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Effects of Differentiation on Purinergic and Neurotensin-Mediated Calcium Signaling in human HT-29 Colon Cancer Cells.

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[1] *Abbreviations:* ALPI, intestinal alkaline phosphatase; ATP,adenosine triphosphate; ER, endoplasmic reticulum; FBS, Fetal Bovine Serum; GPCRs, G-protein-coupled receptors; NaBt, Sodium butyrate; NT, neurotensin; NTS1, neurotensin receptor 1; NTS2, neurotensin receptor 2; NTS3, neurotensin receptor 3

Abstract

Calcium signaling is a key regulator of processes important in differentiation. In colon cancer cells differentiation is associated with altered expression of specific isoforms of calcium pumps of the endoplasmic reticulum and the plasma membrane, suggesting that differentiation of colon cancer cells is associated with a major remodeling of calcium homeostasis. Purinergic and neurotensin receptor activation are known regulators of cytosolic free Ca^{2+} levels in colon cancer cells. This study aimed to assess changes in cytosolic free Ca^{2+} levels in response to ATP and neurotensin with differentiation induced by sodium butyrate or culturing post-confluence. Parameters assessed included peak cytosolic free Ca^{2+} level after activation; time to reach peak cytosolic free Ca^{2+} and the EC_{50} of dose response curves. Our results demonstrate that differentiation of HT-29 colon cancer cells is associated with a remodeling of both ATP and neurotensin mediated Ca^{2+} signaling. Neurotensin-mediated calcium signaling appeared more sensitive to differentiation than ATP-mediated Ca^{2+} signaling.

Key words

Differentiation; colon cancer; purinergic receptors; neurotensin receptors; calcium signaling

Introduction

Calcium signaling has an essential role in various stages of tumorigenesis including angiogenesis, transcription, cell cycle, motility and differentiation [1]. Differentiation therapy has been used to treat some cancers [2], as differentiation can convert malignant cells to a more benign phenotype associated with reduced proliferation and in some cases can induce apoptosis [3, 4]. A feature of the differentiation of colon cancer cells is altered expression of calcium pumps of the endoplasmic reticulum (sarco/endoplasmic reticulum Ca^{2+} ATPases - SERCA) and plasma membrane (plasma membrane Ca^{2+}-ATPase - PMCA) [1]. SERCA3 is elevated in differentiated colon cancer cells and decreased in clinical colon cancers that are poorly differentiated [5, 6]. Similarly, our previous studies show that PMCA4 is increased during HT-29 colon cancer cell differentiation and down regulated in colon cancer compared to normal colon [7, 8]. Despite these studies suggesting that a remodeling of Ca^{2+} signaling occurs as a consequence of colon cancer cell differentiation, there has been no assessment of the consequences of differentiation of colon cancer cells on calcium signals mediated by purinergic and neurotensin receptors.

Adenosine triphosphate (ATP) and neurotensin (NT) activate purinoreceptors and neurotensin receptors respectively, and induce Ca^{2+} signaling [9]. The purinoreceptors stimulated by ATP, are known as P2 receptors and are subdivided into P2X ($P2X_{1-7}$) and P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_{11-14}$) receptors [10-12]. The P2X receptors are Ca^{2+} permeable ion channels that promote the opening of voltage-dependent Ca^{2+} channels [13 36]. P2Y receptors include G-protein-coupled receptors (GPCRs) that increase intracellular calcium by activation of phospholipase C, formation of IP_3 and the subsequent release of Ca^{2+} from intracellular stores [9, 12]. Three neurotensin receptors have been identified, of which neurotensin receptor 1 (NTS1) and

neurotensin receptor 2 (NTS2) are G-protein coupled receptors (GPCR) and induce Ca^{2+} release from internal stores [14] while neurotensin receptor 3 (NTS3) is a non GPCR which modulates NT signaling in HT-29 cells by forming a functional interaction with NTS1 [15].

In the leukemia cell line HL-60, differentiation attenuates the Ca^{2+} signal elicited by both ATP and NT [16, 17]. Increases in intracellular Ca^{2+} ($[Ca^{2+}]_{cyt}$) mediated by ATP are also reduced during the differentiation of both mouse skeletal muscle [18] and C2C12 myoblast cells [19]. Purinoreceptor expression levels are often decreased with differentiation of osteoblasts and C2C12 myoblasts [20, 21]. A relationship between differentiation and NT receptor expression has been observed in primary cultures of prostate cancer cells derived from patient samples and in prostate cancer cell lines [22]. Less differentiated prostate cancer cells lines show increased NTS1, whereas more differentiated cells have higher levels of NTS2 [22]. A similar remodeling of these signaling pathways may also occur in colon cancer cells. Colon cancers overexpress P2Y2, P2Y4 and NTS1 receptors compared to normal colon [23, 24] and decreased NTS1 expression is a feature of sodium butyrate-mediated differentiation of HT-29 colon cancer cells [25]. Despite these observations, Ca^{2+} signaling via NT and ATP has not been explored in the context of HT-29 colon cancer cell differentiation.

In the study presented here, differentiation of HT-29 colon cancer cells was induced chemically (sodium butyrate) or spontaneously (culturing cells post-confluence) [5] to determine if altered Ca^{2+} signaling mediated by ATP and NT is associated with the differentiation of colon cancer cells.

Materials and Methods

Materials

Fetal Bovine Serum (FBS) was purchased from JRH Bioscience (Lenexa, KS, USA) and RPMI-1640 media, L-glutamine, penicillin/streptomycin and fluo-4 AM were from Invitrogen (Carlsbad, CA, USA). Black CellBIND surface 96-well plates were obtained from Corning Inc. (Corning, NY, USA). Sodium butyrate (NaBt), ATP, NT and all other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). TaqMan Universal PCR Master Mix and TaqMan gene expression assays were from Applied Biosystems (Mulgrave, Victoria, Australia).

Cell Culture

HT-29 cells were cultured and maintained in RPMI-1640 media, supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 U/mL)/ streptomycin (100 µg/mL) at 37°C with 5% CO_2 as previously described [7]. Cell lines were tested 6 monthly for mycoplasma contamination using the MycoAlert Mycoplasma Detection Assay (Lonza. Inc., Mt Waverley, Victoria, Australia).

Differentiation in HT-29 cells

Sodium butyrate (NaBt) induced differentiation

For assessment of mRNA, HT-29 cells were seeded at 2.0 X 10^5 cells/well in 6-well tissue culture plates. For measurement of intracellular Ca^{2+} ($[Ca^{2+}]_{cyt}$), HT-29 cells were seeded at 6.3 X 10^3 cells/well in 96-well black CellBIND surface plates. After 48 h cells were treated with 0 or 3 mM NaBt in fresh culture media for 96 h.

Culturing post-confluence induced differentiation

For mRNA assessment, HT-29 cells were seeded in 6-well plates at 2.5 X 10^5 cells/well. For $[Ca^{2+}]_{cyt}$ assessment, HT-29 cells were seeded at 6.3 X 10^3 cells/well in 96-well black CellBIND surface plates. After the cells were 95-100% confluent (72 h) the cells were maintained in growth media for a further 7-14 days with media changes every third day.

Real time RT- PCR

RNA was isolated from undifferentiated and differentiated HT-29 cells using the RNeasy® Plus Mini Kit (Qiagen, Clifton Hill, Victoria, Australia) and reverse transcribed using the Omniscript RT kit (Qiagen). Real time RT-PCR was performed using TaqMan Universal PCR Master Mix and a 7500 Real-Time PCR System. The mRNA expression of target genes were assessed using the following TaqMan gene expression assays; Hs00357578_m1 (ALPI). Levels of mRNA were normalized to 18S rRNA and relative expression levels were determined using the comparative C*t* method [8, 26].

Measurement of $[Ca^{2+}]_{cyt}$

Levels of $[Ca^{2+}]_{cyt}$ in undifferentiated and differentiated HT-29 cells were assessed upon activation with ATP (1 μM – 1 mM) or NT (1 nM – 1 μM), using Fluo-4, as previously described [8]. Data were assessed using ScreenWorks 2.0.0.22 (Molecular Devices) at 1 s intervals for 800 s and fluorescence was normalized for each treatment by assessing response over baseline which is a measure of relative $[Ca^{2+}]_{cyt}$ [8]. Maximum relative $[Ca^{2+}]_{cyt}$ response and time to reach peak was calculated using ScreenWorks 2.0.0.22 (Molecular Devices).

Statistical analysis

Statistical significance was assessed as described in individual figure legends. Data analysis including calculation of EC_{50} values was performed using Prism version 5.0.2 for Windows (GraphPad Inc). The EC_{50} was calculated using a nonlinear regression analysis in Prism version 5.0.2.

Results

Confirmation of HT-29 cells differentiation

To verify that differentiation was induced in the HT-29 colon cancer cells by NaBt and culturing post-confluence, mRNA levels of a well-established differentiation marker, intestinal alkaline phosphatase (ALPI) in HT-29 cells[7, 27, 28], were assessed. NaBt treatment induced ALPI expression by approximately 60-fold ($P < 0.05$) (Fig. 1a), and culturing post-confluence (day 14) increased ALPI mRNA levels by approximately 10 fold ($P < 0.05$) compared to control (Fig. 1b), consistent with previous reports from our group and others [7, 29].

Effect of NaBt induced differentiation on Ca^{2+} signaling

To assess purinergic and neurotensin signaling in differentiated colon cancer cells, ATP and NT were utilized. HT-29 colon cancer cells were differentiated with NaBt and the cytosolic Ca^{2+} responses to the agonists ATP (1.0 mM) and NT (1.0 μM) were assessed. The average $[Ca^{2+}]_{cyt}$ transient shows that differentiation altered the nature of the $[Ca^{2+}]_{cyt}$ transient in response to ATP (Fig. 2a). A significant difference in the maximum response to ATP was not observed (Fig. 2b), however, the time to reach peak $[Ca^{2+}]_{cyt}$ was significantly decreased in differentiated cells (Fig.

2c). NT-mediated increases in $[Ca^{2+}]_{cyt}$ (Fig. 2e) had a similar time to reach peak (Fig. 2g) for differentiated and undifferentiated cells, but the maximum response to NT was significantly reduced (72%) in differentiated cells (Fig. 2f).

Differential effects of differentiation on ATP and NT responses were also reflected in the dose response curves for both ligands. Dose response curves for maximal $[Ca^{2+}]_{cyt}$ response to ATP showed that NaBt induced differentiation shifted the peak Ca^{2+} dose response curve for ATP to the left (Fig. 2d). The EC_{50} decreased from 27.7 µM to 4.31 µM with no change in the maximum $[Ca^{2+}]_{cyt}$. In contrast, when differentiated HT-29 cells were treated with NT, the maximal $[Ca^{2+}]_{cyt}$ response was reduced at all concentrations of NT (1 nM - 1 µM); and the EC_{50} increased from 54.8 nM to 103 nM (Fig. 2h).

Effect of differentiation induced by culturing post-confluence on Ca^{2+} signaling.

An alternative method of differentiation, culturing post-confluence, was used to further assess the effect of colon cancer cell differentiation on Ca^{2+} signaling mediated by ATP and NT. Figure 3a shows the average Ca^{2+} transient for ATP stimulated cells. The maximum response to ATP was reduced in differentiated cells (Fig. 3b). However, the time to reach peak Ca^{2+} with ATP stimulation was not affected by differentiation (Fig. 3c). A similar trend was observed for NT stimulation (Fig. 3e); decreased maximum Ca^{2+} response in differentiated cells (Fig. 3f), but a similar time to reach peak $[Ca^{2+}]_{cyt}$ for differentiated and undifferentiated cells (Fig. 3g). Comparison between the two ligands suggests that the decrease in maximum $[Ca^{2+}]_{cyt}$ response in differentiated cells appears to be more pronounced for NT activation compared to ATP activation (Fig. 3b and 3f). The maximal Ca^{2+} dose response curves for ATP and NT showed that

culturing post-confluence mediated differentiation decreased the $[Ca^{2+}]_{cyt}$ response for both agonists (Fig. 3d and 3h). With ATP the EC_{50} increased from 3.62 µM (2.07×10^{-6} – 6.32×10^{-6}, 95% confidence interval) to 65.6 µM (32.5×10^{-6} – 132.0×10^{-6}, 95% confidence interval) and with NT from 13.4 nM (8.09×10^{-9} – 22.2×10^{-9}, 95% confidence interval) to 160 nM (34.3×10^{-9} – 748.0×10^{-9}, 95% confidence interval) with differentiation.

Comparison of NT and ATP responses

To assesses whether the change in response to NT was more pronounced compared to ATP with differentiation, the maximum $[Ca^{2+}]_{cyt}$ was normalized to ATP for all treatments for NaBt (Fig. 4a) and culturing post-confluence induced differentiation (Fig. 4b). Responses to NT were more significantly reduced than to ATP for both NaBt (Fig. 4a) and culturing post-confluence induced differentiation (Fig. 4b).

Discussion

Altered expression of Ca^{2+} pumps is a well characterized feature of colon cancer cell differentiation [5, 7] and suggests that a remodeling of Ca^{2+} signaling may be one of the consequences of differentiation of colon cancer cells. While there is evidence that increases in $[Ca^{2+}]_{cyt}$ in response to ATP and NT are reduced by differentiation in promyelocytic leukemia cells [16, 17] and muscle cells [18, 19], such alterations in colon cancer cells during differentiation have not been explored.

The current study used two methods to differentiate HT-29 colon cancer cells and assessed the effect of differentiation on Ca^{2+} responses elicited by NT and ATP. The current study

demonstrated that NaBt induced differentiation and culturing post-confluence both modified aspects of the $[Ca^{2+}]_{cyt}$ response to ATP. However, the nature of the ATP effect on $[Ca^{2+}]_{cyt}$ was different between the two methods of differentiation. These result suggest that the changes in ATP-mediated $[Ca^{2+}]_{cyt}$ response may not be a consequence of differentiation *per se*. NaBt induced differentiation shifted the dose response curve to ATP to the left and decreased the time to peak, without significantly affecting the peak $[Ca^{2+}]_{cyt}$ response, whereas, spontaneous differentiation decreased the maximum $[Ca^{2+}]_{cyt}$ response and shifted the dose response curve to the right. NaBt induced differentiation also significantly decreased the time to reach peak $[Ca^{2+}]_{cyt}$ after ATP-stimulation, but this was not a feature of post-confluence induced differentiation. Since NaBt and post-confluence induced differentiation had the opposite effect with ATP stimulation (ie NaBt shifted the dose responses curve to the left, and culturing post confluency shifted the dose response curve to the right), these differences are not likely due to a difference in the degree of differentiation. Furthermore, these differences in the Ca^{2+} response do not parallel with changes in SERCA3 and PMCA4 expression with differentiation [5, 8]. The expression levels of P2Y2 and P2Y4 are lower in normal colon compared to colon cancer [23]. However, the current assessment of ATP mediated increases in $[Ca^{2+}]_{cyt}$ suggests that this potential difference in levels of purinergic receptors between differentiated and undifferentiated colon cells is not reflected in a consistent functional difference in the assessed HT-29 differentiation models. A variance in the effect of different differentiation mechanisms is also been seen with PMA- versus DMSO-induced differentiation in HL-60 cells where ATP potency and efficacy are decreased only in the presence of PMA [16].

In contrast to ATP, aspects of changes in the NT $[Ca^{2+}]_{cyt}$ response were consistently observed between the two differentiation methods. NT-mediated increases in $[Ca^{2+}]_{cyt}$ were attenuated by

both NaBt and post-confluence induced differentiation. The reduction in maximum $[Ca^{2+}]_{cyt}$ response for NT but not ATP after NaBt-mediated differentiation demonstrates that the altered $[Ca^{2+}]_{cyt}$ response is not due to general effects on the receptor activated calcium signaling cascade; for example reduced endoplasmic reticulum Ca^{2+} levels and/or phospholipase C activity and 1,4,5 trisphosphate generation. Future studies should explore an explanation for the more pronounced effects of differentiation on the $[Ca^{2+}]_{cyt}$ response to ATP compared to NT. In conclusion differentiation of HT-29 colon cancer cells is associated with a remodeling of Ca^{2+} signaling and this is in part dependent on the activator, with changes in the $[Ca^{2+}]_{cyt}$ response to NT more pronounced that that for ATP

Acknowledgements

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References

1. G.R. Monteith, D. McAndrew, H.M. Faddy and S.J. Roberts-Thomson, Calcium and cancer: targeting Ca2+ transport, Nat. Rev. Cancer 7 (2007) 519-30.
2. S. Waxman, Differentiation therapy in acute myelogenous leukemia (non-APL), Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K 14 (2000) 491-6.
3. A.I. Spira and M.A. Carducci, Differentiation therapy, Curr. Opin. Pharmacol. 3 (2003) 338-43.
4. R. Lotan: Differentiation Therapy: Alison MR, (ed). John Wiley & Sons Ltd., 2002.
5. P. Gelebart, T. Kovacs, J.P. Brouland, R. van Gorp, J. Grossmann, N. Rivard, Y. Panis, V. Martin, R. Bredoux, J. Enouf and B. Papp, Expression of endomembrane calcium pumps in colon and gastric cancer cells. Induction of SERCA3 expression during differentiation, J. Biol. Chem. 277 (2002) 26310-20.
6. J.P. Brouland, P. Gelebart, T. Kovacs, J. Enouf, J. Grossmann and B. Papp, The loss of sarco/endoplasmic reticulum calcium transport ATPase 3 expression is an early event during the multistep process of colon carcinogenesis, Am. J. Pathol. 167 (2005) 233-42.
7. C.S. Aung, W.A. Kruger, P. Poronnik, S.J. Roberts-Thomson and G.R. Monteith, Plasma membrane Ca2+-ATPase expression during colon cancer cell line differentiation, Biochem. Biophys. Res. Commun. 355 (2007) 932-6.
8. C.S. Aung, W. Ye, G. Plowman, A.A. Peters, G.R. Monteith and S.J. Roberts-Thomson, Plasma membrane calcium ATPase 4 and the remodeling of calcium homeostasis in human colon cancer cells, Carcinogenesis 30 (2009) 1962-9.

9. V. Ralevic and G. Burnstock, Receptors for purines and pyrimidines, Pharmacol. Rev. 50 (1998) 413-92.

10. I. von Kugelgen, Pharmacological profiles of cloned mammalian P2Y-receptor subtypes, Pharmacol. Ther. 110 (2006) 415-32.

11. C. Coddou, Z. Yan, T. Obsil, J.P. Huidobro-Toro and S.S. Stojilkovic, Activation and regulation of purinergic P2X receptor channels, Pharmacol. Rev. 63 (2011) 641-83.

12. L. Erb, Z. Liao, C.I. Seye and G.A. Weisman, P2 receptors: intracellular signaling, Pflugers. Arch. 452 (2006) 552-62.

13. T.M. Egan, D.S. Samways and Z. Li, Biophysics of P2X receptors, Pflugers. Arch. 452 (2006) 501-12.

14. R.M. Myers, J.W. Shearman, M.O. Kitching, A. Ramos-Montoya, D.E. Neal and S.V. Ley, Cancer, chemistry, and the cell: molecules that interact with the neurotensin receptors, ACS. Chem. Biol. 4 (2009) 503-25.

15. S. Martin, V. Navarro, J.P. Vincent and J. Mazella, Neurotensin receptor-1 and -3 complex modulates the cellular signaling of neurotensin in the HT29 cell line, Gastroenterology 123 (2002) 1135-43.

16. K. Adrian, M.K. Bernhard, H.G. Breitinger and A. Ogilvie, Expression of purinergic receptors (ionotropic P2X1-7 and metabotropic P2Y1-11) during myeloid differentiation of HL60 cells, Biochim. Biophys. Acta. 1492 (2000) 127-38.

17. S.Y. Choi, H.D. Chae, T.J. Park, H. Ha and K.T. Kim, Characterization of high affinity neurotensin receptor NTR1 in HL-60 cells and its down regulation during granulocytic differentiation, Br. J. Pharmacol. 126 (1999) 1050-6.

18. G.P. Szigeti, H. Szappanos, T. Deli, J. Cseri, L. Kovacs and L. Csernoch, Differentiation-dependent alterations in the extracellular ATP-evoked calcium fluxes of cultured skeletal muscle cells from mice, Pflugers. Arch. 453 (2007) 509-18.

19. T. Martinello, M.C. Baldoin, L. Morbiato, M. Paganin, E. Tarricone, G. Schiavo, E. Bianchini, D. Sandona and R. Betto, Extracellular ATP signaling during differentiation of C2C12 skeletal muscle cells: role in proliferation, Mol. Cell. Biochem. 351 (2011) 183-96.

20. I.R. Orriss, G.E. Knight, S. Ranasinghe, G. Burnstock and T.R. Arnett, Osteoblast responses to nucleotides increase during differentiation, Bone 39 (2006) 300-9.

21. W. Banachewicz, D. Suplat, P. Krzeminski, P. Pomorski and J. Baranska, P2 nucleotide receptors on C2C12 satellite cells, Purinergic Signal. 1 (2005) 249-57.

22. S.L. Swift, J.E. Burns and N.J. Maitland, Altered expression of neurotensin receptors is associated with the differentiation state of prostate cancer, Cancer Res. 70 (2010) 347-56.

23. G. Nylund, L. Hultman, S. Nordgren and D.S. Delbro, P2Y2- and P2Y4 purinergic receptors are over-expressed in human colon cancer, Auton. Autacoid. Pharmacol. 27 (2007) 79-84.

24. X. Gui, G. Guzman, P.R. Dobner and S.S. Kadkol, Increased neurotensin receptor-1 expression during progression of colonic adenocarcinoma, Peptides 29 (2008) 1609-15.

25. X. Wang, L.N. Jackson, S.M. Johnson, Q. Wang and B.M. Evers, Suppression of neurotensin receptor type 1 expression and function by histone deacetylase inhibitors in human colorectal cancers, Mol. Cancer. Ther. 9 (2010) 2389-98.

26. K.M. Suchanek, F.J. May, J.A. Robinson, W.J. Lee, N.A. Holman, G.R. Monteith and S.J. Roberts-Thomson, Peroxisome proliferator-activated receptor alpha in the human breast cancer cell lines MCF-7 and MDA-MB-231, Mol. Carcinog. 34 (2002) 165-71.

27. B.F. Hinnebusch, A. Siddique, J.W. Henderson, M.S. Malo, W. Zhang, C.P. Athaide, M.A. Abedrapo, X. Chen, V.W. Yang and R.A. Hodin, Enterocyte differentiation marker intestinal alkaline phosphatase is a target gene of the gut-enriched Kruppel-like factor, Am. J. Physiol. Gastrointest. Liver Physiol. 286 (2004) G23-30.

28. J.H. Kim, S. Meng, A. Shei and R.A. Hodin, A novel Sp1-related cis element involved in intestinal alkaline phosphatase gene transcription, The American journal of physiology 276 (1999) G800-7.

29. J.L. Shie, Z.Y. Chen, M.J. O'Brien, R.G. Pestell, M.E. Lee and C.C. Tseng, Role of gut-enriched Kruppel-like factor in colonic cell growth and differentiation, American journal of physiology. Gastrointestinal and liver physiology 279 (2000) G806-14.

Figure legends

Figure 1: Assessment of intestinal alkaline phosphatase (ALPI) mRNA levels as a marker of differentiation in HT-29 cells. ALPI mRNA levels increased during differentiation induced by 3 mM sodium butyrate (NaBt) (A) or culturing post-confluence (B). Data are normalized to 18S rRNA and are relative to control treated cells or 1 day post-confluence. The data are mean ± SEM (n = 3-4) and are representative of two independent experiments and the symbol (*) indicates significant difference; $P < 0.05$ using unpaired t-test.

Figure 2: Effect of NaBt induced differentiation on receptor mediated Ca^{2+} signaling. Assessment of $[Ca^{2+}]_{cyt}$ in HT-29 cells treated with 0 or 3 mM NaBt and stimulated with ATP (A-D) or NT (E-H). A and E, average $[Ca^{2+}]_{cyt}$ transient in cells treated with ATP (A) or NT (E). B and F, Relative peak $[Ca^{2+}]_{cyt}$ with ATP (B) or NT (F) stimulation. C and G, time to peak $[Ca^{2+}]_{cyt}$ with ATP (C) or NT (G) stimulation. D and H, concentration response curves for receptor mediated Ca^{2+} signaling in HT-29 cells stimulated with ATP (1 µM-1 mM) (D) or NT (1 nM – 1 µM) (H). Insert shows average EC_{50} values. Data are mean (± SEM; n = 12) and are representative of 4 independent assays and symbol (*) indicates significant difference; $P < 0.05$ using unpaired *t*-test.

Figure 3: Effect of culturing post-confluence induced differentiation on receptor mediated Ca^{2+} signaling. Assessment of $[Ca^{2+}]_{cyt}$ in HT-29 cells cultured for seven days post-confluence and stimulated with ATP (A-D) or NT (E-H). A and E, average $[Ca^{2+}]_{cyt}$ transient in cells treated with ATP (A) or NT (D). Data are mean ± SEM (n = 12) and are representative of four independent assays. B and E, Relative peak $[Ca^{2+}]_{cyt}$ with ATP (B) or NT (F) stimulation. C and G, time to peak $[Ca^{2+}]_{cyt}$ with ATP (C) or NT (G) stimulation. D and H, concentration response curves for receptor mediated Ca^{2+} signaling in HT-29 cells stimulated with ATP (1 µM-1 mM) (D) or NT (1 nM – 1 µM) (H). Insert shows average EC_{50} values. Data are mean +SEM (n = 12) dose response curves for relative peak $[Ca^{2+}]_{cyt}$ from four independent assays. Insert shows average EC_{50} values. Data are mean (± SEM; n = 12) from four independent assays and symbol (*) indicates significant difference; $P < 0.05$ using unpaired *t*-test.

Figure 4: Comparison of relative peak $[Ca^{2+}]_{cyt}$ in differentiated and undifferentiated HT-29 cells stimulated with ATP or NT. A, normalized relative peak $[Ca^{2+}]_{cyt}$ in cells treated with 0 or 3

mM NaBt. B, normalized relative peak $[Ca^{2+}]_{cyt}$ in cells cultured for seven days post-confluence. Data are mean ± SEM (n = 12) from four independent assays and are normalized to the ATP for each condition. Symbol (*) indicates significant difference; $P < 0.05$ using unpaired t-test.

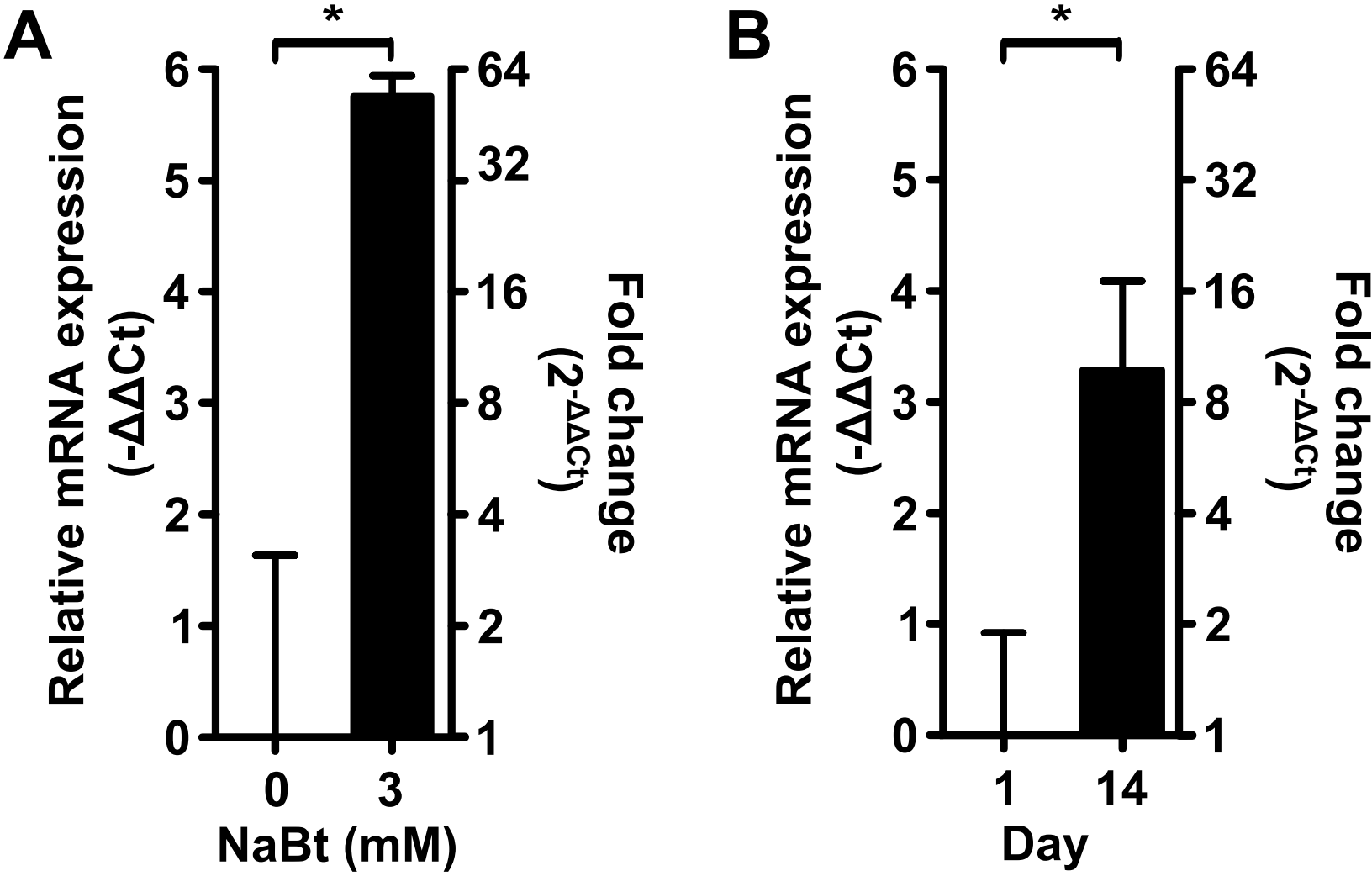
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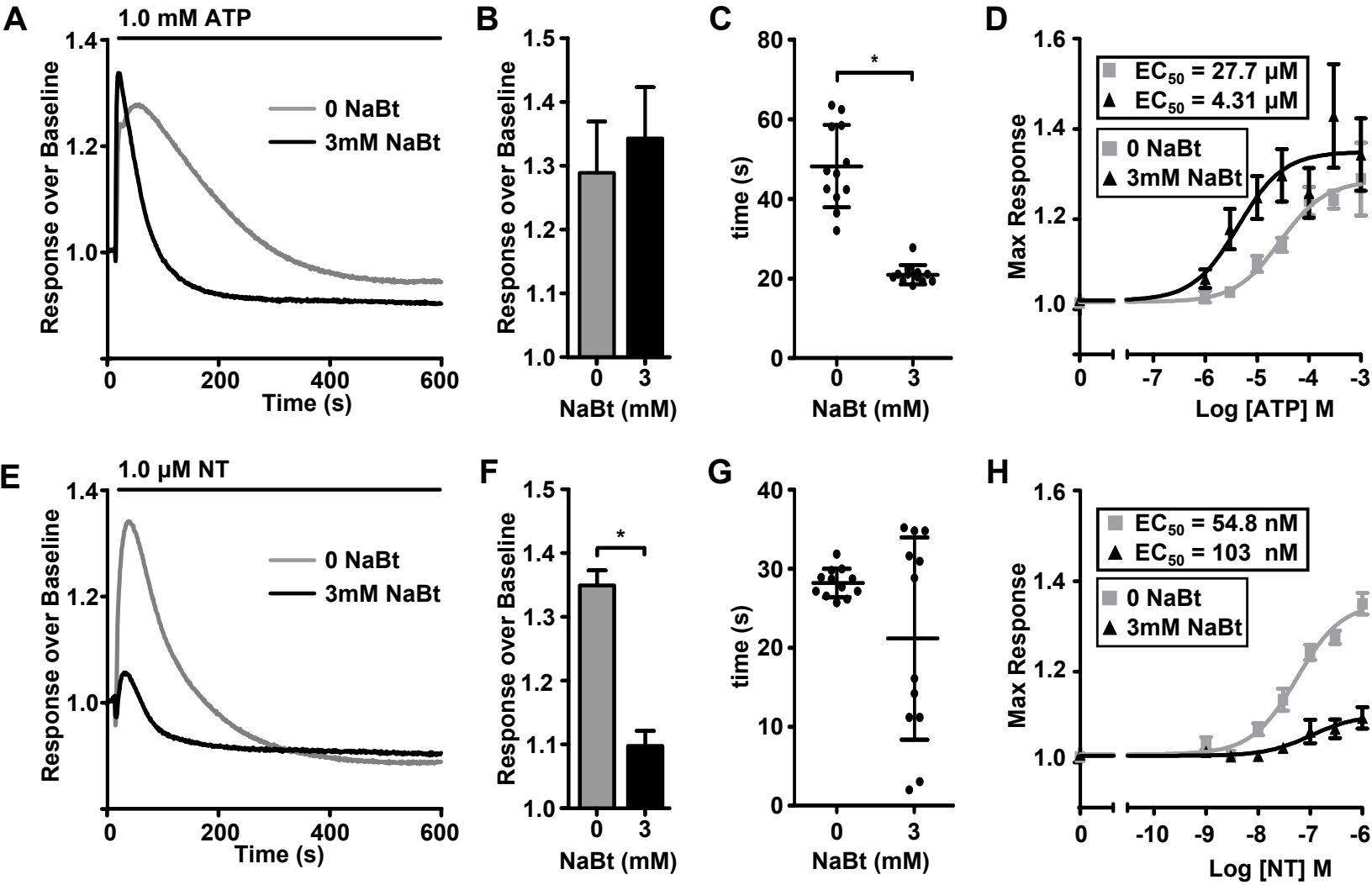
Figure 1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EC50 = 27.7 µM
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0 NaBt
3mM NaBt
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3mM NaBt
Response over Baseline
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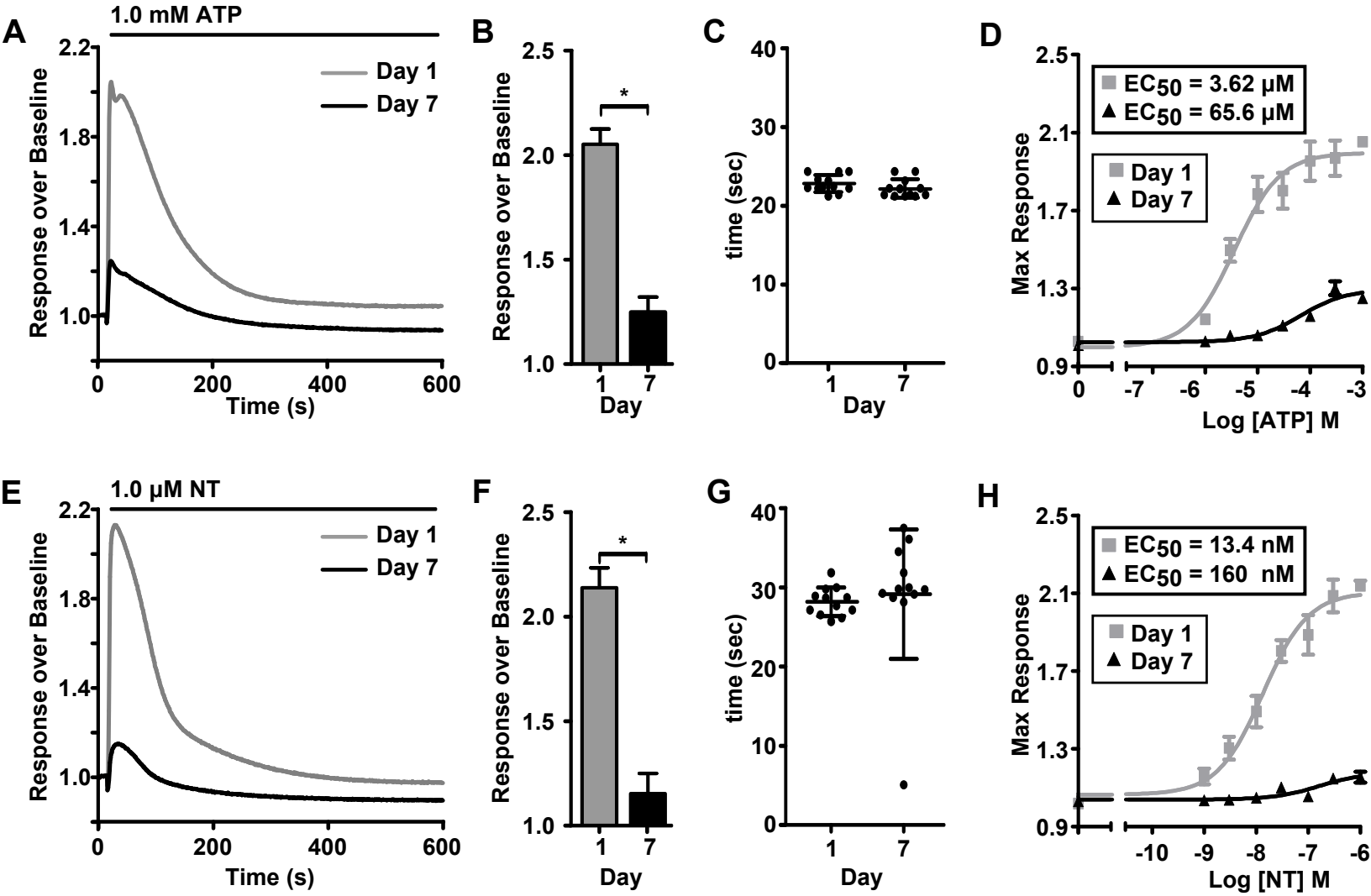
Log [NT] M

Figure 2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Day 1
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Response over Baseline
Time (s)
B
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C
time (sec)
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EC50 = 65.6 µM
Day 1
Day 7
Max Response
Log [ATP] M
E
1.0 µM NT
Day 1
Day 7
Response over Baseline
Time (s)
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Response over Baseline
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Max Response
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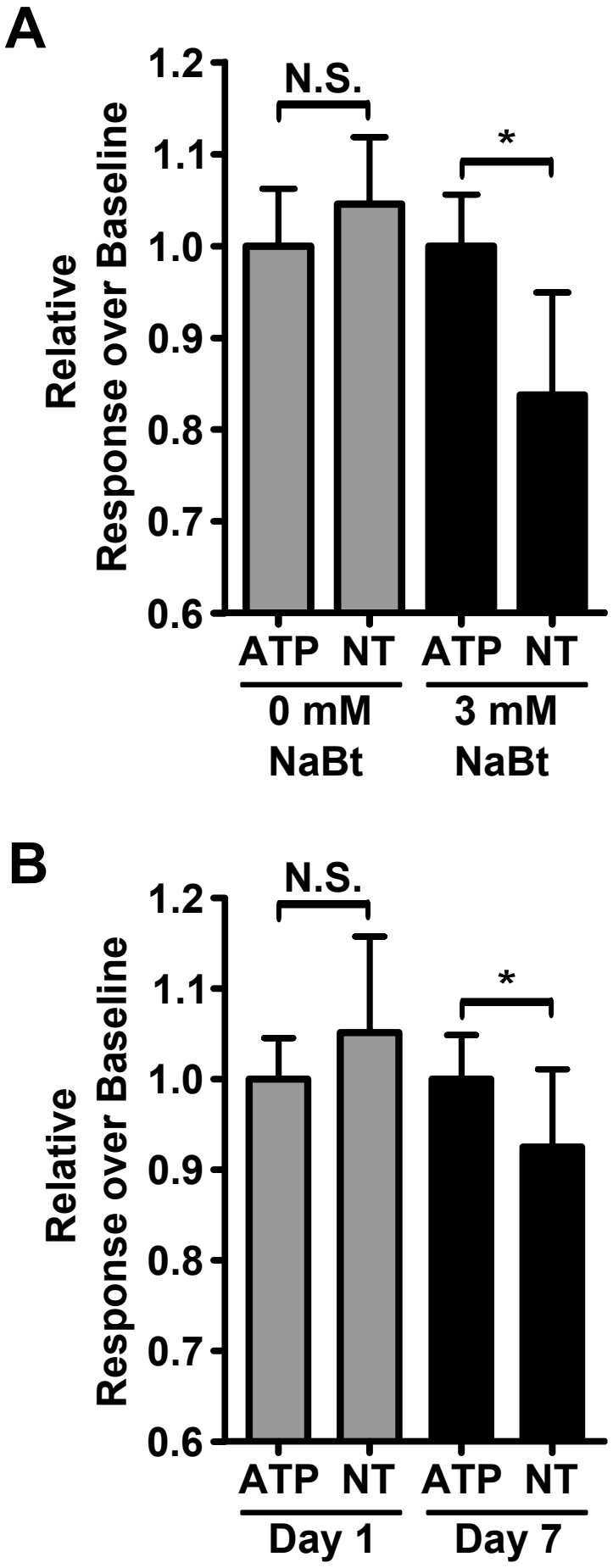
Figure 3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Relative Response over Baseline
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1.0
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ATP NT ATP NT
Day 1
Day 7

Figure 4