigated. MG63 cells were stimulated for 24 hours in the presence of 0-50mM calcium either with or without FCS. Culture medium was removed after 24 hours and assayed for OPG and DKK-1. Calcium significantly increased OPG production in a dose-dependent manner both with and without FCS ($P<0.001$, Fig 6.1A). The lowest effective concentration to exhibit a significant response was 5mM in both culture conditions. OPG production continued to increase up to the maximum concentrations used. At 50mM calcium, OPG production had been significantly increased by 185% and 883% in the presence and absence of FCS respectively compared to own control ($P<0.001$).

Calcium significantly increased DKK-1 production in a dose-dependent manner both with and without FCS ($P<0.001$, Fig 6.1B). The lowest effective concentration to exhibit a significant response was 10mM in the presence of FCS and 2.5mM in the absence of FCS. A maximal effect was observed at 25mM calcium where DKK-1 production had been significantly increased by 56% and 1225% in the presence and absence of FCS respectively compared to own control ($P<0.001$).

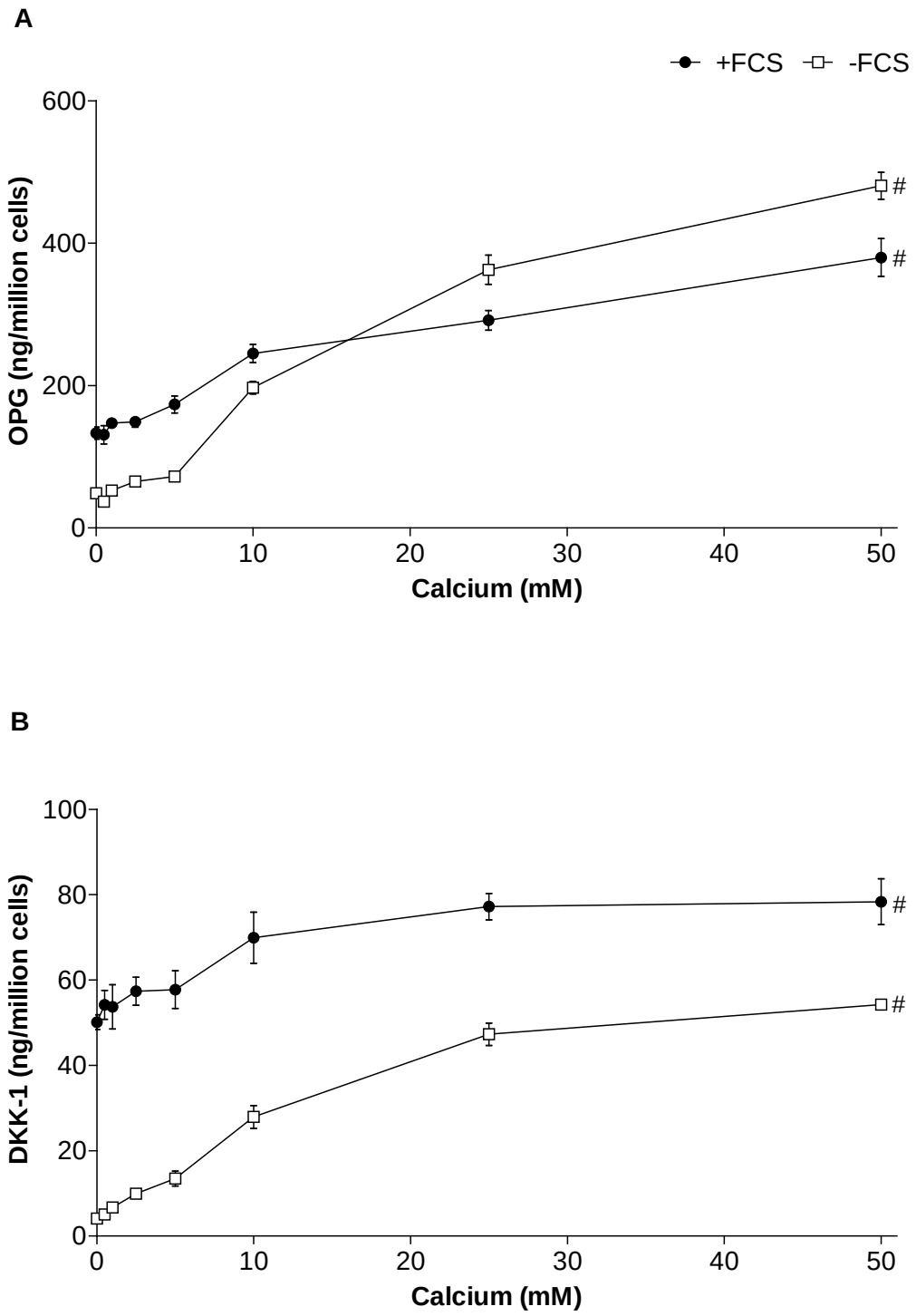

Fig 6.1 The effect of calcium on OPG and DKK-1 production in MG63 cells. MG63 cells were stimulated with 0-50mM calcium for 24 hours with or without FCS. Culture medium was assayed for A) OPG and B) DKK-1. Data shown are mean ± SD, n=4. #P<0.001 versus control (0mM calcium, one-way ANOVA using Dunnett's multiple comparison test).

6.2 The effect of EDTA on OPG and DKK-1 production

MG63 cells were stimulated for 24 hours in the presence of 0-2mM ethylenediaminetetraacetic acid (EDTA). EDTA is a chelator of calcium ions and therefore should deplete any free calcium in the culture medium. EDTA significantly inhibited OPG production in a dose-dependent manner in the presence of FCS ($P<0.01$, Fig 6.2A). The lowest and only effective concentration to exhibit a significant response was 2mM EDTA where OPG production had been significantly inhibited by 13% compared to control ($P<0.05$). In the absence of FCS, EDTA had an overall significant effect in reducing OPG production ($P<0.05$), although no specific concentration was significant in itself.

EDTA significantly inhibited DKK-1 production in a dose-dependent manner both with and without FCS ($P<0.001$, Fig 6.2B). The lowest effective concentration to exhibit a significant response was 2mM EDTA in the presence of FCS and 1mM EDTA in the absence of FCS. A maximal effect was observed at 2mM EDTA where OPG production had been significantly inhibited by 20% ($P<0.05$) and 66% ($P<0.001$) in the presence and absence of FCS respectively compared to own control.

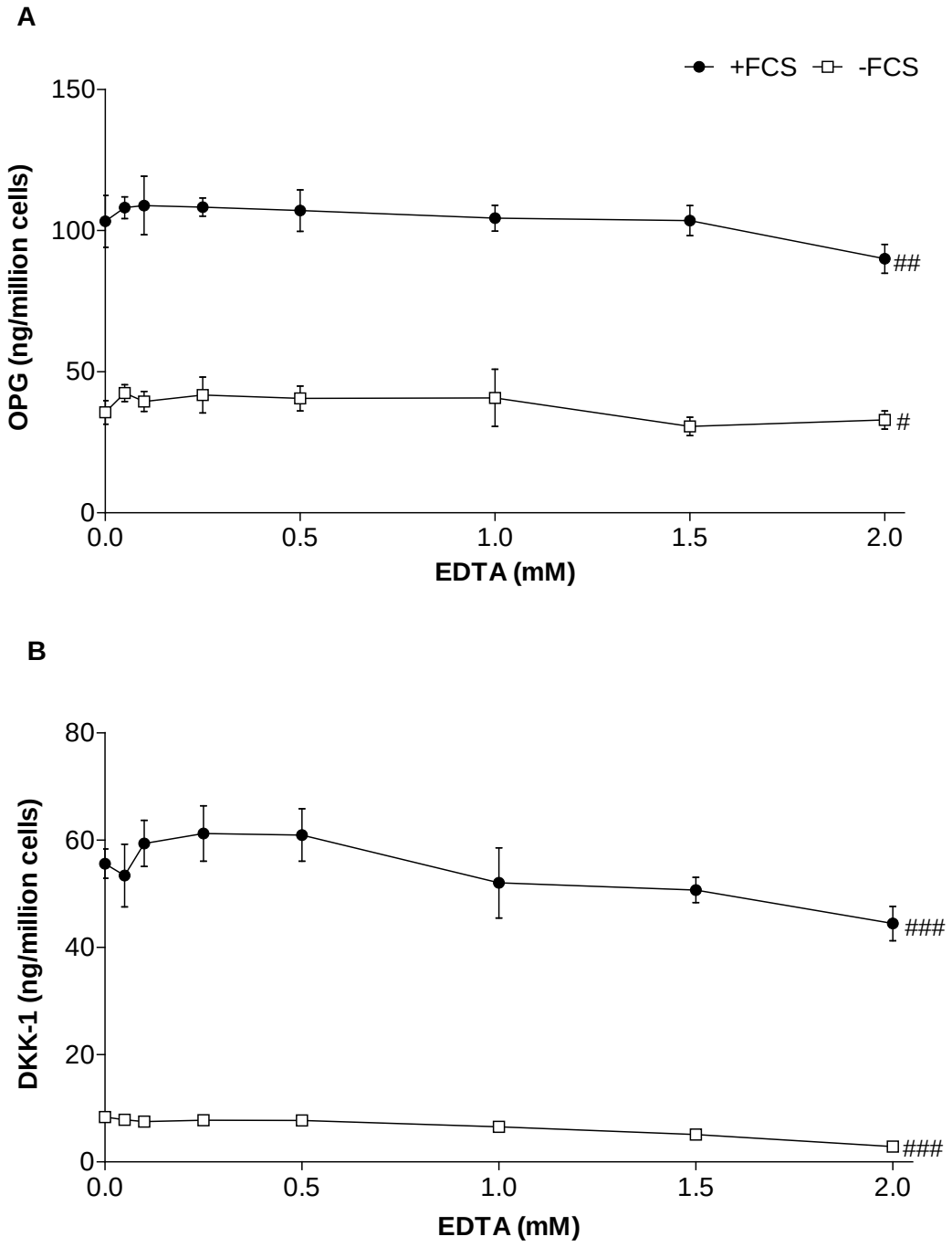

Fig 6.2 The effect of EDTA on OPG and DKK-1 production in MG63 cells. MG63 cells were stimulated with 0-2mM EDTA for 24 hours with or without FCS. Culture medium was assayed for A) OPG and B) DKK-1. Data shown are mean ± SD, n=4. #P<0.05, ##P<0.01 and ###P<0.001 versus control (0mM EDTA, one-way ANOVA using Dunnett's multiple comparison test).

6.3 Time course of calcium-induced DKK-1 production

The time course of DKK-1 stimulation in response to calcium was determined. MG63 cells were cultured for a total of 24 hours with or without 25mM calcium. Medium was removed after 1, 2, 4, 8, 12 and 24 hours, assayed for DKK-1 and values adjusted for rate of production of DKK-1 per hour. In both control and calcium stimulated cells, DKK-1 production per hour significantly reduced in a time-dependent manner ($P<0.001$, Fig 6.3). At all six time points, the rate of DKK-1 production in calcium-stimulated cells was significantly higher than in control cells ($P<0.001$). At 1 hour, the rate of DKK-1 production in calcium-stimulated cells had been significantly increased by 72% compared to control cells at the same time point ($P<0.001$) and remained significantly elevated by 30% above control cells at 24 hours ($P<0.001$).

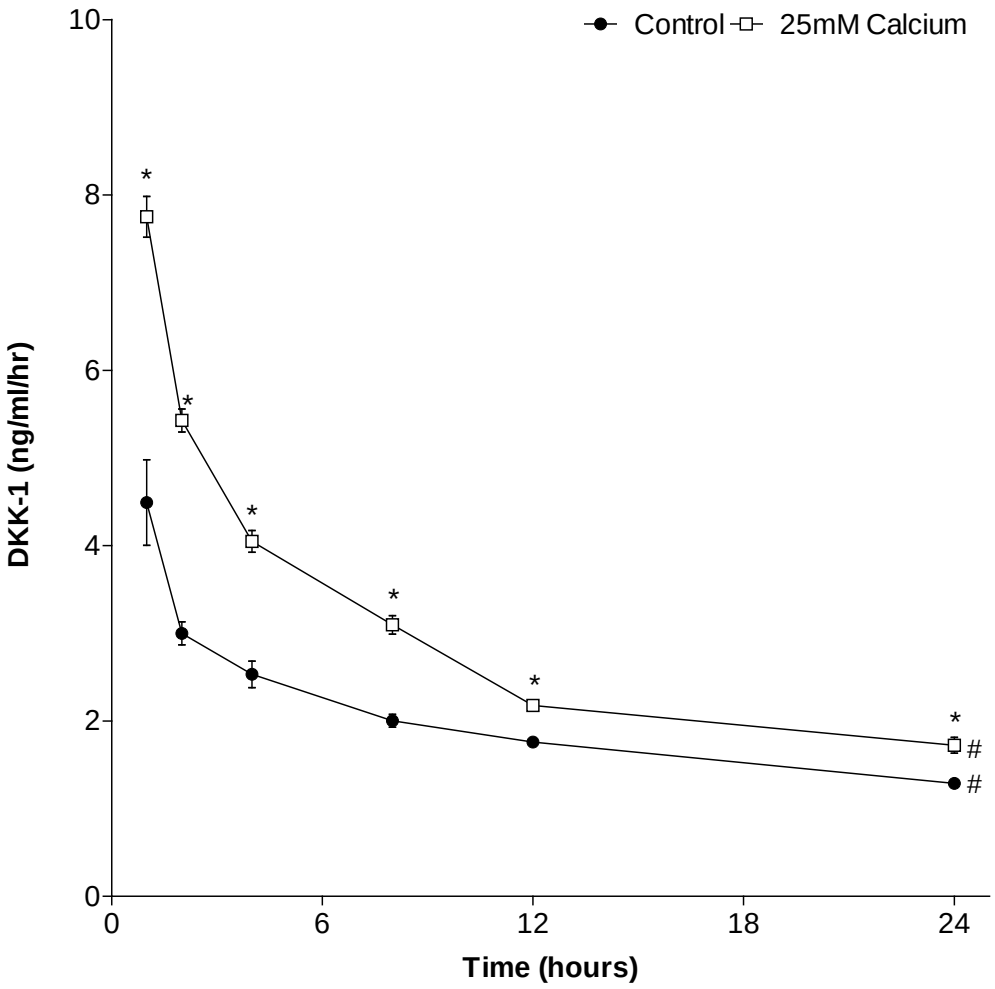

Fig 6.3 The time course for calcium-induced DKK-1 production in MG63 cells. MG63 cells were stimulated with 25mM calcium for 1, 2, 4, 8, 12 and 24 hours. Culture medium was assayed for DKK-1. Data shown are mean rate of DKK-1 production per hour ± SD, n=4. #P<0.001 versus 1 hour time point of same treatment (one-way ANOVA using Dunnett's multiple comparison test and *P<0.001 versus no calcium control at each time point (unpaired t-test)

6.4 The effect of signalling inhibitors on calcium-induced DKK-1 production

Other studies have previously investigated the effects of calcium on OPG production (Takami *et al.*, 2000; Bergh *et al.*, 2004) so the mechanism via which calcium was stimulating DKK-1 production was investigated. MG63 cells were stimulated for 30 minutes with 25µM of the inhibitors for JNK (SP600125), P38MAPK (SB202190), ERK/MEK (U0126), PI3K (LY294002) and NF-kB (BAY 11-7082), 10µM BIM (PKC inhibitor) 10nM STS (wide specificity protein kinase inhibitor) and 10µM BIO (GS3K inhibitor). After 30 minutes, 25mM calcium was added to the medium and cells were left to incubate for 24 hours. Culture medium was removed and assayed for DKK-1.

As previously observed, inhibitors for P38MAPK, ERK, PI3K, PKC and GS3Kβ significantly inhibited DKK-1 production (all $P<0.001$) whilst inhibitors for JNK and NF-kB had no effect (Fig 6.4). None of the inhibitors used however prevented the ability for calcium to induce DKK-1 production. Calcium was able to significantly stimulate the production of DKK-1 in the presence of all inhibitors used by 21-104% compared to own inhibitor control ($P<0.01$-0.001, Table 6.1). Calcium-stimulated DKK-1 production in the presence of SP600125 and U0126 was significantly higher than calcium only control ($P<0.001$) whilst calcium-stimulated DKK-1 production in the presence of SB202190, LY294002, BIM, STS and BIO was significantly less than calcium only control ($P<0.001$).

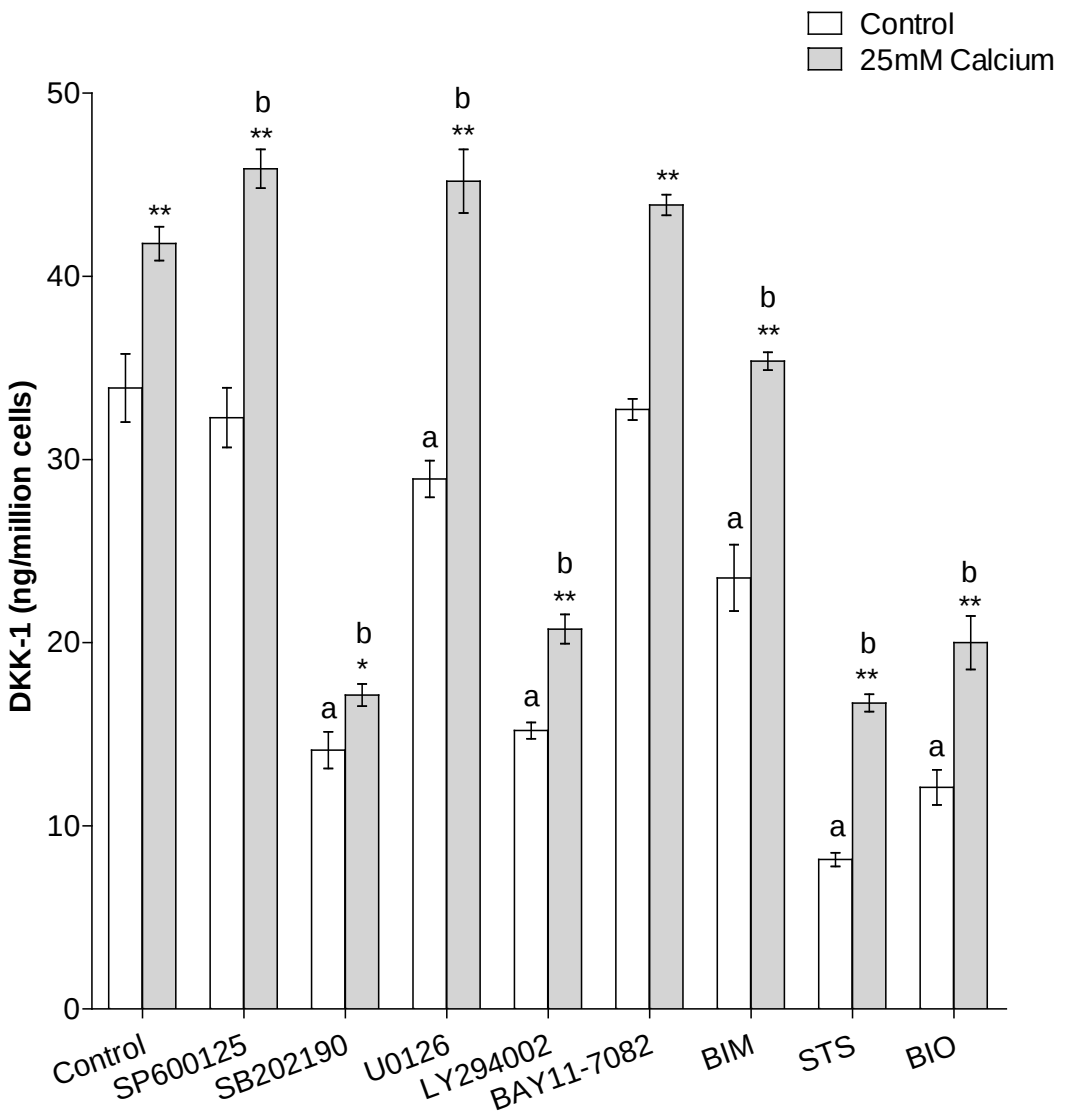

Fig 6.4 The effect of signalling inhibitors on calcium-induced DKK-1 production in MG63 cells. MG63 cells were stimulated with 25mM calcium in complete medium following a 30 minute pre-incubation with either 25µM SP600125, 25µM SB202190, 25µM U0126, 25µM LY294002, 25µM BAY11-7082, 10µM BIM, 10nM STS or 10µM BIO. Culture medium was assayed for DKK-1. Data shown are mean ± SD, n=4. *P<0.01, **P<0.001 versus own treatment control (one way ANOVA using Bonferroni's multiple comparison test). [a]P<0.001 versus no treatment control and [b]P<0.001 versus 25mM calcium control (one way ANOVA using Dunnett's multiple comparison test).

Table 6.1 Percentage stimulation of calcium-induced DKK-1 production in MG63 cells in the presence of various inhibitors. *P<0.01, **P<0.001 versus own treatment control (one way ANOVA using Bonferroni's multiple comparison test).

Treatment	% stimulation of DKK-1 production by 25mM calcium
Control	23**
SP600125	42**
SB202190	21 *
U0126	56**
LY294002	36**
BAY11-7082	34**
BIM	50**
STS	104**
BIO	65**

6.5 The effect of Nifedpine and NPS2390

Other possible mechanisms for calcium-induced DKK-1 production in osteoblastic cells were investigated. MG63 cells were stimulated for 30 minutes in complete medium in the presence of either 0-50µM nifedipine (L-type calcium channel inhibitor) or 0-25µM NPS2390 (calcium sensing receptor (CaSR) inhibitor) prior to addition of 25mM calcium. After 24 hours, culture medium was removed and assayed for DKK-1. Nifedipine significantly inhibited DKK-1 production in both control cells and calcium stimulated cells in a dose responsive manner ($P<0.05$ and $P<0.01$ respectively, Fig 6.5A). The lowest effective dose to exhibit a significant response was 50µM in control cells and 10µM in calcium stimulated cells. A maximum effect was observed at 10µM nifedipine in calcium stimulated cells where DKK-1 production was inhibited by 13% compared to calcium control (no inhibitor). Nifedipine however did not completely abrogate the effects of calcium on DKK-1 production. At 10µM nifedipine, DKK-1 production in calcium stimulated cells was significantly higher by 21% compared to unstimulated cells ($P<0.001$).

In control cells, NPS2390 slightly but significantly inhibited DKK-1 production in a dose-dependent manner ($P<0.01$, Fig 6.5B). The lowest concentration to exhibit a significant effect was 10µM. A maximal effect was observed at 10µM where DKK-1 production was inhibited by 11% compared to control ($P<0.01$). NPS2390 did not have a significant effect on inhibiting calcium-induced DKK-1 production in MG63 cells.

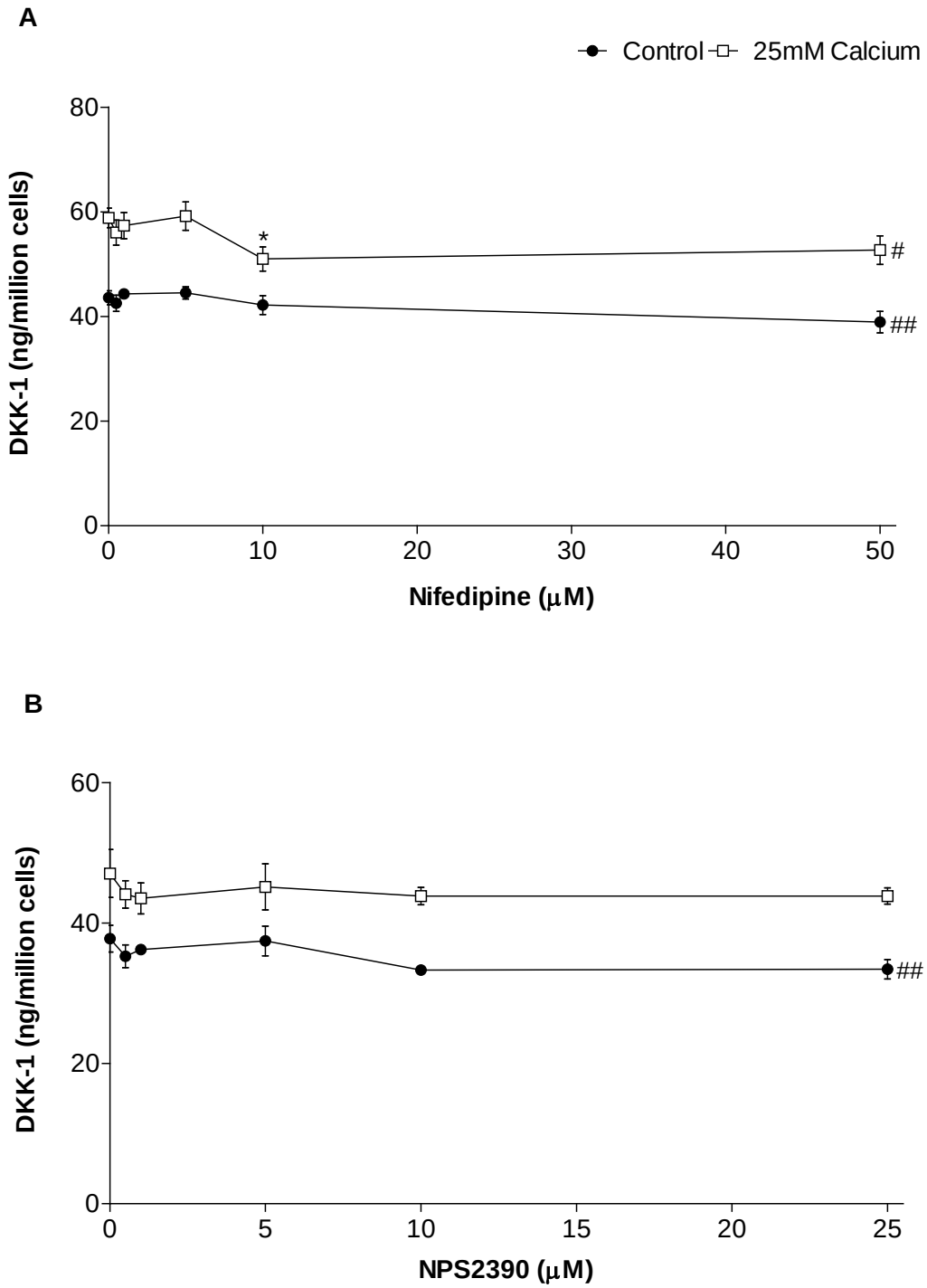

Fig 6.5 The effect of nifedipine and NPS2390 on calcium-induced DKK-1 production in MG63 cells. MG63 cells were stimulated for 30 minutes in complete medium with either A) 0-50μM nifedipine or B) 0-25μM NPS2390 prior to addition of 25mM calcium. Culture medium was assayed for DKK-1. Data shown are mean ± SD, n=4. #P<0.05 and ##P<0.01 versus own control (0μM inhibitor, one-way ANOVA using Dunnett's multiple comparison test). *P<0.001 versus no calcium control at same treatment concentration (unpaired t-test).

6.6 The effect of Ionomycin

Ionomycin is a calcium ionophore and will facilitate calcium ion transport into the cell. Therefore, the effect of ionomycin was investgated for its ability to stimulate DKK-1 production. MG63 cells were stimulated for 30 minutes in complete medium in the presence of 0-100nM ionomycin prior to addition of 25mM calcium. After 24 hours, culture medium was removed and assayed for DKK-1. Ionomycin significantly inhibited DKK-1 production in control cells in a dose responsive manner ($P<0.001$, Fig 6.6). The lowest effective dose to exhibit a significant response was 50nM. A maximal effect was observed at 100nM ionomycin where DKK-1 production was inhibited by 17% compared to control ($P<0.001$). Ionomycin had no effect on calcium-induced DKK-1 production in MG63 cells.

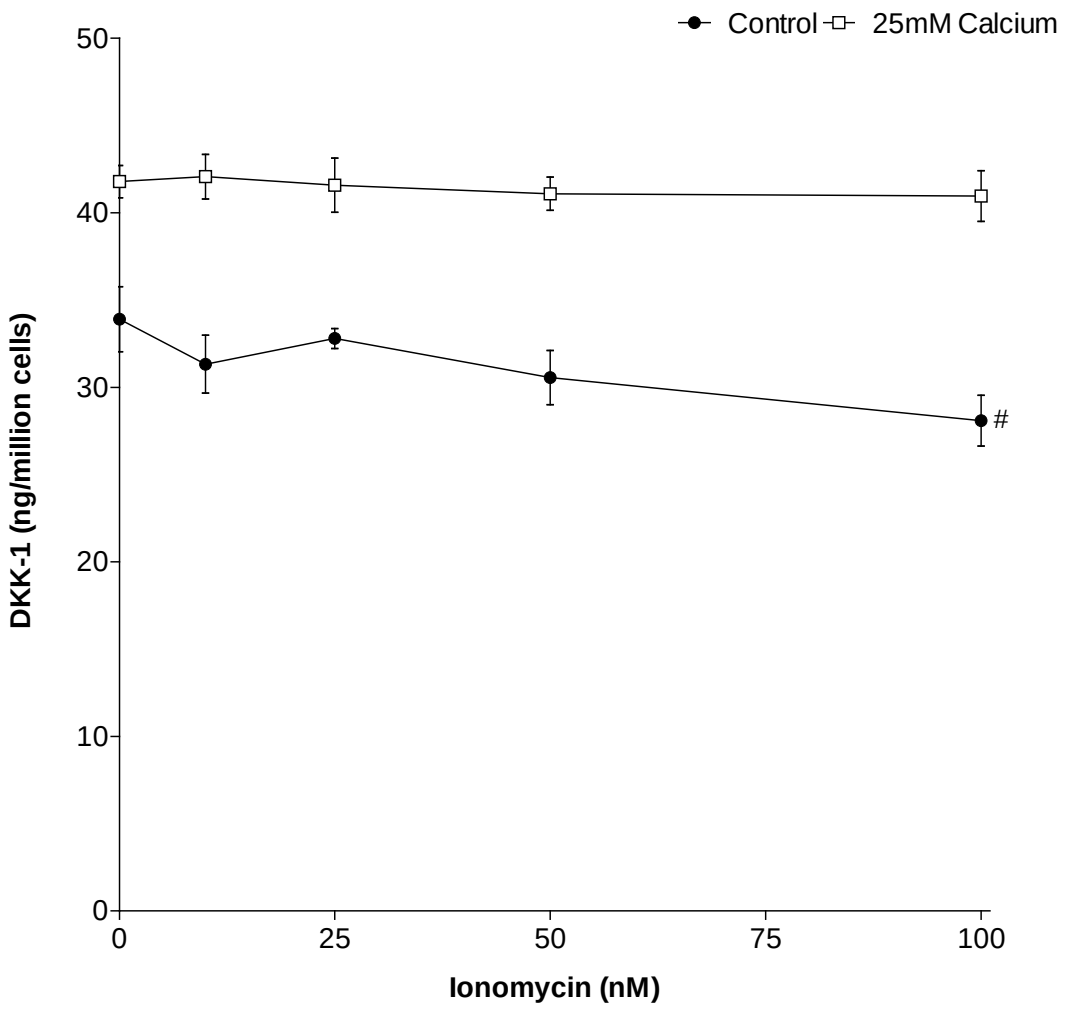

Fig 6.6 The effect of ionomycin on calcium-induced DKK-1 production in MG63 cells. MG63 cells were stimulated with 25mM calcium in complete medium following a 30 minute pre-incubation with 0-100nM ionomycin. Culture medium was assayed for DKK-1. Data shown are mean ± SD, n=4. #P<0.001 versus own control (0nM ionomycin, one-way ANOVA using Dunnett's multiple comparison test).

6.7 The effect of calcium on Wnt expression

Considering an increase in DKK-1 production could be observed after only 1 hour of calcium stimulation, the expression of *DKK-1* was investigated to see if the increase observed required new protein production. The expression of *Wnt3a, Wnt4, Wnt5a, Wnt7b, Wnt10a* and *Wnt11* was also investigated. MG63 cells were cultured for 0.5, 1, 2, 4, 8 and 24 hours in the presence of 25mM calcium. To control for any change in gene expression in response to simply changing the media as was observed in chapter 3 (Fig 3.7B), calcium was added to cells 24 hours after a medium change.

Over the 24 hour time period, it was observed that calcium had significantly affected the expression of *Wnt7b, Wnt10a* and *Wnt11* compared to control (all $P<0.001$, where control is 0 hours calcium stimulation). The earliest time for calcium to have a significant effect on the expression of *Wnt7b* was at 4 hours ($P<0.001$, Fig 6.7A). A maximum response was also observed at 4 hours, where expression of *Wnt7b* was up-regulated 10-fold compared to control in response to calcium ($P<0.001$). *Wnt7b* expression remained significantly elevated 3.4-fold above control after 24 hours of stimulation with calcium ($P<0.001$). The earliest time for calcium to have a significant effect on the expression of *Wnt10a* was at 0.5 hours ($P<0.01$, Fig 6.7B). A maximum response was observed between 0.5 and 2 hours, where expression of *Wnt10a* was down-regulated approximately 50% compared to control ($P<0.01$). Expression of *Wnt10a* returned to control levels by 8 hours. The earliest time for calcium to have a significant effect on the expression of *Wnt11* was at 4 hours ($P<0.05$, Fig 6.7B). A maximum response was observed at 8 hours, where expression of *Wnt11* was down-regulated approximately 70% compared to control ($P<0.001$). Expression of *Wnt11* returned to control levels by 24 hours. There was no significant change in expression for *Wnt3a*, *Wnt4*, *Wnt5a* or *DKK-1* in response to calcium stimulation (Fig 6.8).

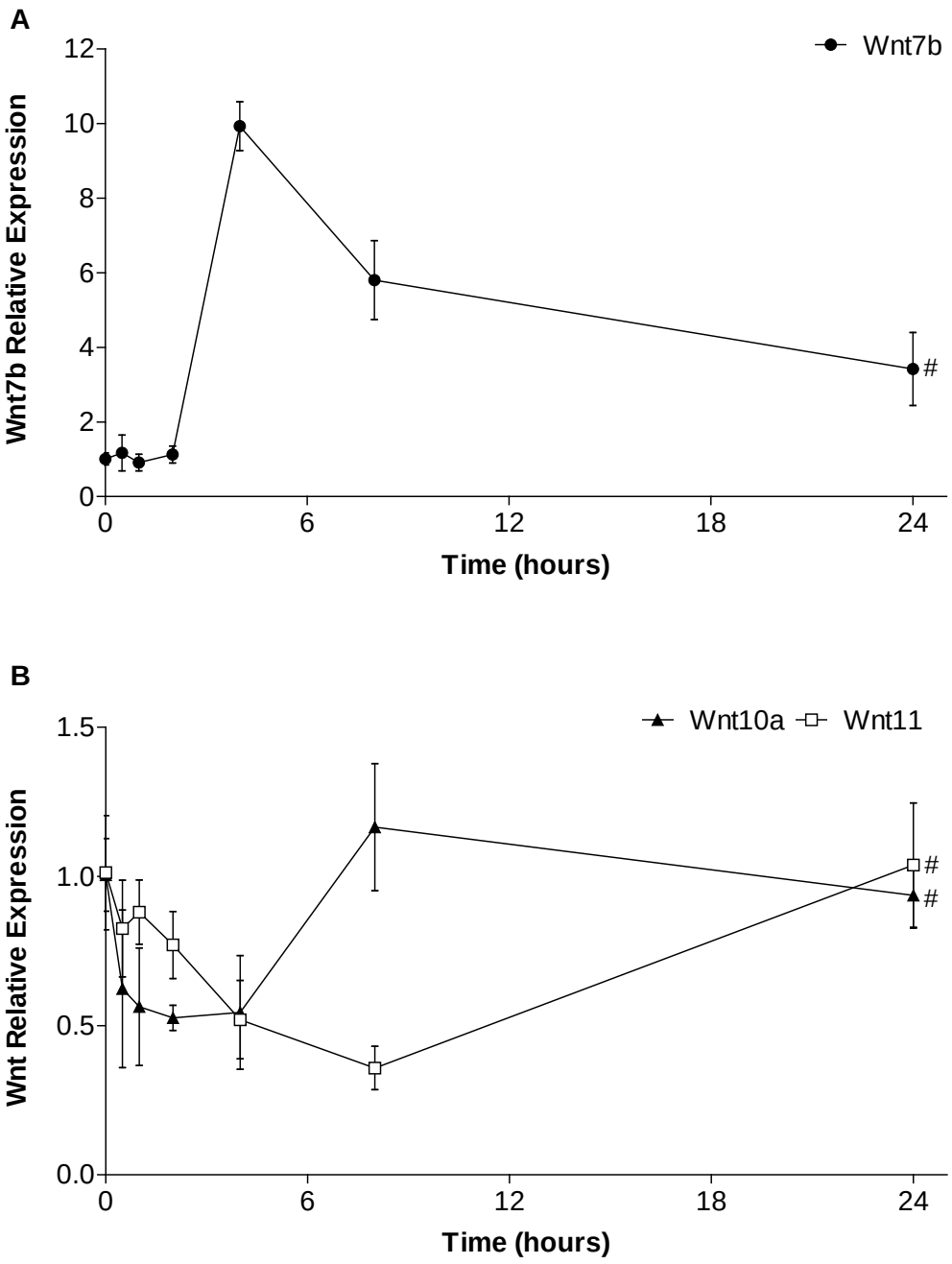

Fig 6.7 The effect of calcium on the expression of *Wnt7b, Wnt10a* and *Wnt11* in MG63 cells. MG63 cells were stimulated for 0.5, 1, 2, 4, 8 and 24 hours with 25mM calcium. RNA was extracted at each time point and mRNA assayed using RT-qPCR. Data shown are expressed relative to 0 hour control (24 hours culture in complete medium) normalised to GAPDH housekeeper expression (mean ± range, n=3). #P<0.001 versus own mRNA control (one-way ANOVA using Dunnett's multiple comparison test).

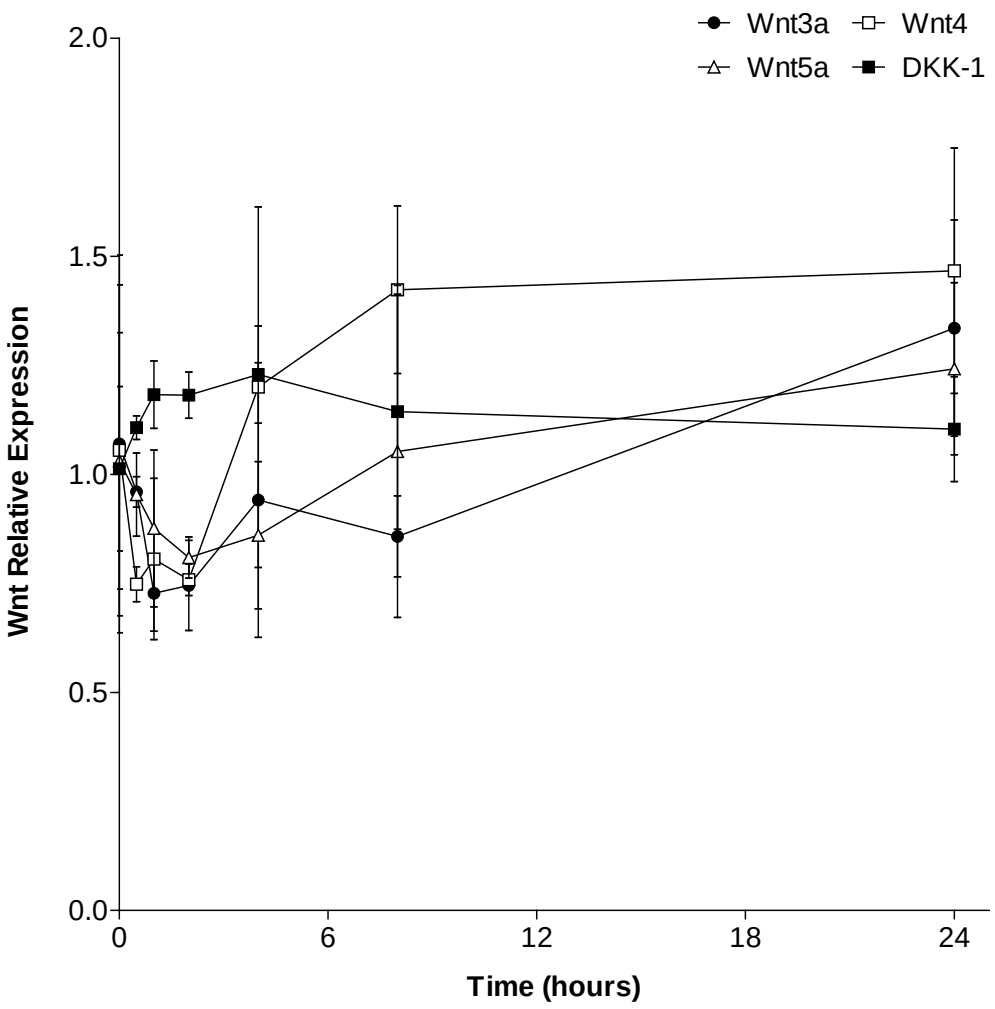

Fig 6.8 The effect of calcium on the expression of *Wnt3a, Wnt4, Wnt5a* and *DKK-1* in MG63 cells. MG63 cells were stimulated for 0.5, 1, 2, 4, 8 and 24 hours with 25mM calcium. RNA was extracted at each time point and mRNA assayed using RT-PCR. Data shown are expressed relative to 0 hour control (24 hours culture in complete medium) normalised to GAPDH housekeeper expression (mean ± range, n=3).

6.8 The effect of cycloheximide and Triton-X on calcium-induced DKK-1 production

As *DKK-1* expression did not alter with the addition of calcium, cycloheximide, a protein synthesis inhibitor, was used to investigate the effects of calcium-induced DKK-1 production. MG63 cells were treated with 5µM cycloheximide for 30 minutes, prior to addition of 25mM calcium. Cells were then left to incubate for 24 hours before assay of the culture medium for DKK-1. In the presence of cycloheximide, DKK-1 production in MG63 cells was attenuated by 56% compared to cells without cycloheximide ($P<0.01$, Fig 6.9). Cycloheximide however did not prevent calcium-induced production of DKK-1. DKK-1 production in the presence of cycloheximide was stimulated by 183% compared to the same treatment control in response to calcium ($P<0.001$, Fig 6.9).

As the observed increase of DKK-1 in the culture medium of MG63 cells treated with 25mM calcium was not a result of increased protein production, the possibility of DKK-1 being held in intracellular stores was investigated. TritonX-100 is a detergent that functions to solubilise cell membranes. MG63 cells were stimulated with 25mM calcium for 24 hours prior to extraction with 0.1% Triton X-100 and the Triton X-100 cell lysate was assayed for DKK-1. Cells that had been stimulated with calcium had significantly less DKK-1 in the Triton X-100 cell lysate than control cells ($P<0.001$, Fig 6.10).

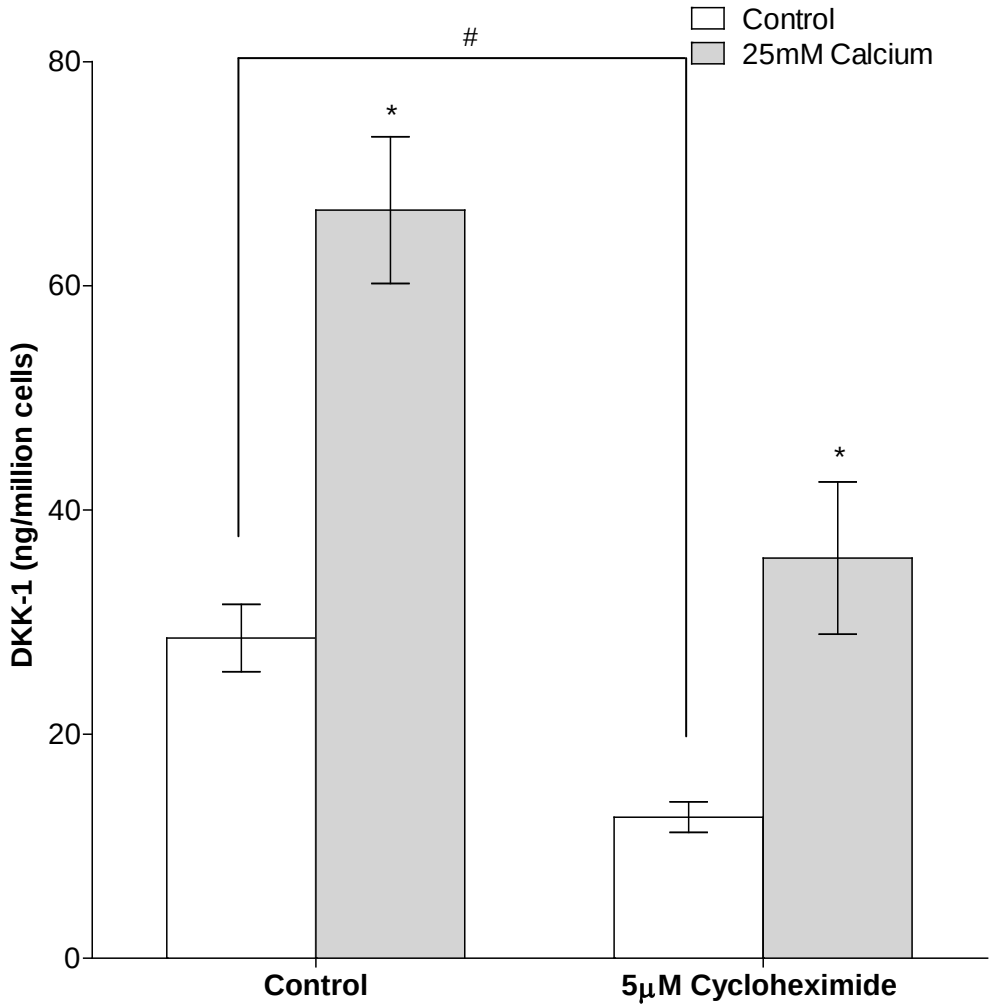

Fig 6.9 The effect of cycloheximide on calcium-induced DKK-1 production in MG63 cells. MG63 cells were treated with 25mM calcium for 24 hours following a 30 minute pre-incubation with 5μM cycloheximide. Culture medium was assayed for DKK-1. Data shown are mean ± SD, n=4. *P<0.001 versus no calcium control and #P<0.01 versus no cycloheximide control (unpaired t-test).

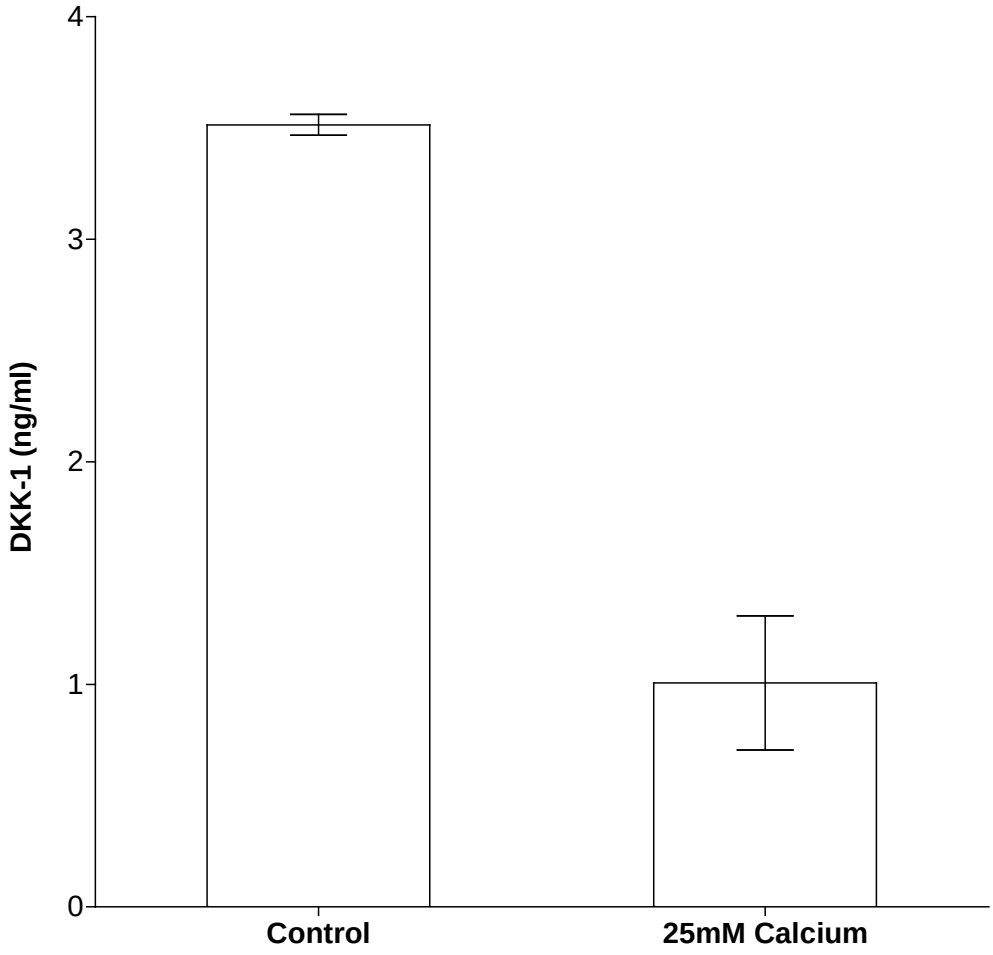

Fig 6.10 The effect of Trition X-100 on calcium-induced DKK-1 production in MG63 cells. MG63 cells were treated with 25mM calcium for 24 hours. Culture medium was removed and Trition X-100 added to cells. Trition X-100 lysate was assayed for DKK-1. Data shown are mean ± SD, n=4. *P<0.001 versus control (unpaired t-test).